Evolutionary Ecology of Host Manipulation by Parasites

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Summary

Many parasites have the ability to change their host's behavior and/ or appearance in a manner that enhances their fitness. It can be difficult to determine whether this is due to true host manipulation that has evolved for this specific purpose or side-effects e.g. from additional energy drain that shifts the host's trade-off between feeding and predation avoidance towards the former. Experimentally, host manipulation has mostly been studied using singly infected hosts in uniform environments. This does not reflect nature. If hosts harbor multiple parasites these parasites might, depending on their aims, be at a conflict over host manipulation or cooperate when it comes to host manipulation.

In this thesis I aim to better understand how parasites manipulate their hosts under complex – and hence more natural conditions, such as infections by multiple parasites. To do so, I use two complex-life cycle parasites, the cestode *Schistocephalus solidus* and both its intermediated hosts, copepods and three-spined sticklebacks and the nematode *Camallanus lacustris* and its first intermediate copepod host. Both parasites alter the behavior of their hosts in a manner likely to enhance their own fitness.

Whether host manipulation by *S. solidus* in three-spined sticklebacks is due to true host manipulation that has evolved for this purpose or presents a side-effect of increased energy drain is the issue of a long standing debate. With true host manipulation an infective and a not yet infective *S. solidus* in the same host should be at a conflict over the direction of host manipulation; the infective parasite should enhance the predation risk of its host, but for the not yet infective one any premature predation would be fatal; it should never enhance its host's risk taking. However, when I experimentally infected sticklebacks in a manner that they harbored one infective and one not yet infective parasite, these were even less risk averse than sticklebacks infected by only an infective parasite. This is only compatible with behavioral changes caused by enhanced energy drain rather than true host manipulation. To verify these findings I tested the effect of hunger status on risk taking in infected sticklebacks. Energy drain should be ineffective in satiated fish but true host manipulation should not. Again the results are more compatible with energy drain rather than true host manipulation. Seemingly adaptive host manipulation need not be caused by mechanisms that have evolved for this specific purpose.

I further investigated a conflict between an infective and a not yet infective parasite in copepod hosts. To do so, I experimentally infected copepods with one or two *S. solidus* and/ or *C. lacustris* at different time points. Not yet infective *S. solidus* or *C. lacustris* reduce their host's activity, infective ones increase it. If a conflict occurred between different developmental stages of the same species, the infective parasite completely sabotaged host manipulation by the not yet infective one in either species. In an interspecific conflict too, the infective parasite performed better. *Camallanus lacustris* was however the stronger manipulator. A conflict between an infective *C. lacustris* and a not yet infective *S. solidus* resulted in complete sabotage by the infective parasite while such a conflict between an infective *S. solidus* and a not yet infective *C. lacustris* resulted in a compromise with regards to host behavior. If there is potential for cooperation, i.e. if multiple *S. solidus* of the same developmental stage co-occur in the same host, they enhance each other's

manipulation but only after they have reached infectivity. I hence show that one parasite can affect host manipulation by both a conspecific and a non-conspecific co-infecting parasite.

In nature, parasites are not only faced with other co-infecting parasites but also with differences in the environment their host experiences. Such differences, for example in differences in resource availability might affect host manipulation. Experimentally infected copepods received different feeding treatments. I measured their parasites' performance in both the first (i.e. copepod) and the second (i.e. stickleback) intermediate host and the behavior of their copepod host. Performance inside the copepod affected performance in the second intermediate fish host. Differences between infected and uninfected copepods (i.e. host manipulation) were larger in a high food environment. Additionally, larger parasites seemed less manipulated. These results could be mediated by changes in host condition, rather than actual differences in host manipulation. Nevertheless, if they lead to differences in the efficiency of host manipulation, they could have ecological consequences.

Zusammenfassung

Viele Parasiten verfügen über die Fähigkeit, Verhalten und/oder Aussehen ihres Wirtes zu verändern um dadurch ihre eigene Fitness zu steigern. Es kann schwierig sein festzustellen, ob dies durch echte Wirtsmanipulation geschieht, die zu diesem Zweck evolviert ist, oder ob es eine Begleiterscheinung darstellt, zum Beispiel aufgrund des zusätzlichen Energieentzuges, der den Trade-off des Wirtes zwischen Fressen und Predationsvermeidung zum ersteren hin verschiebt. Experimentell ist Wirtsmanipulation bisher meist unter der Bedingung einer einheitlichen Umwelt und mit Hilfe von Wirten, die nur mit einem Parasiten infiziert worden sind, untersucht worden. Dies spiegelt jedoch nicht die Natur wider. Wenn Wirte mehrere Parasite beherbergen, können diese sich, abhängig von ihren jeweiligen Zielen, in einem Konflikt um Wirtsmanipulation befinden oder in Bezug auf Wirtsmanipulation kooperieren.

In dieser Dissertation möchte ich besser verstehen, wie Parasiten ihre Wirte unter komplexen – und daher natürlicheren – Bedingungen, wie zum Beispiel Mehrfachinfektionen – manipulieren. Dazu verwende ich zwei Parasiten mit komplexen Lebenszyklen, den Cestoden *Schistocephalus solidus* und seine zwei Zwischenwirte Copepoden und dreistachlige Stichlinge, und den Nematoden *Camallanus lacustris* und seinen ersten Zwischenwirt, Copepoden. Beide Parasiten verändern das Verhalten ihrer Wirte in einer Art, die wahrscheinlich ihre Fitness erhöht.

Ob Wirtsmanipulation durch *S. solidus* in dreistachligen Stichlingen aufgrund von echter Wirtsmanipulation, die zu diesem Zwecke evolviert ist, zu Stande kommt oder eine Begleiterscheinund aufgrund erhöhten Energieentzuges darstellt, ist der Gegenstand einer langanhaltenden Debatte. Im Falle echter Wirtsmanipulation sollten sich ein infektiöser und ein noch nicht infektiöser *S. solidus* in einem Konflikt um Wirtsmanipulation befinden; der infektiöse Parasit sollte das Predationsrisiko seines Wirtes steigern, aber für den noch nicht infektiösen wäre jegliche vorzeitige Predation fatal; er sollte niemals die Risikobereitschaft seines Wirtes erhöhen. Ich habe Stichlinge experimentell so infiziert, dass sie einen infektiösen und einen noch nicht infektiösen Parasiten beherbergten. Diese waren sogar noch risikofreudiger als die nur mit einem infektiösen Parasiten infizierten Stichlinge. Das ist nur mit Verhaltensänderungen, die durch erhöhten Energieentzug verursacht worden sind, und nicht mit echter Wirtsmanipulation erklärbar. Um diese Ergebnisse zu bestätigen, habe ich den Einfluss des Hungerzustandes auf die Risikobereitschaft infizierter Stichlinge getestet. Energieentzug sollte in satten Fischen wirkungslos sein, aber echte Wirtsmanipulation nicht. Wiederum sind meine Ergebnisse eher mit Energieentzug als mit echter Wirtsmanipulation vereinbar. Scheinbar adaptive Wirtsmanipulation wird nicht zwangsläufig durch Mechanismen verursacht, die zu diesem speziellen Zweck evolviert sind.

Darüber hinaus habe ich einen Konflikt zwischen einem infektiösen und einem noch nicht infektiösen Parasiten im Copepoden Wirt untersucht. Dazu habe ich Copepoden zu verschiedenen Zeitpunkten experimentell mit ein oder zwei *S. solidus* und/ oder *C. lacustris* infiziert. Noch nicht infektiöse *S. solidus* oder *C. lacustris* reduzieren die Predationsanfälligkeit ihres Wirtes, infektiöse erhöhen sie. Wenn ein Konflikt

zwischen verschiedenen Entwicklungsstadien derselben Art stattfindet, sabotiert der infektiöse Parasit die Wirtsmanipulation des noch nicht infektiösen in beiden Arten vollständig. In einem interspezifischen Konflikt schlug sich der infektiöse Parasite ebenfalls besser. *Camallanus lacustris* war dabei allerdings der staerkere Manipulateur. Ein Konflikt zwischen einem infektiösen *C. Lacustris* und einem noch nicht infektiösen *S. Solidus* resultierte in der vollständigugn unterdrückung durch den infektiösen Parasiten während ein solcher Konflikt zwischen einem infektiösen *S. Solidus* und einem noch nicht infektiösen *C. Lacustris* zu einem Kompromis bezüglich des Wirtverhaltens führte. Wenn Kooperationspotential besteht, d.h. mehrere *S. solidus* desselben Entwicklungsstadiums im selben Wirt auftreten, verstärken sie ihre Manipulation gegenseitig, aber erst nachdem sie infektiös geworden sind. Ich zeige also, dass ein Parasit die Wirtsmanipulation eines anderen Parasitens derselben oder einer anderen Art beeinflussen kann.

In der Natur sind Parasiten nicht nur anderen Parasiten ausgesetzt, die denselben Wirt infizieren, sondern auch Unterschieden in der Umwelt, in der ihr Wirt lebt. Solche Unterschiede, z.B. im Bezug auf Ressourcenverfügbarkeit könnten Wirtsmanipulation beeinflussen. Experimentell infizierte Copepoden haben verschiedene Nahrungstreatments erhalten. Ich habe den Erfolg ihrer Parasiten sowohl im ersten (Copepoden) als auch dem zweiten (Stichling) Zwischenwirt und das Verhalten des Copepoden Wirtes gemessen. Der Erfolg im Copepoden beeinflusste den Erfolg im Fisch. Unterschiede zwischen infizierten und nicht infizierten Copepoden (d.h. Wirtsmanipulation) waren größer in einer Umwelt mit viel Nahrung. Darüber hinaus schienen größere Parasiten weniger zu manipulieren. Diese Ergebnisse könnten durch Veränderungen im Zustand des Wirtes anstatt durch tatsächliche Unterschiede in der Wirtsmanipulation herbeigeführt worden sein. Nichtsdestotrotz könnten diese zu ökologischen Konsequenzen führen, falls daraus Unterschiede in der Effizienz der Wirtsmanipulation resultieren.

Introduction

The introduction has partly been altered from: Hafer, N., and M. Milinski (submitted). Cooperation or Conflict: Host manipulation in multiple infections in H. Mehlhorn, eds. Parasites and Behavioural Changes. Parasitology Research Monographs. Springer, Heidelberg.

Host manipulation

Complex life cycle parasites are faced with having to pass from one host to the next. Often this goes against the host's interests. For example in trophically transmitted parasites this requires the parasite's current host to be consumed by its next host. Naturally, any host should avoid predation. To overcome this hurdle, parasites can change the behavior of their host to fit their own needs and make them for example more prone to predation. The host may no longer be in full control of its own behavior (Lefèvre et al. 2009a; Milinski 2014). More generally, such host manipulation occurs if a parasite changes the behavior or other aspects of the host's phenotype in a manner that enhances its own fitness beyond the benefits it gains from normal exploitation. It usually serves to enhance parasite transmission, dispersal or survival. It occurs in a wide variety of host and parasite taxa including most mayor linages and has evolved several times independently (reviewed by e.g. Holmes and Bethel 1972; Poulin 1994a, 2010; Poulin and Thomas 1999; Moore 2002, 2013). The forms it can take are very divers and by no means restricted to complex life cycle parasites. Vector born animal parasites such as malaria (e.g. Koella et al. 2002; Koella 2005; Lacroix et al. 2005; Cator et al. 2012) and different vector born plant parasites (e.g. Maitland 1994; Fereres and Moreno 2009; Mauck et al. 2012) enhance transmission between hosts; They increase encounter rates between their vectors and their hosts and alter the attractiveness of their hosts. Parasites that require a different habitat for reproduction and dispersal than normally used by their host induce their host to seek out such a habitat (e.g. Thomas et al. 2002b; Andersen et al. 2009; Hughes et al. 2011), even if this is fatal to the host (e.g. Thomas et al. 2002). Parasitoids cause their hosts to guard them after emergence to avoid predation (e.g. Grosman et al. 2008; Maure et al. 2011; Dheilly et al. 2015). Such a host may eventually recover, albeit it does suffer a reduction in fitness (Maure et al. 2014). Prior to reaching infectivity to a subsequent host, complex life cycle parasites can increase host behaviors likely to reduces predation to avoid fatal premature predation (Hammerschmidt et al. 2009; Parker et al. 2009c; Thomas et al. 2010; Dianne et al. 2011). Thereafter, they often increase behaviors likely to enhance predation susceptibility of their host to facilitate transmission (reviewed by e.g. Holmes and Bethel 1972; Poulin 1994a, 2010; Poulin and Thomas 1999; Moore 2002, 2013).

Humans too, can be affected by host manipulation. *Toxoplasma gondii* for example causes mice to lose all fear of its definite host, cats. It can also infect humans and lead to changes in their behavior and personality up to severe psychological disorders such as schizophrenia (reviewed e.g. by Flegr et al. 1996; Lafferty 2006;

Alvarado-Esquivel et al. 2011; Flegr 2013). Host manipulation might also have important consequences for the ecosystem (Thomas et al. 1998b, 1999, 2005; Lefèvre et al. 2009b; Lafferty and Kuris 2012). It is, for example, likely to affect the energy flow in food webs. It will strengthen or even create certain links between prey and predators if a parasite alters the predation susceptibility of its hosts (Lefèvre et al. 2009b; Lafferty and Kuris 2012).

Distinguishing between actual host manipulation, host compensatory responses, and side-effects can be challenging (Milinski 1990; Poulin 1995; Poulin and Thomas 1999; Thomas et al. 2005; Lefèvre et al. 2009a; Cézilly et al. 2010; Moore 2013, Figure 1). For example, animals are usually faced with a trade-off between consuming energy and avoiding predation. Parasites impose an additional energy drain on their host shifting this trade-off further away from predation avoidance. This can resemble predation enhancement often attributed to parasites increasing their chances to be trophically transmitted to a subsequent host (Milinski 1990). In order to be considered adaptive, host manipulation needs to convey fitness benefits to the parasite, e.g. via increased transmission (Poulin 1995, 2010). Unfortunately, even the existence of such fitness benefits cannot conclusively answer whether any host manipulation has really evolved for this purpose since host compensatory responses or side-effects can provide "coincidental" or "fortuitous benefits" to the parasite (Figure 1) on which selection will act just as well as on host manipulation that has originally evolved for this purpose (Thomas et al. 2005; Poulin 2010; Dubois et al. 2013; Moore 2013).

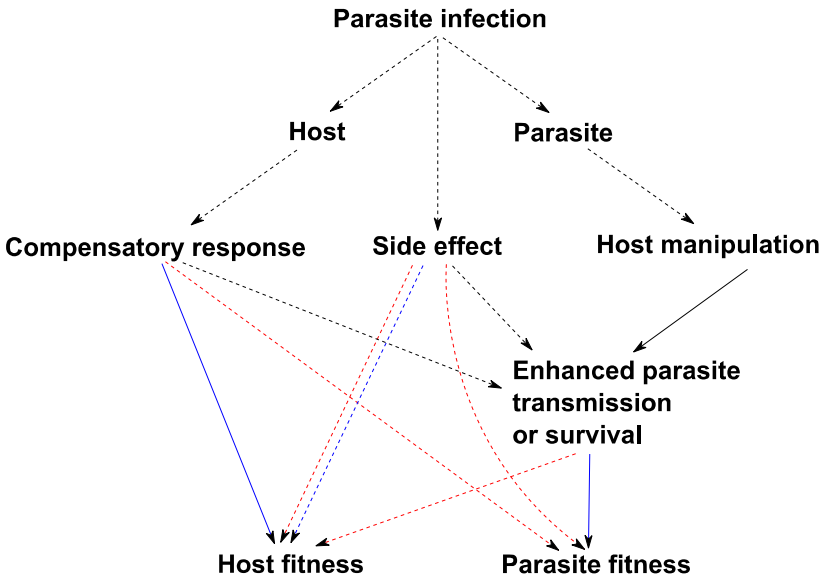

Figure 1: Potential effects of parasite infection on host behavior. Distinctions between different effects can sometimes be blurred since different effects can lead to similar outcomes. Selection will act on any behavioral alteration that changes host and/or parasite fitness irrespectively of its origin. Blue lines indicate positive fitness effects; red lines indicate negative fitness effects; dashed lines indicate optional consequences.

Understanding the mechanisms underlying host manipulation may help to distinguish between true host manipulation, side-effects and host compensatory responses. Unfortunately, we are only starting to do so. Besides host manipulation caused by parasites exploiting compensatory responses or side-effects (see above), host manipulation can occur through encystment at a certain site, hitchhiking the immune system or neuromodulation (reviewed by Adamo 2002, 2012; Lafferty and Shaw 2013; Perrot-Minnot and Cézilly 2013).

A number of studies have for example shown enhanced levels of neuroactive substances in infected animals (e.g. Øverli et al. 2001; Tain et al. 2006; Shaw et al. 2009; Helluy 2013). In gammarids, injections of the neurotransmitter serotonin can mimic the behavior of gammarids infected with an acanthocephalan parasite (Helluy and Holmes 1990; Tain et al. 2006; Helluy 2013; Perrot-Minnot et al. 2014). More insights into how parasites manipulate their hosts are starting to emerge as transcriptomics and proteomics are becoming increasingly available. They can serve to identify genes and molecules potentially involved in host manipulation (reviewed by Biron et al. 2005a; Thomas et al. 2005; Lefèvre et al. 2009a; Hughes 2013; van Houte et al. 2013), whose role can then be confirmed experimentally for instance by knocking-out these candidate genes (Hoover et al. 2011).

Host manipulation often comes at a cost. Such costs emerge from two different avenues; enhanced parasite mortality for example via dead-end predation and energetic costs. Predation enhancement by complex life cycle parasites does not always seem to specifically target the correct subsequent host. Rather, it unspecifically increases the host's predation susceptibility to any predator, including dead-end predators (e.g. Milinski 1985; Mouritsen and Poulin 2003; Seppälä et al. 2008; Cézilly et al. 2010; Thomas et al. 2010; Dianne et al. 2014). This should nevertheless be adaptive as long as predation by the correct host is improved over proportionally compared to all other causes of mortality, i.e. the benefits of host manipulation outweigh its costs (Cézilly and Perrot-Minnot 2005; Poulin 2010). Direct evidence for energetic costs has remained elusive to date. Nevertheless, such costs are usually assumed to be associated with the process of host manipulation itself (e.g. Poulin 1994b; Biron et al. 2005b; Thomas et al. 2005, 2011). For example, if a parasite manipulates by excreting a neuroactive substance, this could impose such energetic costs on the parasite. In two different systems, Franceschi et al. (2010) and Maure et al. (2011) found that the strength of host manipulation was negatively associated with other fitness related traits. This could indicate a trade-off due to potential energetic costs of host manipulation. Such trade-offs could enhance variation in host manipulation (Thomas et al. 2011, 2012).

Host manipulation can vary within the same host and parasite species. Such differences can be due to genetic differences within both hosts (e.g. Cox and Holland 2001; Franceschi et al. 2010b) and parasites (e.g. Franceschi et al. 2010a; Leung et al. 2010; Khudr et al. 2013). Non-genetic factors such as the hunger status of the host (e.g. Giles, 1987; Barber *et al.*, 1995; Jakobsen and Wedekind, 1998) and parasite age (e.g. Koella et al. 2002; Benesh et al. 2009a; Hammerschmidt et al. 2009; Dianne et al. 2011) and size (e.g. Seppälä et al. 2005; Benesh et al. 2009b; Dianne et al. 2012) also play a role. In addition, parasites seem to adapt their host manipulation to the abiotic environment their host experiences. Host manipulation can differ over the course of the day (McCurdy et al. 1999) and between different seasons (Brodeur and McNeil 1989; Benesh et al. 2009a). Abiotic factors that do influence host manipulation have received little attention to date but could include resource availability (Thomas et al. 2011, 2012), pollution (Thomas et al. 2011), light conditions and temperature (Benesh et al. 2009a). The influence of the biotic environment on host manipulation has received somewhat more attention. Host manipulation can for example alter in response to the presence of different predators (e.g. Jakobsen and Wedekind 1998; Durieux et al. 2012; Dianne et al. 2014) or other co-infecting

parasites (reviewed by Rigaud and Haine 2005; Koella et al. 2006; Thomas et al. 2010, 2011, 2012; Mauck et al. 2012; Syller 2012; Cézilly et al. 2014) and potentially also commensals and mutualists (Cézilly et al. 2014).

Multiple infections and host manipulation

Most studies of host manipulation (using experimentally infected hosts) to date have focused on infections by single parasites. This does not reflect nature where nearly every host is infected by multiple parasites (e.g. Petney and Andrews 1998; Kalbe et al. 2002). There is increasing evidence that even symbionts or commensals may influence their host's behavior (Feldhaar 2011; Ezenwa et al. 2012). Their interaction with co-infecting macroparasites remains largely unexplored (reviewed by Cézilly et al. 2014). Given the ubiquity of parasites, symbionts and commensals, hosts may no longer be in full control of their own behavior but rather might display a compromise of all parties' interests (Milinski 2014). So, how do parasites deal with co-infecting parasites with the same or different aims? From an ecological perspective, an already infected potentially manipulated host may no longer be the same environment as an uninfected non-manipulated host. Parasites could hence act as ecosystem engineers, modifying the host environment for all co-infecting parasites (Thomas et al. 1998b; Poulin and Thomas 1999; Lefèvre et al. 2009).

Depending on the aims of each of the parasites involved, co-infections might lead to cooperation or conflict over host manipulation (Figure 2). This could affect infection patterns if parasites seek out hosts co-infected by suitable manipulators and avoid co-infections by unsuitable ones. Once two parasites enter the same host with at least one of them able to manipulate host behavior, cooperation or conflict might ensue. Cooperation can occur between parasites with the same interest. However, agreeing interests could also be exploited by free-riders saving the cost of manipulation. Conflict can exist between parasites with different aims. This includes two parasites of different species with different definitive hosts, different developmental stages of the same or different parasite species or two different parasite species with different transmission strategies (e.g. vertically transmitted parasites and parasites that require trophic transmission). Any such conflict might result in one parasite sabotaging the other parasite's manipulation.

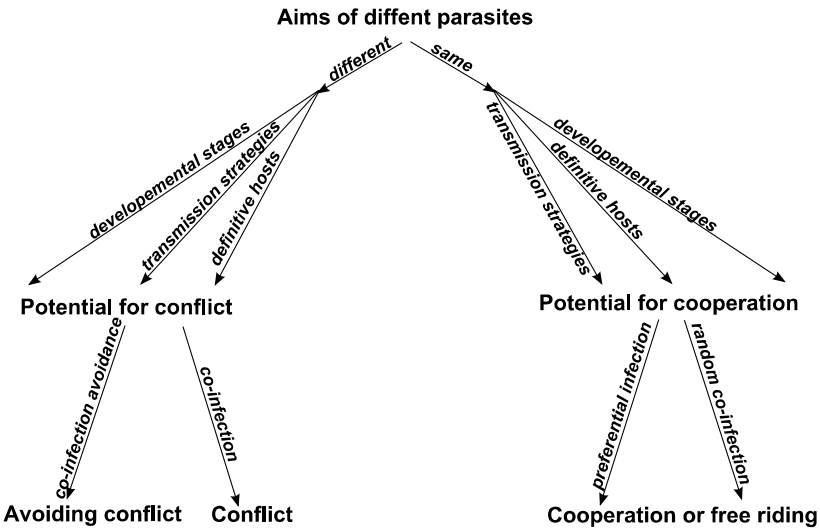

Figure 2: Overview of potential consequences of multiple infections with regards to host manipulation

Cooperation – when parasites agree

Facilitating cooperation: Hitch-Hiking

If host manipulation is beneficial but costly and baseline transmission rates are low, then parasites should seek out opportunities to co-infect hosts already infected by other parasites with appropriate host manipulation (Thomas et al. 1998b). This should result in non-random associations between parasites (Cézilly et al. 2014). Preferential infection of suitably manipulated hosts, termed hitch-hiking, can be considered an adaptation if it is genetically determined and has become prevalent by conferring a selective advantage (Thomas et al. 1998b). In order to show that hitch-hiking occurred, preferential infections of already infected hosts need to be shown to increase fitness through enhanced transmission, ideally under experimental conditions (Thomas et al. 1998b). To my knowledge, this has never been done. Indeed, experimental studies testing whether parasites associate with each other according to their manipulation are missing entirely. Studies on naturally infected hosts suggest that associations between parasites with the same consecutive hosts do occur (Thomas et al. 1997; Dezfuli et al. 2000; Poulin et al. 2003; Leung and Poulin 2007; Wisenden et al. 2012), but not always (Thomas et al. 1998a; Poulin et al. 2003; Lagrue and Poulin 2008; Leung and Poulin 2010; Rauque et al. 2011). Hitch-hiking is not the only possible explanation for associations between parasites with parallel life cycles. The concurring life cycles themselves could cause associations between parasites. Parasites accumulate in their definite host. They (or their eggs) leaving it via defecation are likely to do so together with parasites using the same definite host. Hence, such parasites are more likely to develop in the same area making it more likely that they will eventually infect the same host (Poulin and Valtonen 2001). Since associations have only been studied using naturally infected hosts, we cannot conclude anything about how such associations may come about.

Cooperation

Once two manipulating parasites with the same aim with regards to host manipulation have co-infected the same host, they have a high potential to cooperate. The simplest case of cooperation involves multiple parasites of the same species at the same developmental stage. A number of theoretical models have predicted what should happen in this case. They all assume that host manipulation bears both costs and benefits (Poulin 1994b; Brown 1999; Vickery and Poulin 2009). Benefits, i.e. altered predation risk, have been shown in a few systems (Wedekind and Milinski 1996; Bakker et al. 1997; Mouritsen and Poulin 2003; Dianne et al. 2011; Weinreich et al. 2013). Evidence for costs, especially energetic costs, has remained largely elusive (see above).

If costly, host manipulation should not be maximized but optimized (Poulin 1994b). Accordingly, parasites should adjust the extent of manipulation to the (expected) presence of other parasites. Thus, individual investment into host manipulation should decrease as within host parasite population size increases (Poulin 1994; Brown 1999; Vickery and Poulin 2009), though exceptions may occur if both benefits and relatedness between parasites are very high. In this case the already very high benefits are received by close kin likely to share the genes responsible for this manipulation with the manipulator (Vickery and Poulin 2009). In either case, total manipulation should still increase with increasing parasite number (Poulin 1994; Brown 1999; Vickery and Poulin 2009). Individual investment into host manipulation has never been measured and doing so might proof challenging. By contrast, total host manipulation has often been recorded as the total extent of (behavioral) change induced by parasites. In naturally infected hosts, total host manipulation often correlates with parasite number for at least some traits (Giles 1987; LoBue and Bell 1993; Brown et al. 2001; Latham and Poulin 2002; Mouritsen 2002; Addino et al. 2010; Seppälä et al. 2011; Fredensborg and Longoria 2012; Rode et al. 2013; Corrêa et al. 2014b). This seems to hold in both mass exposed (Urdal et al. 1995; Seppälä et al. 2005; Luong et al. 2011) and individually infected (Cox and Holland 1998, 2007; Franceschi et al. 2008; Benesh et al. 2009; Santos et al. 2011; Dianne et al. 2012; Santos and Santos 2013) hosts. Some effect of parasite number on the strength of host manipulation, though it does not always seem to be linear, seems particularly consistent in parasites that usually infect their hosts in high numbers (Cox and Holland 1998, 2007; Latham and Poulin 2002; Mouritsen 2002; Seppälä et al. 2005, 2011; Addino et al. 2010; Luong et al. 2011; Santos et al. 2011; Fredensborg and Longoria 2012; Santos and Santos 2013; Corrêa et al. 2014b). Parasite number should also correlate with manipulation strength, if host manipulation results from the impairment of a certain organ (Thomas et al. 2011). Brown (1999) predicted that host manipulation should only occur once a certain threshold in parasite population size within a host was reached. Such a threshold might be automatic if host manipulation depends on the impairment of a certain organ, which cannot be accomplished by a single parasite. The trematode *Diplostomum* sp. encysts in the eyes of fish obscuring their vision and thereby making them more vulnerable to predation especially at higher parasite numbers within one host (Seppälä et al. 2005). This, however, only takes effect when a considerable number of parasites have encysted inside the eye (Seppälä et al. 2011).

In parasite species in which the number of individual parasites per host is usually very low and single infections may be the rule, evidence for an effect of parasite number on host manipulation is less clear. Some studies did find it (Brown et al., 2001; Dianne et al., 2012; Franceschi et al., 2008; Giles, 1987; LoBue & Bell, 1993; Rode et al., 2013; Urdal et al., 1995), but others did not, including both studies using naturally infected (Cézilly et al. 2000; Latham and Poulin 2002) and experimentally exposed (Poulin et al. 1992; Benesh et al. 2005; Franceschi et al. 2010; Dianne et al. 2014) hosts. In cestodes energy drain might be a frequent mechanism resulting in host manipulation (Lafferty and Shaw 2013). Interestingly, most of the studies that found a correlation between parasite number and manipulation in systems with usually low parasite numbers per host used cestodes (Brown et al., 2001; Giles, 1987; LoBue & Bell, 1993; Rode et al., 2013; Urdal et al., 1995). Energy drain should increase as parasite size and number increases. This might result in similar patterns (correlation between parasite number and host manipulation) as predicted by theoretical models (Poulin 1994b; Brown 1999; Vickery and Poulin 2009) without fulfilling one of their assumptions, i.e. energetic costs associated with host manipulation: energy drain comes at no extra cost.

A correlation between parasite number and host manipulation might also be caused by more parasites having a larger combined volume. Dianne et al. (2012) found that experimentally infected gammarids harboring a single acanthocephalan parasite were more strongly manipulated if that parasite was larger. In parasites that can vary strongly in their mass, increased individual mass might have the same effect as increased parasite number. Only a limited number of studies has taken this possibility into consideration and found that parasite size seemed to be responsible for stronger manipulation (Seppälä et al. 2005; Benesh et al. 2009).

Benefits of manipulation will change with size and composition of the parasite community within a host. Increased parasite number also leads to increased competition. This can decrease the potential gains of successful manipulation and transmission to the subsequent host (Poulin 1994b; Vickery and Poulin 2009). Hence, in some cases, individual decrease in host manipulation might be so strong that even overall host manipulation decreases as parasite number increases (Vickery and Poulin 2009). This represents a bystander dilemma as studied in humans. If an emergency occurs, an increased number of bystanders do not increase the chances that at least one of them will aid the victim. Instead, the victim is less likely to receive any help at all, because from the perspective of any one bystander there are others that could provide the required help (reviewed by Fischer & Krueger, 2011; Latané & Nida, 1981). In gammarids, experimental double infection with an acanthocephalan parasite leads to increased phototaxis compared to single infections. Nevertheless, in infections with more than two parasites this host manipulation does not seem to increase any further (Franceschi et al. 2008). In mice infected with the nematode *Toxocara canis*, exploratory behavior is increased if parasite burdens are low but not if they are high compared to control mice (Cox and Holland 1998). Such an effect could also be caused by reduced ability of parasites to manipulate due to resource limitations or damages imposed on the host (Rigaud and Haine 2005; Franceschi et al. 2008).

Cooperation is not necessarily limited to individuals of the same species but could also occur between members of different species if they have the same aim. This hypothesis has received much less attention.

Observational field evidence suggests that such co-infections can also facilitate cooperation (Lafferty and Morris 1996; Poulin et al. 2003; Santiago Bass and Weis 2009; Rode et al. 2013). For example, *Artemia* naturally infected with two microsporidian parasites are found more likely to be swarming, which is thought to enhance transmission via spores to other *Artemia*, than those infected only with one (Rode et al. 2013).

Free riding

If multiple parasites with agreeing aims manipulate a host, host manipulation becomes a public good: An individual parasite that manipulates bears the costs of manipulation but all co-infecting parasites will benefit equally (Brown 1999; Vickery and Poulin 2009). As discussed above, parasites could increase their chances of encountering a suitably infected host by preferentially infecting already infected hosts. To my knowledge, the existence of free riding, i.e. benefits of increased transmission through co-infecting without contributing to manipulation, has not been investigated explicitly in complex life cycle parasites. Salvaudon et al. (2013) identified a potential case in vector transmitted plant viruses. Two viruses, the zucchini yellow mosaic virus and the watermelon mosaic virus infect cucurbits and are transmitted by aphids. The former virus modifies aphid-plant interaction in a way that enhances its transmission; the latter does not but probably benefits from these modifications. The existence of free-riding raises the question of how host manipulation can be maintained if it is potentially exploited by other parasites (Brown 1999). Two trematode parasites, *Acanthoparyphium* sp. and *Curtuteria australis* infect cockles and require birds to prey upon them and serve as the parasites' definite host. They can enhance the likelihood of bird predation by encysting at the tip of the cockle's foot and thereby impairing its burrowing ability. However, encysting there comes at a cost: It also enhances dead end predation by fish that chew off the tip of the foot (Mouritsen and Poulin 2003). Both trematodes can also encyst in other body parts where they do not pay the cost from potential dead end predation, but cannot manipulate. *Acanthoparyphium* sp. only rarely encysts at the tip of the foot and would hence benefit from associating with *C. australis* which encysts at the tip of the foot much more often (Leung and Poulin 2010). Leung and Poulin (2010), however, found the opposite result when they exposed field collected cockles to both parasites in the lab: The stronger manipulator, *C. australis* is also the stronger competitor. It sometimes excludes *Acanthoparyphium* sp. from co-infections.

Conflict – when parasites disagree

Avoiding conflict

Akin to seeking out suitably manipulated hosts, parasites would benefit from avoiding unsuitably manipulated ones. This not only applies to complex life cycle parasites with different subsequent hosts but also to parasites that benefit most from a non-manipulated host, e.g. because they need to remain in it or rely on vertical transmission. There are two ways parasites can avoid co-infections. Already present parasites could protect their host from subsequent infection by unsuitably manipulating parasites. There is some evidence that vertically transmitted parasites or symbionts offer protection against parthenogenetic parasites (reviewed by

Haine 2008; Brownlie and Johnson 2009; Feldhaar 2011). Such parasites would be fatal for the host's reproduction and fitness. Vertically transmitted parasites directly depend on their host's reproduction for transmission. Hence, any reduction in host reproduction would also result in reduced parasite fitness (reviewed by Haine 2008; Brownlie and Johnson 2009; Feldhaar 2011). Accordingly, Haine et al. (2005) tested whether a vertically transmitted parasite would succeed in protecting its gammarid host from a manipulating trematode by recording the prevalence of single and co-infections in naturally infected hosts. They found no evidence that the vertically transmitted parasite prevented co-infections by the trematode.

If a host is already infected by an unsuitably manipulating parasite, a second parasite could avoid infecting this host. Distinguishing such avoidance from avoidance that evolved to eliminate resource competition could however proof difficult (Fauchier and Thomas 2001). Studies looking at negative associations between parasites with mutually exclusive life cycles have, to my knowledge, been limited to amphipod hosts (Dezfuli et al. 2000; Fauchier and Thomas 2001; Outreman et al. 2002; Haine 2008; Lagrue and Poulin 2008; Rauque et al. 2011). With one exception (Fauchier and Thomas 2001), they failed to find any negative association. Instead of protection or avoidance, parasites might respond to co-infections by a parasite with unsuitable manipulation by sabotaging that manipulation (Dianne et al. 2010; Haine et al. 2005).

Conflict

Conflict between parasites with different definitive hosts

Two parasites with mutually exclusive definitive hosts should be at a conflict over host manipulation. Cézilly et al. (2000) used field infected gammarids to investigate this conflict between two acanthocephalan parasites, the fish infecting *Pomphorhynchus laevis* and the bird infecting *Polymorphus minutus*. *Pomphorhynchus laevis* strongly changed the gammarids' vertical distribution in the water column while *P. minutus* infected individuals became more photophilic. Double infections seemed to result in some compromise: The host's vertical distribution was intermediate compared to singly infected hosts while their photophilia was stronger than that of only *P. minutus* infected hosts but not significantly different from that of hosts infected only by *P. laevis*. In another study using naturally infected amphipods harboring a manipulating acanthocephalan fish parasite co-infection with a non-manipulating bird infecting trematode had no significant effect on host manipulation (Rauque et al. 2011). However, since these studies used naturally infected hosts their results should be interpreted with caution. *Toxoplasma gondii* and the trematode *Toxocara canis* both change the behavior of their current rodent host to increase its susceptibility to their successive hosts, cats and dogs, respectively. Queiroz et al. (2013) and Corrêa et al. (2014a) studied their interactive effect on rats and mice using experimental infections. Since both parasites have different definitive hosts, one might expect a conflict even though this was not the focus of either study. However, even the effects of single infections with either parasite did not significantly differ from each other (Queiroz et al. 2013; Corrêa et al. 2014a) and thus the question of how such a conflict would be resolved becomes obsolete.

A conflict over which of two different definitive hosts has to be reached will only occur if manipulation is sufficiently specific (Cézilly et al. 2014). Not all mutually exclusive life cycles might also lead to mutually exclusive manipulation. Predation enhancement seems often very unspecific. For example, three-spined sticklebacks infected by the cestode *Schistocephalus solidus* not only show an reduced fear response when presented with a simulated bird predator which presents a suitable definitive hosts to *S. solidus* (e.g. Giles 1983; Barber et al. 2004), but also when exposed to piscivorous predatory fish which would be dead-end predators (e.g. Milinski 1985). Other means, e.g. spines in sticklebacks might help reduce dead-end predation (Hoogland et al. 1956). Nevertheless, such a general increase in predation susceptibility is not likely to result in any conflict over host manipulation even between parasites with very different definitive hosts. Such parasites might even benefit from each other's manipulation. Hence, in order to investigate conflict over host manipulation, significant differences between manipulation effects by different parasites should be shown in singly infected hosts as a prerequisite. Only Cézilly et al. (2000) managed to do so, though, compared to uninfected hosts, both parasites manipulated into the same direction albeit to varying extends. To proof potential for a conflict it should ideally be shown that one behavioral alteration benefits one parasite but is costly (through a relative increase in dead-end predation) to the other. This has never been done.

Conflict between parasites with different transmission strategies

A conflict will also occur between parasites if one parasite has to remain within the host and the other has to pass to a subsequent host. Such a conflict is inevitable when a vertically transmitted parasite, or indeed any vertically transmitted organism, and a manipulating parasite co-infect the same host. There is probably no host without any vertically transmitted parasite, symbiont or commensal. Accordingly, a conflict due to different transmission strategies should be universal whenever a manipulating parasite infects a host. Nevertheless, this conflict has rarely been studied. In naturally infected gammarids co-infection by a vertically transmitted microsporidian parasite reduced manipulation by a bird infecting acanthocephalan parasite (Haine et al. 2005). In another study with naturally infected gammarids, Thomas et al. (2002) observed that hosts harboring a manipulating bird trematode were less manipulated when they also harbored a non-manipulating nematode which has to remain inside the gammarid. However, when they experimentally cured the host from and re-infected it with nematodes, they were unable to reverse or reintroduce this effect. This illustrates the urgent need for studies that use experimentally infected hosts.

Conflict between different developmental stages

A conflict between two different developmental stages of the same parasite species will occur if parasites need to spend a certain time within their intermediate host before they become infective to their next host and manipulate accordingly. In this case, parasites will switch their manipulation upon reaching infectivity to the subsequent host. This has been predicted theoretically (Parker et al. 2009c) and shown experimentally (Koella et al. 2002; Hammerschmidt et al. 2009; Dianne et al. 2011). Complex life cycle parasites should suppress predation of their intermediate host prior to such a switch and enhance it thereafter (Hammerschmidt et al.

2009; Parker et al. 2009c; Dianne et al. 2011). Studying this conflict has been restricted to a conflict between parasites of the same species. In isopods, an acanthocephalan parasite induces color changes thought to enhance predation. However, only infective parasites will benefit from predation. Accordingly, this color change is stronger in hosts with infective parasites but also occurs, albeit to a lesser extent, in hosts with not yet-infective ones. Unsurprisingly, hosts naturally infected by both stages seem to resemble those with infective parasites (Sparkes et al. 2004). Dianne et al. (2010) investigated the same conflict by using wild caught gammarids experimentally infected at two different time points and measuring their reaction to light. Again, the infective parasite seemed to win the conflict, but there was some indication of sabotage by the not yet-infective one. More studies will be needed to determine whether the parasite that comes first and becomes infective first has an advantage. And if so, is this something that is typical of intraspecific conflict between different developmental stages or would it also occur in a similar interspecific conflict?

Study system

Schistocephalus solidus and its effect on host behavior

The cestode *Schistocephalus solidus* is a complex life cycle parasite that has to pass through three different hosts in order to reproduce (Figure 3 A). Initially, *S. solidus* hatches from an egg into a coracidium which then has to be taken up by a cyclopoid copepod (Clarke 1954; Dubinina 1980). In the laboratory, *Macrocyclops albidus* is often used as an experimental host (Figure 3 C), since it can be cultured easily (Orr and Hopkins 1969; van der Veen et al. 2002). Infected copepods have to be eaten by three-spined sticklebacks (*Gasterosteus aculeatus*, Figure 3 E) for *S. solidus* to be passed on to the next host (Clarke 1954; Dubinina 1980). The interaction between *S. solidus* and both copepods (e.g. Wedekind 1997; Hammerschmidt and Kurtz 2005a,b; Benesh 2010b, 2011) and sticklebacks (Barber and Wright 2008; Barber and Scharsack 2010; Barber 2013) is the focus of ongoing research. The three-spined stickleback itself has been used as a model species in behavioral biology for some decades (reviewed by Huntingford & Ruiz-Gomez 2009) and hence its biology is very well known. Final reproduction of *S. solidus* takes place in a bird by which the stickleback has to be eaten (Clarke 1954; Dubinina 1980). In the laboratory the bird can easily be replaced by an in vitro culture allowing the whole cycle to be maintained in the laboratory (Smyth 1946, 1954).

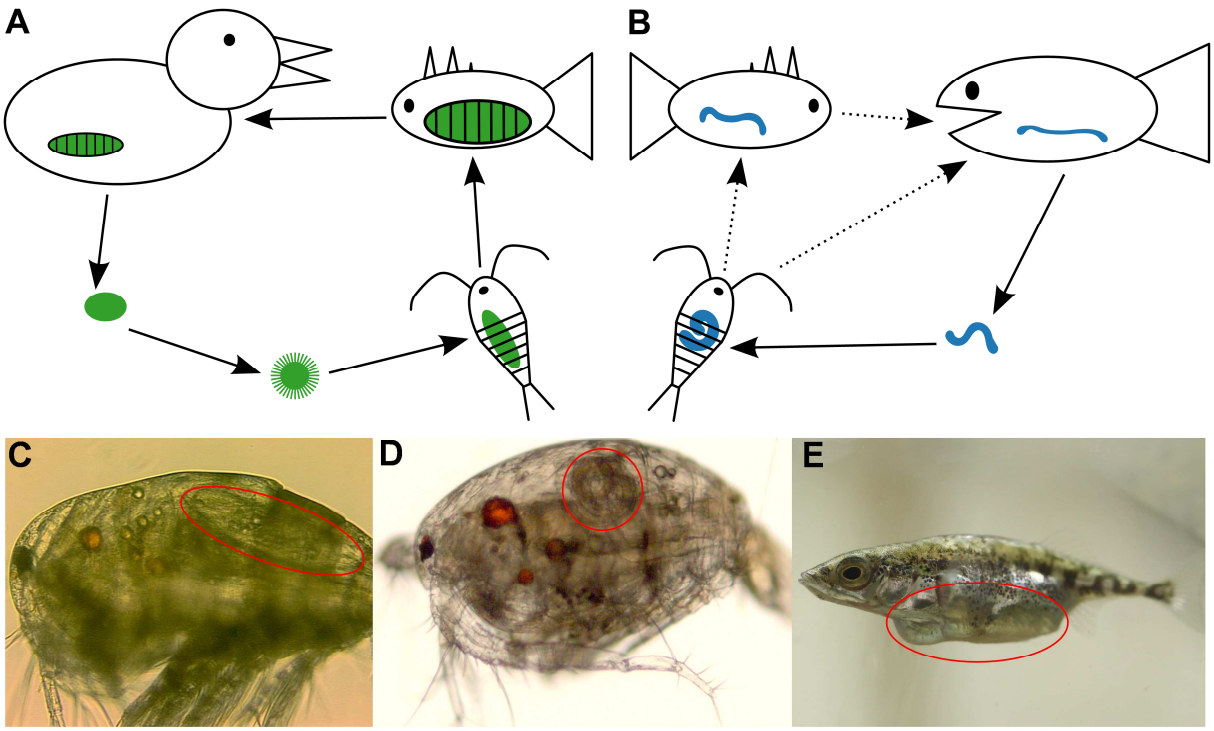

Figure 3: Life cycle of *Schistocephalus solidus* (A) and *Camallanus lacustris* (B); *Macrocyclops albidus* infected by *S. solidus* (C) and *C. lacustris* (D) and three-spined stickleback infected by *S. solidus* (E). Life cycle of *S. solidus* according to Clarke (1954) and Dubinina (1980). Lifecycle of *C. lacustris* simplified after Moravec (1994). Dashed arrows indicate alternative routes. In panel C-E, parasites are encircled in red.

Host manipulation by *S. solidus* has been the subject of ongoing study and occurs in both its intermediate hosts. In the copepod, *S. solidus* initially engages in predation suppression (see Parker et al. 2009c). This behavioral alteration sets in within 4 days post infection and consists of decreased activity and an increased recovery period after a simulated predator attack (Hammerschmidt et al. 2009). It coincides with reduced predation susceptibility (Weinreich et al. 2013). Once *S. solidus* has reached infectivity, the decrease in activity that occurs during predation suppression is reversed: Infected copepods are more active (Urdal et al. 1995; Wedekind and Milinski 1996; Hammerschmidt et al. 2009) and recover faster from a simulated predation attack (Hammerschmidt et al. 2009) than uninfected ones and do not seek a dilution effect in the presence of stickleback odor but rather avoid it in favor of the absence of competition from conspecifics (Jakobsen and Wedekind 1998). They are also more likely to be consumed by three-spined-sticklebacks (Wedekind and Milinski 1996). The extent of this predation enhancement (see Parker et al. 2009c), however, seems to differ between populations (Benesh 2010b).

Schistocephalus solidus also alters the behavior of its second intermediate host. Naturally infected sticklebacks have been found to react less strongly to a simulated predation attack by correct consecutive hosts (Giles 1983, 1987b; Godin and Sproul 1988; Tierney et al. 1993) and the presence of dead-end predators (Milinski 1985), through this effect can disappear when fish are fed to satiation (Giles 1987b). Infected fish also show an impaired escape behavior (Blake et al. 2006) and react sooner to oxygen deprivation (Giles 1987a) and are found closer to the shore (Lester 1971) and higher up in the water (LoBue and Bell 1993). They shoal less (Barber and Huntingford 1995; Barber et al. 1995) and choose less profitable prey assumedly

because their competiveness is reduced (Milinski 1984, 1986). These behavioral changes seem to correlate with parasite mass (Giles 1983, 1987a; Godin and Sproul 1988). A reduced reaction to a simulated bird predator has also been demonstrated in experimentally infected fish and seems to occur only after parasites have arguably reached infectivity (Barber et al. 2004). Before, no significant behavioral changes have been found in experimentally infected fish (Aeschlimann et al. 2000; Barber et al. 2004).

Nothing definite is known about the mechanisms *S. solidus* employs to manipulate either of its intermediate hosts, but some effort has been made to investigate this question in sticklebacks. In laboratory infected sticklebacks, a burst in the immune system occurs at the same time when the parasite reaches infectivity (Scharsack et al. 2007). Various reviews have suggested that parasites could exploit preexisting links between the immune system and the neuronal system of their host to manipulate host behavior (Thomas et al. 2005; Poulin 2010; Adamo 2012; Lafferty and Shaw 2013). In wild caught fish, an infection with *S. solidus* is associated with altered monoamine levels in the brain (Øverli et al. 2001). Due to the correlational nature of this study it can, however, not proof any causality. Altered monoamine levels could for example also be caused by chronic stress likely to be experienced by fish infected with a parasite like *S. solidus* (Øverli et al. 2001). The lack of any conclusive and unambiguous evidence has fueled an ongoing debate on whether or not altered host behavior in *S. solidus* infected sticklebacks is due to true host manipulation or increased energy drain (Milinski 1990; Barber and Huntingford 1995; Barber et al. 2000; Barber and Wright 2008; Barber and Scharsack 2010). Side-effects could for example be caused by increased energetic needs and a change in the trade-off between feeding and predator avoidance when it comes to increased risk taking (Giles 1983, 1987b; Milinski 1984, 1990) or increased oxygen consumption causing infected fish to stay closer to the surface (Lester 1971; Giles 1987a; LoBue and Bell 1993).

Camallanus lacustris

The nematode *Camallanus lacustris*, like *S. solidus*, uses cyclopoid copepods such as *Macrocyclops albidus* as its first intermediate host (Figure 3 D). Perch are needed as definitive hosts in whose blind sacks the parasite matures. Life larvae are released into the environment and consumed by copepods. Small fish, such as three-spined sticklebacks can serve as paratenic hosts when they eat infected copepods. The life cycle is then continued when those fish are ingested by perch (Moravec 1969, 1994, Figure 3 B). Similarly to *S. solidus*, *C. lacustris* has to spend a certain amount of time inside its intermediate host before becoming infective. In the case of *C. lacustris* this takes place after it has undergone two moldings inside the copepod, the second of which takes place 10 to 11 days post infection at room temperature (Moravec 1969). Behavioral changes themselves have never been investigated in *C. lacustris* directly, however, not yet infective larvae reduce the predation susceptibility of copepods to similar levels as not yet infective *S. solidus* (Weinreich et al. 2013).

Thesis outline

The main focus of my PhD is to understand host manipulation under more complex – and hence more natural – conditions using controlled laboratory experiments. In this context I mainly focus on three different aspects:

1) Has what appears to be adaptive host manipulation by *S. solidus* in three-spined sticklebacks evolved for this specific purpose or can it also be caused by side-effects (Chapter II)?
2) What effect do multiple infections have on host manipulation (Chapter III, IV)? Is there cooperation (Chapter III)? How are intra- and interspecific conflicts over host manipulation resolved (Chapter III, IV)?
3) Does the abiotic environment, e.g. resource availability, affect parasite performance (Chapter V, VI) and more particularly host manipulation (Chapter II, VI)?

This thesis is divided into six different chapters each of which represents a different independent study. These chapters are outlined below. Each of these projects was conducted in cooperation with other scientists. Their and my contributions varied between chapters and are outlined at the end of this section.

Chapter I: Effects of VIE tagging and partial tissue sampling on the immune response of three-spined stickleback *Gasterosteus aculeatus*

To investigate the behavior of animals it is often necessary to be able to recognize individuals. This can require marking them in a manner that does not have any long term effects. Accordingly, the first chapter investigates the effect of VIE tagging and spine-clipping on the three-spined stickleback *G. aculeatus*.

Chapter II: An experimentally induced conflict of interest between parasites reveals the mechanism of host manipulation

Whether or not host manipulation has specifically evolved to enhance transmission or whether it results from side-effects of an infection has been under debate for a long time in *S. solidus* infected sticklebacks. In the second chapter, I use sequential infections in which the parasites should be at a conflict over the direction of host manipulation to solve this puzzle. An infective parasite should enhance risk taking to facilitate trophic transmission, but a not yet infective one should not since any premature predation would be fatal for its fitness. By contrast, if a side-effect due to increased energy drain shifting the trade-off between predation and

feeding towards the latter is responsible for altered host behavior, a not yet infective parasite should add to the risk taking of its host since it, too, drains energy from the host. In an additional experiment, I tested the risk averseness of infected and uninfected stickleback when they had either been fed to satiation or staved. True host manipulation should act independently of hunger levels, but energy drain should not.

Chapter III: When parasites disagree: Evidence for parasite-induced sabotage of host manipulation

In nature hosts are usually infected by multiple parasites whose aims with regards to host behavior might agree or disagree resulting in potential for conflict or cooperation over host manipulation. In chapter III, I study both using laboratory bred and experimentally infected copepods and the cestode *S. solidus*. *Schistocephalus solidus* has to spend a certain amount of time in its copepod host before it is ready for transmission to the next host and manipulates accordingly; not yet infective parasites reduce host activity, infective ones increase it. Hence, infective and not yet infective parasites should be at a conflict over host manipulation if they infect the same host. I infected copepods at two different time points to obtain copepods that harbored either one or two infective or not yet-infective parasite or one infective parasite plus one or two not yet infective parasites and measured their activity after a simulated predator attack.

Chapter IV: Inter- and intraspecific conflict over host manipulation

Hosts are not only infected by multiple parasites from one species but also by parasites from different species. To investigate the outcome of such an interspecific conflict and compare it to the outcome of an interspecific conflict, I used different developmental stages of *S. solidus* and the nematode *Camallanus lacustris* and their common host, copepods. *Camallanus lacustris*, like *S. solidus*, has to spend a certain amount of time in its copepod host before it can infect the next host and should manipulate accordingly. Hence, infective *S. solidus* and not yet infective *C. lacustris* should be at a conflict over host manipulation and *vice versa*. I experimentally infected lab-bred copepods in a manner that resulted in copepods harboring an infective *C. lacustris* plus a not yet infective *C. Lacustris* or *S. solidus* or an infective *S. solidus* plus a not yet infective *C. lacustris* and recorded their behavior.

Chapter V: Growth and ontogeny of the tapeworm *Schistocephalus solidus* in its copepod first host affects performance in its stickleback second intermediate host

Chapter V focuses on how the performance of *S. solidus* in the first intermediate copepod host affects its performance in its second intermediate stickleback host. To do so, experimentally infected copepods received different feeding treatments. Subsequently, different life history traits (i.e. growth and ontogeny in the copepod and infection success at different ages, growth and energy storage in the stickleback) were measured in both intermediate hosts.

Chapter VI: Does resource availability affect host manipulation? - An experimental test with *Schistocephalus solidus*

Not only differences in the biotic environment such as multiple infections but also differences in the abiotic environment could affect host manipulation. Chapter VI investigates the effect of resource availability on host manipulation and tries to identify potential trade-offs between host manipulation and other fitness related traits using copepods experimentally exposed to *S. solidus*. Additionally to traits measured in chapter V, I measured the behavior of infected and uninfected copepods under different feeding regimes.

Author's contributions

Chapter I

Tina Henrich conceived the study, Nina Hafer, Tina Henrich and Kenyon B. Mobley contributed to the design of the study and performed the experiment. Tina Henrich analyzed the data and drafted the manuscript. All authors contributed to the revision of the manuscript.

Chapter II

Nina Hafer conducted the experiment, performed the statistical analysis and drafted the manuscript. Nina Hafer and Manfred Milinski conceived and designed the experiment, interpreted the data, wrote the manuscript and gave final approval for publication.

Chapter III

Nina Hafer and Manfred Milinski designed the experiments. Nina Hafer performed the experiments and analyzed the data. Nina Hafer and Manfred Milinski wrote the paper.

Chapter IV

Nina Hafer conducted the experiment, performed the statistical analysis and drafted the manuscript. Nina Hafer and Manfred Milinski designed the experiment and revised the manuscript.

Chapter V

Daniel P. Benesh conceived the study. Daniel P. Benesh and Nina Hafer performed the experiment, collected the data, and outlined the manuscript. Daniel P. Benesh wrote the manuscript. Both authors read and approved the final manuscript.

Chapter VI

Daniel P. Benesh conceived the study; Daniel P. Benesh and Nina Hafer designed and performed the experiment. Nina Hafer analyzed the data with advice from Daniel P. Benesh and drafted the manuscript. Both authors contributed to the writing of the manuscript.

Chapter I

Effects of VIE tagging and partial tissue sampling on the immune response of three-spined stickleback *Gasterosteus aculeatus*

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Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.3s7r7/2

Abstract

A 14 day experiment on effects of visible implant elastomer (VIE) tagging and spine-clipping of three-spined stickleback *Gasterosteus aculeatus* showed significant increases in immune response, particularly in the granulocyte:lymphocyte ratio, in both treatments and the sham control. A minimum two-week recovery after handling, anaesthesia, tagging and spine-clipping is recommended to minimize effect of manipulation on the immune system.

Marking individuals for unique identification is an important tool in eco-evolutionary studies of fishes and conservation and management of fisheries, *e.g.* to track individuals for behavioural studies, to monitor population sizes and stock levels and to aid the identification of hatchery *v*. wild fishes (Skalski et al. 2009).

A variety of methods are currently available that do not require the sacrifice of the animal. These techniques can be generally classified as either non-invasive or invasive (Nielsen 1992). Non-invasive techniques, such as recognizing individuals using photographic methods, are generally preferred over invasive techniques that require physically manipulating or removing tissue. Invasive marks can, however, be indispensable when dealing with many individuals that appear similar or tracking the movements of individuals in large open populations. In addition, marking fishes with invasive individual tags allows individuals to be monitored remotely or recaptured and quickly returned to the environment unharmed. Examples of such techniques include traditional multicoloured banding tags, electronic passive integrated transponder (PIT) tags and, increasingly popular, visible implant elastomer (VIE) tags. VIE tags consist of a coloured silicone-based material that is injected subcutaneously and visible through transparent or lightly pigmented skin. They have been used to mark a variety of vertebrate and invertebrate taxa, with no negative effects on growth, movement and behavior (Malone et al. 1999).

The advent of DNA fingerprinting permits the identification of individuals *via* genetic tagging for a variety of purposes, to study mating and migratory patterns, fish breeding and conservation programmes and even aid identification of hatchery-raised escapees in wild populations (Palsbøll 1999; Glover et al. 2010; Andreou et al. 2012). A small amount of DNA must be harvested from individuals and a diversity of sources such as epidermal mucus, spines, scales, blood, barbels, sperm or buccal tissue have been used (Woodall et al. 2011). By far, the most widely used is fin-clipping. This does not appear to impair natural swimming or inhibit survival, growth, behaviour or sexual maturity (Churchill 1963; Gjerde and Refstie 1988; Woodall et al. 2011). The consequences of fin-clipping for the fish probably depend, however, on the amount of tissue removed, size, age, and stress experienced during the procedure and environmental conditions during recovery.

In this study, the effect of VIE tagging and tissue sampling on several measures associated with the immune response of the three-spined stickleback *Gasterosteus aculeatus* L. 1758 was investigated. As both methods may cause tissue damage, this may lead to alterations of the immune system. An immune response is particularly relevant to studies involving experimental manipulation of the immune system or infection with parasites, as marking the fish may influence the experimental outcome. For example, a study by (Wedekind and Little 2004) showed that experimental removal of spines (*i.e*. spine-clipping) of *G*. *aculeatus* 7 days after exposure to the parasite *Schistocephalus solidus* resulted in a 50% reduction in the risk of parasite infection and concluded that acute stress may enhance the host antiparasite response. With this hypothesis in mind, the aim of this study was to investigate if and when the immune system returned to baseline levels after handling and tagging.

Four measures were used to assess the overall health and immune response of fish including (1) condition factor (*K*) which is a measure of overall nutritional status and health, (2) spleno-somatic index (I_S) which correlates with overall immune system health, (3) granulocyte:lymphocyte ratio (G:L) which is an estimate of differences between the level of activation of the innate and adaptive immune system and (4) respiratory burst activity of head kidney leucocytes which is used to measure the reaction of the innate immune system.

Laboratory-bred F1 generation *G*. *aculeatus* from several families with parental stock originating from Großer Plöner See, Germany (54° 09′ 21″ N; 10° 25′ 50″ E) were used. Fish from four families were pooled into groups of eight fish and placed into individual 16 l tanks (15 tanks, five tanks per treatment) in a flow-through freshwater system maintained at 18° C and a 16L:8D cycle. Fish in each tank were systematically assigned to one of the three treatments: VIE tags, spine-clipped or handling control. Tanks were interspersed so that there was no spatial separation of treatments. Fish were fed a diet of frozen and live chironomid larvae three times per week before and during the experiment.

On the first day of the experiment (day 0), each fish was anaesthetized with MS-222) for *c*. 2 min, weighed to the nearest mg, measured for standard length (tip of rostrum to tip of caudal peduncle, L_S) to the nearest 0·5 mm and immediately processed according to the treatment. For the VIE-tagged treatment, two fluorescent yellow tags *c*. 1 cm long were injected subcutaneously (injection needle diameter: 0·30 mm) according to the

manufacturer's instructions (Northwest Marine Technology; www.nmt.us) in the dorsal musculature on the left and right side behind the second dorsal spine. In the spine-clipped treatment, the first dorsal spine was gently lifted with forceps and the tip was cut off with sterile scissors. After anaesthesia, control fish were gently handled for 1 min out of water to simulate the handling time in the other treatments. All fish were allowed to recover for a minimum of 10 min in an aerated recovery tank before placing them back into their original tanks.

Dissections and immune assays were carried out at days 1, 3, 7 and 14 after the initial treatment. On each day, two fish per treatment were selected from each tank and euthanized with an overdose of MS-222 (200 mg l^{-1}). This resulted in 10 individuals per treatment per sampling day. Measurements included L_S, mass (M) and sex of fish as well as mass of individual organs (head kidneys, spleen, liver and gonads). The condition factor was calculated after (Frischknecht 1993). The I_S was calculated as $I_S = 100\ M\ M_S^{-1}$, where M_S is spleen mass. Head kidneys were immediately placed on ice and processed to estimate the number of lymphocytes and monocytes with flow cytometry (Scharsack et al. 2004; Kalbe and Kurtz 2006). Respiratory burst activity was measured with a lucigenin-enhanced chemiluminescence assay in relative light units (RLUs) after adjusting the cell number for each sample to 1×10^5 cells per well (Kalbe and Kurtz 2006). Since this measurement is highly variable between days, the mean RLU of 32 control wells per plate (including all components except head kidney cells) was measured. The RLU of each sample was then divided by the mean RLU of the negative controls for that day and the total number of vital granulocytes to give a standardized value of the RLU per single granulocyte on each day measured.

R 2.12.2 (R Development Core Team; www.r-project.org) was used to select the best statistical model explaining variation in the response variable. All statistics were carried out using PASW Statistics 18 (IBM; www.spss.com.hk/statistics).

Two fish died during the experiment, one in the VIE-tagged treatment and one in the spine-clipped treatment between days 7 and 14; these were not included in analyses. In five samples, head kidney cell numbers were too low to measure the respiratory burst and were therefore excluded. One extreme outlier was dismissed due to high leverage in Cook's distance (RLU granulocyte^{-1}, VIE-tagged treatment, day 14).

The overall estimate of fish health, K, did not differ significantly between treatments (analysis of variance (ANOVA), $F_{2,108} = 0{\cdot}26$, $P > 0{\cdot}05$) or sex (ANOVA, $F_{1,108} = 0{\cdot}80$, $P > 0{\cdot}05$). There was a significant increase, however, in K over time (ANOVA, $F_{3,108} = 5{\cdot}35$, $P < 0{\cdot}01$; Figure I. 1 a). There was no significant correlation between K and I_S (Pearson's $r = 0{\cdot}154$, $n = 116$, $P > 0{\cdot}05$), G:L (Pearson's $r = 0{\cdot}118$, $n = 116$, $P > 0{\cdot}05$) or RLU granulocyte^{-1} (Pearson's $r = -0{\cdot}029$, $n = 110$, $P > 0{\cdot}05$).

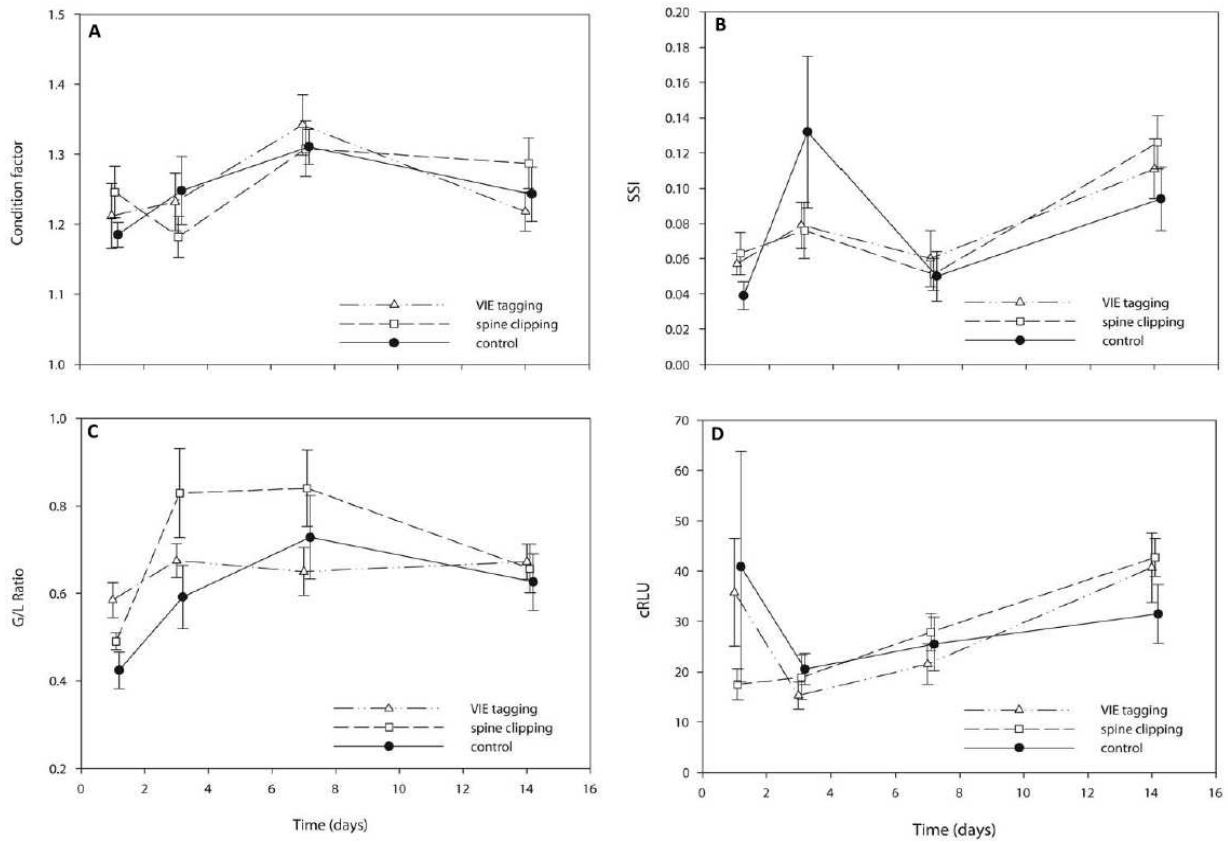

Figure I. 1: Changes in condition and immune relevant measurements over time after spine-clipping (❑) and visible implant elastomer (VIE) tagging (△) and control (●) in *Gasterosteus aculeatus*. Mean ± s.e. are given for (a) condition factor (*K*), (b) spleno-somatic index (I_S), (c) granulocyte and lymphocyte ratio (G:L) and (d) controlled respiratory burst activity (relative light unit, RLU granulocyte^{-1}) for all treatment groups and sampling days ($n = 10$ in all groups except: $n = 9$ for *K*, I_S and G:L, day 14 VIE-tagged and spine-clipped fish, RLU granulocyte^{-1}, day 1 VIE-tagged fish and day 14 spine-clipped fish; $n = 8$ for RLU granulocyte^{-1}, day 14 VIE-tagged fish; $n = 7$ for RLU granulocyte^{-1}, day 1 control fish).

The best model to explain variance in the I_S used the factors treatment, time and sex. The ANOVA showed significant effects of time ($F_{3,110} = 6{\cdot}98$, $P < 0{\cdot}001$) and sex ($F_{1,110} = 3{\cdot}45$, $P < 0{\cdot}05$) but not for treatment ($F_{2,110} = 0{\cdot}47$, $P > 0{\cdot}05$). The I_S was significantly higher in males than in females. A *post hoc* Tukey honest significant difference (HSD) test showed significant differences between days 1 and 7 as well as between 7 and 14 irrespective of the treatment. There was an increase in IS at day 14 for all treatments, which was highest for the spine-clipped fish (Figure I. 1 b). Looking into each treatment group using ANOVAs with time as a factor, there were only significant effects of the factor time in the spine-clipped and control group (Table I. 1). In spine-clipped fish, the IS was highest on day 14 of the experiment, while it was highest on day 3 in the control group (Figure I. 1 b). The VIE-tagged fish did not show any significant differences in the IS over time (Figure I. 1 b).

Table I. 1: Changes in condition factor (*K*), spleno-somatic index (I_S), granulocyte to lymphocyte ratio (G:L) and respiratory burst activity (relative light unit, RLU) granulocyte^{-1} of *Gasterosteus aculeatus* in response to spine-clipping and visible implant elastomer (VIE) tagging. A summary of *F* and *P*-values obtained from analyses of variance (ANOVAs) using time as factor for different measurements and treatment groups is shown

	Control		Spine-clipped		VIE-tagged	
	F	p	F	p	F	p
K	F3,36 = 2·188	>0·05	F3,35= 2·402	>0·05	F3,33= 2·360	>0·05
IS	F3,36 = 2·939	<0·05	F3,35 = 4·024	<0·05	F3,35 = 2·653	>0·05
G:L	F3,36 = 3·309	<0·05	F3,35 = 4·646	<0·01	F3,35 = 0·984	>0·05
RLU granulocyte−1	F3,32 = 5·069	<0·01	F3,35= 6·382	<0·001	F3,33= 7·536	<0·001

The best model for G:L included the factors treatment and time. This ANOVA showed significant effects for both factors, treatment ($F_{2,112} = 3·72$, $P < 0·05$) and time ($F_{3,112} = 7·61$, $P < 0·001$). A *post hoc* Tukey HSD showed that the G:L was significantly higher in spine-clipped than in control fish when data from all days were combined. It also revealed differences between day 1 and all other dissection days (3, 7 and 14) that were further analysed using time as a factor (Table I. 1). This result indicated that the difference between days is mainly due to the control group and the spine-clipped treatment, which showed significant effects. Only considering day 1, the treatment groups differed significantly from each other (ANOVA, $F_{2,27} = 4·62$, $P < 0·05$) and a *post hoc* Tukey HSD test showed that the VIE-tagged fish had a higher G:L than the spine-clipped and control fish. Both control and spine-clipped treatments showed an elevation of G:L on days 3 and 7 and returned to baseline levels on day 14 of the experiment (Figure I. 1 c). In control fish only, day 7 showed a significant elevation of G:L (Tukey HSD, $P < 0·05$). In spine-clipped fish, G:L was significantly higher on days 3 and 7 (Tukey HSD, $P < 0·05$) than on days 1 and 14. On day 14, there was no significant difference to day 1 in all treatments (Tukey HSD, $P > 0·05$).

The best model explaining the RLU granulocyte^{-1} included the factors treatment and time. The only significant factor in this ANOVA was time ($F_{3,106} = 17·18$, $P < 0·001$), while the treatment group was not significant ($F_{2,106} = 0·21$, $P > 0·05$). A post hoc Tukey HSD showed significant differences between day 1 and all other days. The RLU granulocyte−1 values were highest on day 1 for all treatments (Figure I. 1 d) and significantly different from all other days (Tukey HSD, $P < 0·05$), except for day 7 in control fish (Tukey HSD, $P > 0·05$). In all treatment groups, the RLU granulocyte^{-1} did not change significantly between days 3 and 14 (Tukey HSD, $P > 0·05$).

The results of the 14 day experiment demonstrated a significant influence of VIE tagging and spine-clipping on the immune response of *G. aculeatus* and these responses were independent of the condition factor. Significantly higher levels in the G:L at day 1 for VIE-tagged fish and the elevation on days 3 and 7 for spine-clipped fish indicated an initial immune response to these techniques. After 14 days, however, these treatments were no longer distinguishable from control fish. Further, slightly elevated values for the I_S and respiratory burst activity of the head kidney leucocytes at 14 days suggest that there may be lingering effects on the immune system of tagging fish with both methods. Whether or not these elevated levels persist for longer periods will require further investigation.

Spleen enlargement is assumed to be a valuable indicator of immune system status and active processes. The differences in the I_S were mainly due to the different dissection days, but did not show differences among treatment groups. Increased size of the spleen can also be a reaction to parasitism in fishes (Lefebvre et al. 2004; Seppänen et al. 2009) possibly as a consequence of increased leucocyte synthesis. It is not clear from these data if the I_S would return to its former value and how long this would take. Looking at the G:L, in all treatment groups, the proportion of granulocytes increased over time, although this was not significant for the VIE-tagged fish. This increase is consistent with the findings for the I_S which may be due to increased leucocyte synthesis.

The respiratory burst is responsible for rapid innate immune reactions and results from the swift release of reactive oxygen species, mainly by granulocytes (Baldridge and Gerard 1932). Measurement of the respiratory burst activity, the RLU granulocyte^{-1}, showed an initial elevation for all treatments on day 1 of the experiment. The RLU granulocyte^{-1} then levelled off in all treatments, but since the relative number of granulocytes increased (G:L), the overall respiratory burst in head kidney leucocytes increased as well. It is possible that the initial elevation of the RLU was due to an activation of the granulocytes in the head kidneys after which the cells migrate to the peripheral tissue where the interaction between immune system and an antigen takes place.

The results also demonstrate an elevated immune response in the control treatment particularly in the G:L. This indicates that anaesthesia and fish handling stimulates an immune response. Therefore, this study also highlights the importance of proper handling controls and sufficient time for recovery after handling, anaesthesia and tagging.

The use of individual marking techniques is a useful tool in fish biology. This study demonstrates that VIE tagging, partial tissue sampling and handling and anaesthesia all appear to influence the immune response in *G. aculeatus*. Based on the results of this experiment, it is recommended that such marking and handling techniques should be carried out at least 2 weeks in advance of the actual study. VIE tagging, however, does not evoke as strong a response as spine-clipping. Therefore, VIE tagging should be preferred to spine clipping for individual identification marks particularly as spine-clipping is limited by the number of unique individuals that can be distinguished and may require more than one clip per fish as was measured here. If, however, DNA and genetic fingerprinting is required, care should be taken to limit the amount of tissue taken, and adequate recovery time should be provided before experimentation. In the future, additional invasive techniques such as partial fin clips and other types of tagging should be examined for their effects on the immune responses.

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).

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Chapter II

An experimentally induced conflict of interest between parasites reveals the mechanism of host manipulation.

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Abstract

Parasites can increase their host's predation susceptibility. It is a long-standing puzzle, whether this is caused by host manipulation, an evolved strategy of the parasite, or by side-effects due to, e.g., the parasite draining energy from its host thereby changing the host's trade-off between avoiding predation and foraging towards foraging. Here we use sequential infection of three-spined sticklebacks with the cestode *Schistocephalus solidus* so that parasites have a conflict of interest over the direction of host manipulation. With true manipulation the not yet infective parasite should reduce rather than enhance risk taking since predation would be fatal for its fitness; if host behaviour is changed by a side-effect the two parasites would add their increase of predation risk because both drain energy. Our results support the latter hypothesis. In an additional experiment, we tested both infected and uninfected fish either starved or satiated. True host manipulation should act independently of the fish's hunger status and continue when energy drain is balanced through satiation. Starvation and satiation affect the risk averseness of infected sticklebacks similarly to that of uninfected starved and satiated ones. Increased energy drain rather than active host manipulation dominates behavioural changes of *S. solidus* infected sticklebacks.

Introduction

Parasites have the potential to change the behaviour of their hosts. They can actively manipulate host behaviour thereby improving their own fitness. In complex life cycle parasites such host manipulation often takes the shape of increased predation susceptibility (Holmes and Bethel 1972; Poulin and Thomas 1999; Moore 2002, 2013; Poulin 2010). However, a similar shift in host behaviour can result from side-effects of an infection. Most animals are faced with a trade-off between predation avoidance and energy consumption. A parasite, by definition, drains energy from its host. Therefore an infected host needs more energy than an uninfected one, shifting the trade-off away from predation avoidance in favour of feeding. Hence an infected host could become more prone to predation without any host manipulation that would have evolved

specifically to enhance transmission (Milinski 1990). If the side effect achieves an optimal behavioural change from the parasite point of view, there would be no selection for an additional manipulation mechanism.

Under natural conditions, studying potential host manipulation in hosts infected by a certain parasite is complicated by the fact that hosts rarely harbour only a single parasite. Rather, hosts are normally infected by a multitude of different parasites from the same and/or different species (e.g. Petney and Andrews 1998; Kalbe et al. 2002). These parasites might have the same or different optima when it comes to how their host should behave. If these optima differ, a conflict over host manipulation can ensue and affect host behaviour (Rigaud and Haine 2005; Thomas et al. 2010, 2011). Such a conflict can also occur between parasites of the same species if one has just entered the host and the other is ready for transmission to the next host; accordingly one needs to suppress and the other one to enhance the predation risk of their shared intermediate host. This conflict has been studied using hosts that were either naturally (Sparkes et al. 2004; Dianne et al. 2010) or experimentally (Dianne et al. 2010; Hafer and Milinski 2015) infected by different stages of the same manipulating parasite species. All these studies found that it was the already infective parasite that dominated the resulting behaviour.

Schistocephalus solidus has a complex life cycle with two intermediate hosts, cyclopoid copepods and three-spined sticklebacks (*Gaterousteus aculeatus*). It reproduces in the gut of birds to which *S. solidus* is transmitted when its stickleback host is eaten by a bird. *S. solidus* is well known to be associated with changes in various aspects of host behaviour in its stickleback host (Milinski 1990; Barber and Huntingford 1995; Barber et al. 1995, 2000; Barber and Wright 2008; Barber and Scharsack 2010) including increased risk taking in the face of both, the correct subsequent bird host (Giles 1983, 1987b; Barber et al. 2004) and dead-end fish predators (Milinski 1984). Any altered behaviour potentially leading to increased predation susceptibility of the host due to true host manipulation rather than side-effects should not be visible before parasites reach infectivity at a weight of 50 mg (Tierney and Crompton 1992). In naturally infected fish the level of behavioural changes often correlates positively with parasite load and no sudden switch seems to occur as would be expected for host manipulation that abruptly sets in when the parasite reaches 50 mg (Giles 1983, 1987a; Godin and Sproul 1988). In experimentally infected sticklebacks no change in reaction to a fish predator occurs when their parasite is still very small and not yet-infective (Aeschlimann et al. 2000). Barber et al. (Barber et al. 2004) repeatedly measured the response of experimentally infected sticklebacks to a simulated bird predator. They found no changes prior to when the parasites assumedly reached 50 mg, but significant changes thereafter. In laboratory infected sticklebacks, an activation of the innate immune system coincides with when the parasite reaches infectivity (Scharsack et al. 2007). Parasites could exploit pre-existing links between the immune system and the neuronal system of their host to manipulate their behaviour (Thomas et al. 2005; Poulin 2010; Adamo 2012; Lafferty and Shaw 2013), e.g. infection is associated with altered levels of monoamine in the brain of naturally infected sticklebacks (Øverli et al. 2001). However, the correlative nature these findings cannot proof any causal link. Accordingly, whether or not altered behaviour in *S. solidus* infected sticklebacks is caused by a side-effect via energy drain or active host manipulation that has evolved specifically for this purpose has been the subject of an ongoing debate (Milinski 1990; Barber and

Huntingford 1995; Barber et al. 1995, 2000; Barber and Wright 2008; Barber and Scharsack 2010), though the recent literature tends to favour true host manipulation (Barber et al. 2004; Barber and Wright 2008; Barber and Scharsack 2010).

In this study, we take advantage of a potential conflict over host manipulation between infective and not yet infective parasites to solve the puzzle of whether host manipulation by *S. solidus* is true manipulation or the consequence of a side-effect. Such a conflict should be abundant in nature where sticklebacks often become infected by multiple parasites which will coexist until their host dies. Several, if not all of them, can become large enough within a single fish to reproduce once they reach their final bird host (e.g. Arme and Owen 1967; Pennycuick 1971; Heins et al. 2002, 2011). A conflict between infective and not yet infective parasites should be mirrored in altered host behaviour only if there is true manipulation: If a not yet infective parasite shares a host with an already infective conspecific, it is expected to sabotage the older parasite's manipulation since any predation at this point would be fatal for it. Hence we should see a compromise in the fish's behaviour reflecting the conflicting parasite interests. Even if such sabotage was to fail completely, combined active host manipulation of two disagreeing parasites should never increase risk taking of their host beyond what an infective parasite would achieve when alone. Any such increase in risk taking is thus likely to be a side-effect of enhanced energy drain caused by two compared to one parasite rather than active host manipulation. In a second experiment, using only singly infected hosts, we investigated the effect of both infective and not yet infective parasites on predation avoidance when fish were either hungry or fed to satiation. If only energy drain is responsible for the alteration in host behaviour, satiated fish, irrespective of being parasitized, should behave in a more risk adverse manner than hungry ones. By contrast, true host manipulation should act independently of the fish's hunger status and continue when energy drain is balanced through satiation. It is in the interest of the infective parasite that its host exposes itself to predation also when it is not hungry.

Material and Methods

Hosts

Sticklebacks were bred from fish caught in the Große Plöner See, northern Germany. For experiment I, fish were about 3 months old at the beginning of the experiment. We used 113 fish from 4 different families. Two weeks prior to the first parasite exposure, they were distributed to 8 different tanks, with 15 fish each, two tanks per family. For experiment II, we used 188 fish from 6 families, which were about 7 months old. We used older (and hence larger) fish in experiment II in order to ensure that parasites could reach maturity without potentially compressing the fish's gut to such an extent that infected fish would be unable to become satiated (Milinski 1985). For both experiments fish families were randomized with regards to treatment. On the day before the (first) infection, we placed each fish in a separate 16-L tank visually isolated from any other fish. Throughout the experiment fish remained in this home tank and were fed with red fly larvae (*Chironomus*

sp.). In experiment I, fish were fed daily except on the day before and during the experiment. For experiment II, fish were randomly assigned to two different feeding treatments. Prior to the experiment they were either fed to satiation ("satiated") or starved for three days ("starved").

Parasites

Schistocephalus solidus were bred in an *in vitro* system in the laboratory (Smyth 1946; Wedekind 1997) from parents dissected from naturally infected fish caught at the "Neustädter Binnenwasser", northern Germany. We used two different families for each experiment and stored the eggs in the fridge at 4°C until use. Prior to infection they were incubated for three weeks at 20°C in the dark and then exposed to light overnight to induce the coracidia to hatch (Dubinina 1980). One coracidium each was administered to lab-bred copepods (*Macrocyclops albidus*). In the copepods they were allowed to grow for 17 days. After one to two weeks copepods were checked for infection by placing them on a microscope slice. Copepods are translucent allowing identification of an infection visually without having to kill the copepod.

Treatments

Experiment I

For experiment I we conducted two rounds of infections, 31 days apart. Infections took place inside the fish's home tank. When fish were placed in individual home tanks on the day prior to the first infection, the home tank was only half filled and water was only turned on 2 days after infections had taken place. For the second infection, water was turned off and water levels were lowered on the day before the infection. This prevented copepods form escaping through the outflow before the sticklebacks could have consumed them.

For experimental infections a stickleback was offered one copepod that was either infected (to obtain infected sticklebacks) or not (to obtain sham-infected sticklebacks). Since this was repeated twice, it resulted in four different treatments: Fish receiving only uninfected copepods (0_0), fish receiving one infected copepod either on day 0 (1_0) or on day 31 (0_1) and an uninfected one on the other day and fish receiving one infected copepod on day 0 plus on day 31 (1_1). If a fish received two parasites, they always originated from two different families. This allowed us to unambiguously determine the infection time for each parasite after dissection in multiply exposed fish by identifying which family they came from using microsatellites.

In experiment I, 3 fish died during the experiment (1 uninfected, two infected only on day 0) and were hence excluded from the analysis. We pooled fish according to the treatment they resembled according to the types of parasite they contained even if they had received more parasites than had managed to establish themselves. This resulted in a total of 110 fish that we could include in the final analysis (0_0: 36, 1_0: 29, 0_1: 19, 1_1: 26).

Experiment II

In experiment II infections took place in the same manner described above for the first round of infections in experiment I. Fish were either infected or uninfected. In order to test fish with parasites that were either not yet infective or infective we conducted the behavioural tests at two different time points, about six weeks (early) and about ten weeks (late) post infection, respectively. For each test we used a different set of fish to enable us to measure parasite weight just after the behavioural tests, which requires dissection. In total, we exposed or sham exposed 188 fish (64 sham exposed, 124 exposed, half of each early and late), 4 of which died (1 early, exposed but uninfected, 3 late, 2 exposed but uninfected, 1 sham exposed). One additional fish (late, exposed but uninfected) developed mould and was hence excluded. Again we pooled sham exposed and exposed but uninfected fish. Infection treatment and early and late fish were combined with the feeding treatment (satiated or starved) described above in a fully factorial manner (early: 36 uninfected, satiated, 36 uninfected, starved, 10 infected, satiated, 11 infected, starved, late: 37 uninfected, satiated, 35 uninfected, starved, 8 infected, satiated, 11 infected, starved).

Behavioural experiments

Experiment I

Experiments took place in a separate experimental tank, 44 by 44 cm and filled with water to a height of about 20 cm. The ground was covered with sand. An array of 4 times 16 small pots which contained one red fly larvae (*Chironomus* sp.) each was placed in the middle of the tank embedded in sand (Milinski 1985). On one side of the tank a model heron head was installed and clamed with a rubber band in a manner that when the rubber band was released, the heron quickly dipped into the water before returning to an upright position to simulate a predation attack (Giles 1983, 1987b; Barber et al. 2004). Opposite to the model heron four plastic water plants were placed in the tank to provide hides (Figure II. 1 A). On one side of the tank a mirror was placed roughly in a 45 degree angle that allows recording a side view of the tank while recording from above. A black curtain to minimise disturbance surrounded the entire set up. Above the experimental tank an HD-camera (MHD-13MG6SH-D, Mintron, Taiwan) was located which allowed us to videotape all behavioural trials and to monitor them on a screen without disturbing the fish. During a few trials problems occurred with the recording. Before we conducted any experiments, we accustomed the fish to the experimental set up and procedure. Prior to the first infection were transferred twice to the experimental tank in groups of 7 or 8 fish and allowed to feed for at least one hour. That way fish knew where to find the food in the experimental tank prior to the actual experiments. Following the first infection and isolation in individual tanks each fish was accustomed once more alone for 45 minutes to the experimental tank.

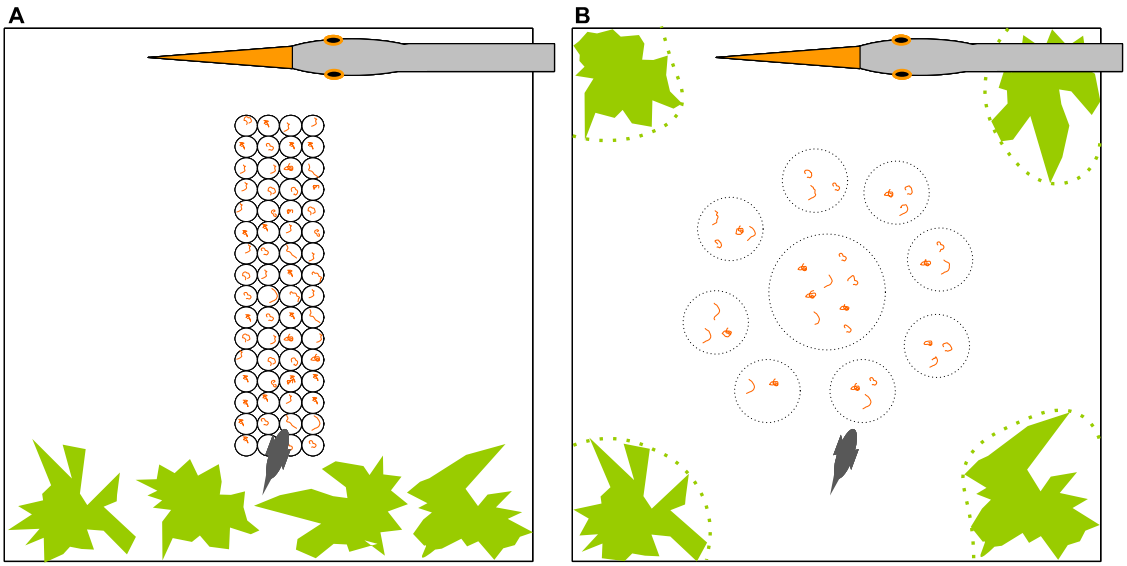

Figure II. 1: Set up of the experimental tank. A: experiment I, B: experiment II. On one side of the tank a model heron head was installed in a manner that it could be dropped into the water in a standardized manner to simulate an attack by a heron. In experiment 1 (A) plants for hiding were placed on the opposite end of the tank, for experiment II (B) plants were placed in all four corners of the tank. A fish was considered hiding if it was within the dashed lines encircling the plants on the video screen. Small feeding pots were placed in the tank in experiment I. In experiment II food was provided in half buried petri dishes in the centre of the tank. This food was accessible during training but covered with translucent lids during the experiment.

The actual behavioural trials consisted of gently transferring a single fish to the experimental tank within a glass pipe filled with water to minimise disturbance. A timer was started as soon as a fish left the glass pipe. Each fish was then allowed to consume 2 food items. As soon as it had done so, a mechanism was released dipping the model heron head into the water. If a fish failed to consume 2 food items, the simulated heron attack took place after 15 minutes. Trials in which fish were hidden underneath the plants at this time were discarded from analysis since this might have prevented them from perceiving the simulated heron attack (Time point 1: 1 0_0, 2 1_1, Time point 2: 2 0_0, 2 1_1, 1 0_1, Time point 3: 3 0_0, 1 1_0, 1 0_1, Time point 4: 5 0_0). After the simulated heron attack, each fish remained in the experimental tank until it had consumed 10 food items, but for at least 5 minutes and at most for 15 minutes. In nature a predator that fails to catch a fish it attacked might remain close by for some time, ready to strike again. Hence, following the simulated predator attack, fish should perceive an enhanced predation risk. We recorded when fish resumed feeding after the simulated heron attack, how much food they consumed within the subsequent five minutes and where they consumed the first two food items before and after the simulated heron attack. Thereafter fish were gently returned to their home tank. From the recordings we estimated the position of each fish every 2 seconds over the course of five minutes starting 10 seconds after the simulated heron attack using the manual tracking plugin within image J (Rasband 2008). We conducted 4 trials, stretching over 5 days each, starting every 10 days. The first trial took place 7.5 weeks after the first infection (parasite age during trials: parasite from day 0/ parasite from day 31: time point 1: 52-56/21-25 days, time point 1: 62-66/31-35 days, time point 1: 72-76/41-45 days, time point 1: 82-86/51-55 days).

Experiment II

We used the same experimental tank with the same model heron as in experiment I, but the exact layout of the tank differed and the mirror was absent. Since we had observed no preference for fish to stay away from the heron side of the tank in experiment I, we equipped the tank more symmetrically in experiment II. Red fly larvae were provided in petri-dishes placed in the centre of the tank. During the actual experiments, these petri-dishes were covered with translucent lids. Fish were able to perceive but not to access food to prevent them from becoming satiated. Unlike in experiment I where food had been accessible that allowed us to also observe fish behaviour before the simulated heron attack without any food sources becoming depleted or fish satiated, but it did not allow us to count the number of food items fish consumed. Plants were provided in all four corners (Figure II. 1 B). Four times over the course of about 10 days fish were transferred to the test tank to become accustomed to this set up. Each fish was allowed to feed for 15 to 30 minutes. Food inside the petri dishes was accessible during this training and no additional food was provided in the home tank.

As in experiment I, fish were transferred to the experimental tank in a glass pipe and a timer started as soon as they left it. We then measured the time fish spent hiding for 5 minutes starting 30 seconds after a fish had left the glass pipe or as soon as a fish emerged from hiding if it had been hiding at that time. A fish was considered hiding as soon as it was partially within immediate proximity to the plants defined by a line had been drawn on the screen prior to the experiments encircling the entire plant but smoothed out its uneven structure (Figure II. 1 B). Once fish had been recorded for 5 minutes, the simulated heron attack occurred. If fish were hiding at that time, we waited until they left the hide. If fish were still hiding 30 minutes after the initial recording started, we discarded them (6 early fish: 2 infected and satiated, 1 infected and starved, 3 uninfected and satiated and 6 late fish: 2 infected and satiated, 4 uninfected and satiated). Starting 10 seconds after the simulated heron attack, we again recorded how much time fish spent hiding and when they first re-emerged from hiding. If fish did not re-emerge from hiding within 15 minutes we stopped the trial.

Dissection

After the experiment (i.e. after the fourth time point (experiment I) or directly after the behavioural trial (experiment II)), fish were killed by placing them in an overdose of an anaesthetic MS222. Their body cavity was opened and any parasite found was removed from the body cavity and weighted.

Statistical analysis

All statistical analysis took place and all plots were created in R (R Development Core Team 2010). We only present relevant p-values in the result section for better readability. For exact statistical outputs please refer to the supplementary information (Table II. S3 – Table II. S7)

Experiment I

To investigate the latency with which fish resumed feeding after the simulated heron attack, we performed a survival analysis by fitting a parametric survival regression model for each time point. We used the survreg function in the survival package (Therneau 2015) with Weibull distribution and the time to emerge from hiding as response. For fish that did not emerge within 15 minutes we set this time to 15 minutes (900 s). We additionally included whether an event occurred (fish emerged from hiding) or not (data censored after 15 minutes) into the response. To investigate the effect of each treatment more closely, we conducted pairwise comparisons, using the same models but including data from only two treatments at each time with bonferroni corrections.

We used generalized linear mixed models in the lme4 package (Bates et al. 2014) with poison error family to analyse how much food fish consumed and where they fed on average. To analyse fish activity (i.e. the average distance fish moved within 2 seconds) we used linear mixed models (lme4 package) (Bates et al. 2014) after log transforming the data. We included fish identity including time point as a random factor and time point as fixed factor. For the position where fish fed we included the time interval in the recording (i.e. before vs. after the simulated heron attack) both in the random factor and as a fixed effect. We then stepwise included treatment and its interaction with time point and, if appropriate, the time interval and its interaction. Subsequently, we performed likelihood ratio tests to compare models. A model was accepted if it was significantly better than a less complex model at explaining the data. For each time point we performed a separate Tukey test using general linear hypotheses within the multcomp package (Hothorn et al. 2008) to determine when treatments differed.

Experiment II

We analysed fish from the early and late group separately. To investigate the fish's latency to re-emerge from hiding after the simulated heron attack we again fitted a parametric survival regression model in the survival package using the survreg function (Therneau 2015). Similarly to described above, we used the time to emerge from hiding (set to 15 minutes if fish failed to remerge) and whether or not they did emerge as response.

To analyse how much time fish spent hiding, we log transformed the data and then applied linear mixed models from the lme4 package (Bates et al. 2014) using fish identity as random factor and the time interval (i.e. before vs. after the simulated heron attack) as fixed effect. For both models, we stepwise added feeding treatment, infection and time interval (only for time hiding) and all two way interactions. Subsequently, we performed likelihood ratio tests (see above).

Results

Experiment I

Prior to all further analysis we confirmed that fish reacted to the simulated heron attack, which they did (Supplementary information II. 1, Figure II. S1) and checked for differences between treatment in the reaction to the simulated heron attack (Supplementary information II. 2, Figure II. S2). We expect adaptive host manipulation enhancing the host's predation susceptibility e.g. by decreasing the host's risk averseness) to occur only once the parasites are infective to their subsequent host and would hence benefit from transmission. In our experiment this was the case from time point 2 or 3 onwards in fish infected only on day 0 and during time point 4 in fish infected on day 31 (Supplementary information II. 3, Figure II. S3). The behaviour of fish infected on both days should depend on the outcome of a potential conflict between their parasites, if active host manipulation was involved. If the parasite from day 0 wins, they should reduce predation avoidance from time point 2 or 3 onwards, just like fish only infected on day 0. If the parasite from day 31 wins, sequentially infected fish should never show any enhanced predation susceptibility since this parasite never became infective (Supplementary information II. 3, Figure II. S3). Any intermediate behaviour indicative of a compromise would also be possible.

Treatment significantly influenced how long it took fish to resume feeding after the simulated heron attack during all four time points (Time point 1: $ChiSq_{104,3}=14.96$, $p=0.0019$, Time point 2: $ChiSq_{103,3}=42.82$, $p<0.0001$, Time point 3: $ChiSq_{102,3}=46.79$, $p<0.0001$, Time point 4: $ChiSq_{101,3}=55.09$, $p<0.0001$). Likewise, treatment ($p=0.0005$) and its interaction with time point ($p=0.0002$) influenced how much food fish consumed in the five minutes following the simulated heron attack (Table II. S3). Neither treatment nor its interaction with time point had any effect on how far fish moved or where they fed ($p>0.2$, Table II. S3). Accordingly, we focus on the latency to resume feeding and the amount of food fish consumed, and conduct post hoc tests to disentangle when and between which treatments significant differences occurred.

Is there predation suppression?

Prior to reaching infectivity, any predation would be fatal. Hence, parasites should reduce the predation risk of their host (Parker et al. 2009c). This should occur prior to time point 2 or 3 in fish infected on day 0 and prior to time point 4 in those infected only on day 31 and should be marked by increased risk averseness in those fish. Fish with not yet infective parasites never took significantly longer to resume feeding or consumed less food than uninfected fish. If there were any differences, fish with not yet infective parasites were even more risk prone (Table II. S4, II. S5). Hence, we found no evidence of increased risk averseness in infected fish indicating predation suppression.

Is there predation enhancement?

Once *S. solidus* is infective (on average from time point 2 or 3 onwards for those in fish infected only on day 0 and during time point 4 for those in fish infected only on day 31) it should increase the predation susceptibility of its host and thereby enhance transmission. From time point 2 onwards fish infected on day 0 resumed feeding sooner than uninfected fish ($p<0.02$, Table II. S4, Figure II. 2 A), through this was only a trend during the fourth time point ($p=0.0612$, Table II. S4), and consumed more food ($p<0.0003$, Table II. S5, Figure II. 2 B). Surprisingly, fish infected on day 31 also started to increase their risk taking during the second time point. They resumed feeding significantly sooner than uninfected fish during the second time point ($p=0.0392$, Table II. S4, Figure II. 2 A) and consumed more food from time point 3 onwards ($p<0.004$, Table II. S5, Figure II. 2 B). Hence we did see increased risk taking likely to result in predation enhancement. It seems to occur about the same time in fish infected on day 31 and day 0 even so parasites in fish infected on day 31 are younger and become infective later. There are two different but not mutually exclusive explanations for these findings which we discuss further in the supplementary information II.

Is there conflict?

A potential conflict between infective and not yet infective parasites would exist if fish with infective and not yet infective parasites differed in their behaviour. In our experiment, we expected such a conflict to occur from time point 2 or 3 onwards when parasites from day 0 are already infective but parasites from day 31 in sequentially infected fish should not yet be infective. As a proxy for each of these parasites' effect on host behaviour we used parasites of the same age that did not have to share their host. There we expect conflict only during time point 2 and 3 since in singly infected fish parasites from day 31 were already infective during time point 4. We did not find any significant differences for any of the traits we measured between fish singly infected either on day 0 or on day 31 ($p>0.4$, Table II. S4, II. S5, Figure II. 2). As discussed above, when either is compared to controls, both cause alterations of host behaviour into the same direction. Though there should be a conflict, it did not show up in the fish's behaviour

What is the outcome of sequential co-infections?

Sequentially infected fish resumed feeding significantly sooner than uninfected fish ($p<0.03$, Table II. S4, Figure II. 2 A) and ate more food ($p<0.0007$, Table II. S5, Figure II. 2 B) at each time point. They also resumed feeding significantly sooner than fish infected only on day 31 ($p=0.0002$, Table II. S4, Figure II. 2 A) during the fourth time point and consumed significantly more food throughout the experiment ($p<0.03$, Table II. S5, Figure II. 2 B). They even resumed feeding sooner during time point 4 ($p=0.0013$, Table II. S4, Figure II. 2 A) and consumed significantly more food during time point 1 and 2 ($p<0.004$, Table II. S5, Figure II. 2 B) than fish infected only on day 0. During this time the parasite from day 31 in sequentially infected fish could not yet have been infective. It should not enhance predation susceptibility beyond the level of fish harbouring only the day 0 parasite if active host manipulation is responsible for increased risk proneness in sequentially infected fish. However, it does drain additional energy from its host. The additional changes in

the behaviour of sequentially infected hosts caused by the parasite from day 31 do not present true manipulation by the parasite from day 31.

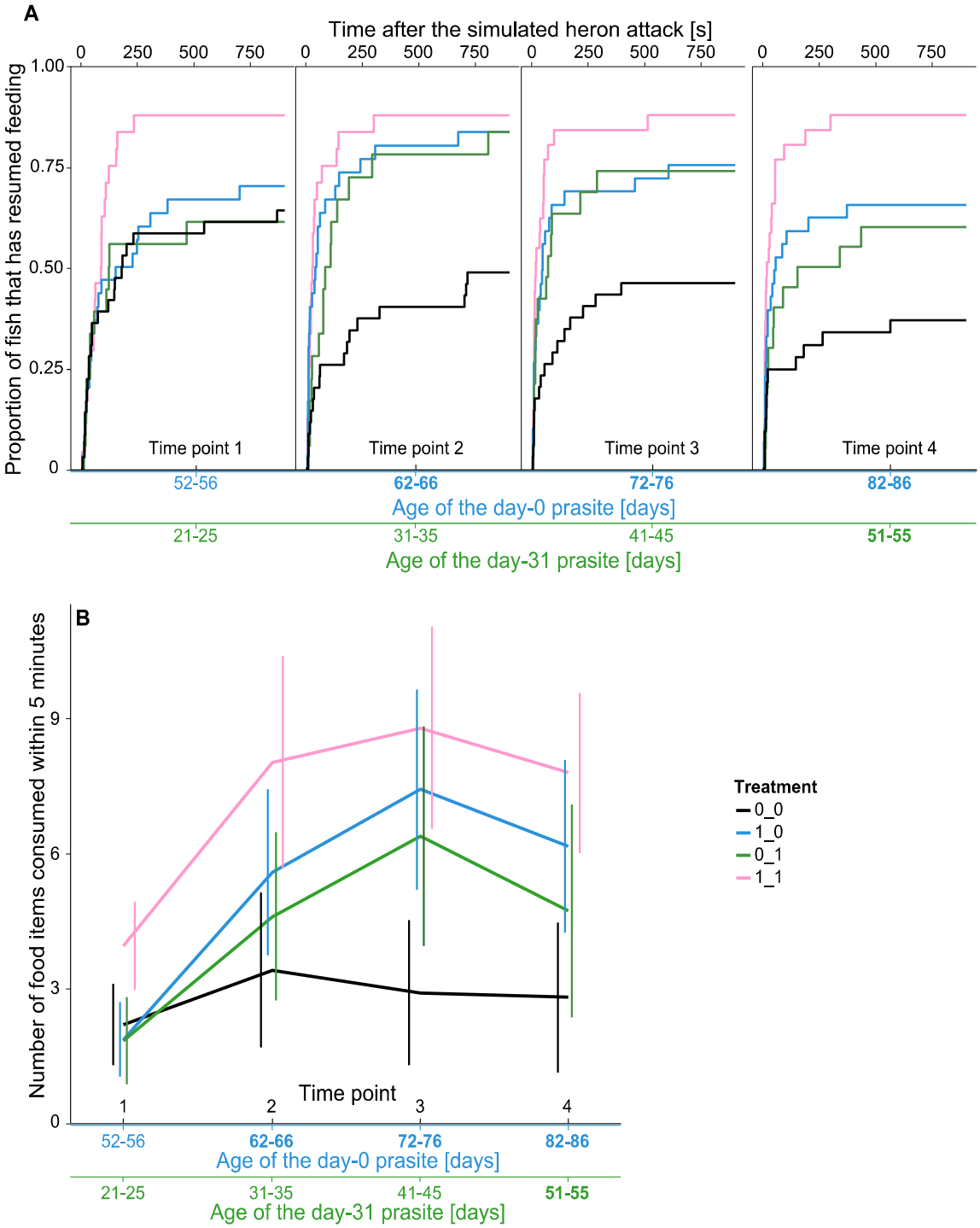

Figure II. 2: Behavioural observations after a simulated heron attack. A: Latency to resume feeding, B: Number of food items consumed within 5 minutes. Bold numbers on the x-axis indicate that a parasite of that age was infective. 0_0: Fish not infected by any parasite. 1_0: Fish infected by one parasite on day 0, 0_1: Fish infected by one parasite on day 31, 1_1: Fish infected by one parasite on day 0 plus one on day 31. N: Time point 1: 0_0: 35, 1_0: 29, 0_1: 19, 1_1: 24, Time point 2: 0_0: 34, 1_0: 29, 0_1: 18, 1_1: 24, Time point 3: 0_0: 33, 1_0: 28, 0_1: 18, 1_1: 26, Time point 4: 0_0: 31, 1_0: 29, 0_1: 19, 1_1: 26.

Experiment II

Effect of parasite infection

In the early group (i.e. prior to reaching infectivity) infection did not significantly affect the fish's latency to re-emerge from hiding ($ChiSq_{85,1}$=1.459, p=0.2271). In the late group (i.e. after reaching infectivity) infection did have a significant effect on when fish re-emerged from hiding ($ChiSq_{82,1}$=10.511, p=0.0012). Contrary to the manipulation hypothesis, infected fish were less likely to re-emerge than uninfected fish. The time fish spend hiding both before and after the simulated heron attack was never significantly affected by infection (p>0.9, Table II. S6, Figure II. 3).

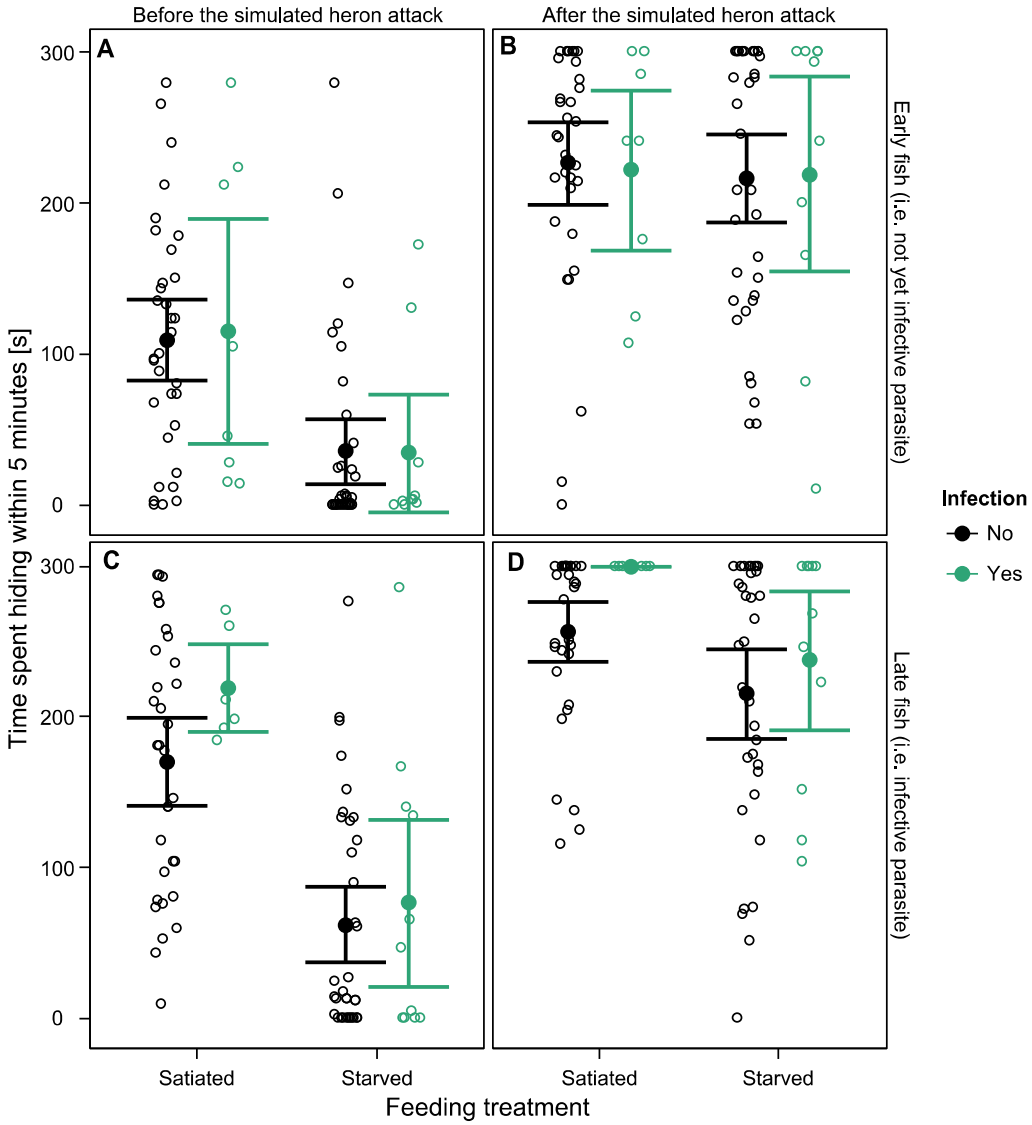

Figure II. 3: Time spent hiding before (A, C) and after (B, D) a simulated heron attack for early (A, B) and late fish (C, D). We recorded the time fish spent hiding for 5 minutes starting 30 seconds after the fish had entered the tank or, if it was hiding at that time, once it had left hiding (before the simulated heron attack) and for 5 minutes starting 10 seconds after the simulated heron attack irrespective of fish were hiding at that time or not (after the simulated heron attack). Error bars indicate 95% CI. N: Early: Satiated: Uninfected:33, infected:8, Starved: Uninfected: 36, infected:10, Late: Satiated: Uninfected:33, infected:6, Starved: Uninfected: 35, infected:11.

Effect of feeding treatment

How long after the simulated heron attack, fish re-emerged from hiding was not affected by the feeding treatment in early fish ($ChiSq_{84,1}$=1.238, p=0.2659), but in late fish starved fish emerged sooner ($ChiSq_{81,1}$=13.815, p=0.0002). The time fish spent hiding was affected by the feeding treatment and its interaction with the time interval (before vs. after the simulated heron attack) both in early and late fish (p<0.0001, Tables II. S6). Post hoc tests revealed that starved fish spent more time hiding but only before the simulated heron attack (p<0.0001, Table II. S7, Figure II. 3 A, C) and not thereafter (p>0.6, Tables II. S7, Figure II. 3 B, D).

If energy drain is responsible for behavioural alterations by *S. solidus*, hungry fish should be less risk averse than satiated fish irrespective of infection. By contrast, hunger levels should not matter in infected fish if active manipulation mechanisms were responsible. There was no significant interaction between infection and feeding treatment, neither for when fish re-emerged from hiding (early: $ChiSq_{83,1}$=1.113, p=0.2915, late: $ChiSq_{80,1}$=3.422, p=0.064), nor for the amount of time they spend hiding (p>0.7, Table II. S6). The risk averseness of sticklebacks seems similarly affected by starvation in both infected and uninfected fish. Thus, also the infective parasite did not truly manipulate its host.

Discussion

If two parasites with different interests share the same host, there is potential for conflict. One parasite might win this conflict (Sparkes et al. 2004; Dianne et al. 2010; Hafer and Milinski 2015), but the other parasite should never enhance the winning parasite's manipulation. However, in the present study the loosing parasite enhances the winner's manipulation when three-spined sticklebacks were experimentally infected by two *Schistocephalus solidus* at different times. Fish infected by both an already infective and a not yet infective *S. solidus* show a stronger reduction in risk averseness than fish infected by either parasite alone. Bird predation upon their shared host will allow the infective parasite to complete its life cycle and reproduce, but for the not yet infective parasite it will be fatal. It cannot reproduce, yet. Why should the not yet infective parasite enhance manipulation that is potentially fatal to it? No active host manipulation should evolve to such an effect. However, active host manipulation is not the only way by which parasites affect their host's behaviour; they also drain energy from it, forcing it to consume more food even if this comes at the cost of exposing themselves to increased predation. Such energy drain will also be exerted by a not yet infective parasite. It can, unlike true host manipulation explain why fish infected with both an infective and a not yet infective parasite behave in a more risk prone manner than those infected by the infective parasite only. Thus, this experiment shows that the not yet infective parasite does not manipulate the stickleback's behaviour.

In order to test this hypothesis further we compared fish with not yet infective or infective parasites to uninfected fish when they had either been starved for three days or fed to satiation. In our first experiment only fish that had been starved for two days have been used to test the effect of *S. solidus* on host behaviour.

The most decisive experiment to test for true host manipulation versus increased energy drain, however, is to test satiated fish. Increased energy drain should not increase the risk taking of fish that do not require any additional energy because they are satiated. By contrast, host manipulation that has evolved for this specific purpose should be independent of hunger levels and also alter the risk taking of satiated fish. In the second experiment we do not observe any altered behaviour due to a parasite infection in fish that have been fed to satiation. Thus, also infective *S. solidus* do not manipulate their stickleback host's behaviour. Previous studies that have tested for an effect of satiation on host manipulation by *S. solidus* have reported that also naturally infected fish seem to act just as risk averse as uninfected ones when satiated (Giles 1987b; Barber et al. 1995). This is in perfect agreement with our hypothesis that host manipulation in *S. solidus* infected sticklebacks is due to increased energy drain but inconsistent with active host manipulation.

Surprisingly, unlike in experiment I and other previous studies (Giles 1983, 1987b; Barber and Huntingford 1995; Barber et al. 1995, 2004) we do not observe any effect of *S. solidus* infection even in fish that have been starved for three days. In heavily infected fish *S. solidus* probably compresses the gut to such an extent that there is not enough space left for food preventing such fish from ever becoming satiated (Milinski 1985), this is again a side effect caused by the parasite and no active manipulation. To avoid this side effect in experiment II that depended on having infected fish that could become fully satiated we used older (and hence larger) fish. In juvenile fish parasites affect host performance more easily because even uninfected juveniles might be closer to their physiological and morphological limits than adults (McElroy and de Buron 2014). This might make them particularly prone to energy drain. This together with their size difference to juvenile fish larger parasites could have made fish in our first experiment more prone to behavioural changes caused by side-effects than those in our second experiment (supplementary information II. 3). Active host manipulation, however, should not stop in larger and older hosts.

Even if apparent host manipulation is caused by side-effects, selection might still act on it. Selection will favour behavioural changes that enhance transmission at the right time and select against traits that do not, irrespective of their underlying mechanisms (Thomas et al. 2005; Poulin 2010; Moore 2013). In the present study several aspects of host manipulation by *S. solidus* in its stickleback host appear suboptimal. Host manipulation should set in once an optimal time for transmission is reached (Parker et al. 2009c). *Schistocephalus solidus* is only rarely able to become reproductive before reaching roughly 50 mg in its fish host (Tierney and Crompton 1992). Accordingly, any increase in predation susceptibility before that time would not be adaptive in terms of transmission, though it might be adaptive to some extend because the parasite needs the fish to provide extra energy. If *S. solidus* increases hunger levels by restricting the space in the body cavity (Milinski 1985), this might cause in addition to energy drain satiation independent extra apparent host manipulation once *S. solidus* has reached a certain relative size compared to its host. In nature hosts are usually much smaller than those that we used to avoid the compression effect in our second experiment. Copepods have the optimal prey size for juvenile sticklebacks; large sticklebacks are less likely to attack copepods, the first intermediate host of *S. solidus* (Christen and Milinski 2005). From the infective parasite's point of view it would also be ideal if host manipulation is independent of hunger levels since this

would increase predation even more. In the laboratory conditions can often be very benign and food readily available. By contrast in nature complete satiation might be much rarer especially in infected fish whose competitive ability is impaired (Milinski 1986; Barber and Ruxton 1998). In addition, as long as hosts have the usual small size, their hunger is maintained at a high level because of the parasite's compressing their gut. There might not be much potential for improving apparent manipulation by additional true host manipulation.

Our results strongly suggest that apparent host manipulation by *S. solidus* in its stickleback host occurs as inevitable side-effect of infection. Through draining energy from the host and restricting space in the gut the parasite moves the fish's trade-off between feeding and avoiding predation (Milinski and Heller 1978) towards feeding thus exposing it to predators. Selection might not be able to improve the resulting "manipulation" effect by adding an extra mechanism. The not yet infective parasite appears to be the looser. Multiple infections of *S. solidus* in three-spined sticklebacks are frequent in nature (Arme and Owen 1967; Heins et al. 2002). Selection should favour parasites that can counteract enhanced predation susceptibility before they reach infectivity. This would require a true manipulation mechanism, which obviously does not exist. On the contrary through its additional energy drain a not yet infective parasite aggravates its problem. In the first intermediate host, the copepod, the not yet infective *S. solidus* actively suppresses predation risk, but only when alone. A co-infecting infective *S. solidus* sabotages the not yet infective parasite's manipulation (Hafer and Milinski 2015), which is the looser again. In the present study we found that the not yet infective parasite does not reduce predation risk of its stickleback host, not even when it is alone, depicting a new puzzle. Upon infection the stickleback is normally too small to allow *S. solidus* to grow large enough to become infective. Therefore is has to restrict its growth to allow the fish to grow until big enough (Christen and Milinski 2005). Letting the fish follow its optimal growth strategy and risk taking might thus prevent the parasite from manipulative interference. No true manipulation of stickleback behaviour seems to be adaptive for both un-infective and infective *S. solidus*, side-effects of infection fulfil the latter's needs.

Ethical statement

Animal experiments were conducted with permission of the 'Ministry of Energy, Agriculture, the Environment and Rural Areas' of the state of Schleswig-Holstein, Germany (reference number: V 313-72241.123-34).

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Chapter III

When parasites disagree: Evidence for parasite-induced sabotage of host manipulation

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Abstract

Host manipulation is a common parasite strategy to alter host behavior in a manner to enhance parasite fitness usually by increasing the parasite's transmission to the next host. In nature, hosts often harbour multiple parasites with agreeing or conflicting interests over host manipulation. Natural selection might drive such parasites to cooperation, compromise or sabotage. Sabotage would occur, if one parasite suppresses the manipulation of another. Experimental studies on the effect of multi-parasite interactions on host manipulation are scarce, clear experimental evidence for sabotage is elusive. We tested the effect of multiple infections on host manipulation using lab bred copepods experimentally infected with the trophically transmitted tapeworm *Schistocephalus solidus*. This parasite is known to manipulate its host depending on its own developmental stage. Co-infecting parasites with the same aim enhanced each other's manipulation but only after reaching infectivity. If the co-infecting parasites disagree over host manipulation, the infective parasite wins this conflict: the non-infective one has no effect. The winning (i.e. infective) parasite suppresses the manipulation of its non-infective competitor. This presents conclusive experimental evidence for both cooperation in and sabotage of host manipulation and hence a proof of principal that one parasite can alter and even neutralize manipulation by another.

Introduction

Parasites can modify their host's phenotype to their own benefit. Such host manipulation is known from a wide range of both host and parasite taxa (Holmes and Bethel 1972; Poulin and Thomas 1999; Moore 2002, 2013; Poulin 2010), including humans (Flegr 2013). In parasites with complex life cycles, it usually enhances a parasite's chances to pass on to the next host at the appropriate time point (Parker et al. 2009c). Before being able to infect the next host, some parasites lower their present host's predation susceptibility: premature predation even by the correct consecutive host would be fatal to the parasite (Koella et al. 2002; Hammerschmidt et al. 2009; Thomas et al. 2010; Dianne et al. 2011). Once the parasite is infective to the next host, manipulation increases transmission to that host, e.g. by increasing the current host's predation

susceptibility (Holmes and Bethel 1972; Poulin and Thomas 1999; Moore 2002, 2013; Poulin 2010). Such predation enhancement can also be a mere side-effect of the parasite's draining energy from the host, forcing it to shift its trade-off between avoiding predation and decreasing hunger towards the latter (Milinski 1990).

Most experimental studies on host manipulation investigated the effect of a single infection on host behaviour. In nature, hosts are usually infected by multiple parasites, typically from different species encountered sequentially (e.g. Kalbe et al. 2002). Manipulation by one parasite will affect every co-infecting parasite, even non-manipulating ones (Milinski 2014). Do parasites react to manipulation of a co-infecting parasite? If interests coincide, the presence of a second manipulator might be beneficial. Potential costs could be shared or manipulation be enhanced increasing transmission probability. Correlational evidence suggests that multiple parasites may indeed strengthen each other's manipulation if they have the same aim (reviewed by Cézilly et al. 2014).

By contrast, two co-infecting parasites with incompatible aims have a conflict over host manipulation, either because they manipulate in different directions or one parasite manipulates whereas the other one does not manipulate if its interest is best served by the host's normal behaviour. In both cases one parasite would benefit from "sabotaging", i.e. partly or completely suppressing the manipulation by the other parasite (Thomas et al. 2002a). Several studies provide correlational evidence for parasites being able to alter manipulation by another parasite. Most of these studies, however, used exclusively naturally infected hosts, making it impossible to decide whether the parasite really caused the observed alteration of host behaviour (reviewed by Cézilly et al. 2014). To our knowledge, only two studies used experimental infections to obtain hosts with parasites that had different aims. Thomas et al. (2002) experimentally cured and re-infected trematode-infected gammarids, i.e. small crustaceans, with nematodes that had appeared to sabotage manipulation by the trematodes in natural infections. The authors did, however, not find the previously observed sabotage. Dianne et al. (2010) experimentally infected gammarids with different stages of an acanthocephalan parasite and found suggestive evidence that the not yet infective stage might have sabotaged manipulation by the infective one. Both studies used wild caught hosts, which might have encountered various other parasites before. One preliminary study infected lab bred rats with two parasites known to affect the host's nervous system – including *Toxoplasma* – a common parasite also capable of manipulating human behavior. One parasite partly influenced the effect of manipulation by another through since no significant differences were found between the different parasites, it remains elusive to which extend there was actual conflict between the parasites or whether this observation was a mere side-effect (de Queiroz et al. 2013). Thus, sabotage may exist but was not stringently shown under experimentally controlled conditions.

Here we use the cestode *Schistocephalus solidus* and its copepod host to compare the effect of single with multiple infections on host behaviour. We study especially the outcome of a conflict between co-infecting parasites over host manipulation using lab bred, hence parasite free, hosts. *S. solidus* has a three-host life cycle. From the first intermediate host, a copepod, the parasite is trophically transmitted to the next host, the three-spined stickleback, a fish, which has to be subsequently consumed by a bird for the parasite to complete its life-cycle (Clarke 1954; Dubinina 1980). In the copepod, *S. solidus* initially reduces the activity of its host (Hammerschmidt et al. 2009) and thus the host's risk of being preyed upon (Weinreich et al. 2013). Once

ready for transmission, *S. solidus* switches the direction of host manipulation to increasing its host's activity (Wedekind and Milinski 1996; Hammerschmidt et al. 2009), risk taking (Jakobsen and Wedekind 1998), and predation susceptibility (Wedekind and Milinski 1996). We show that two co-infecting parasites (i) with the same aim cooperate, but (ii) with a conflict of interest sabotage each other's manipulation.

Materials and Methods

Hosts

Copepods (*Macrocyclops albidus*) came from a laboratory culture originated from populations from the "Neustaedter Binnenwasser", northern Germany, where sticklebacks are naturally infected by *S. solidus*. One day prior to the first exposure to the parasites, copepods were filtered from their home tanks and each individual copepod was transferred to a well in a 24-well plate with about 1 mL of water. To reduce variation with regards to the host, only adult male copepods were used. We used a total of 1992 copepods in two separate experiments (1248 in experiment 1, 744 in experiment 2). During both experiments, copepods were fed with five *Artemia* sp. nautili and the wells cleaned if necessary every other day (always a day on which no infections or behavioural recordings took place, i.e. day 1, 3, 5, 8, 10, 12, 14, 16, 18, 20 and 22 after the first infection). The copepods were kept at 18°C in a 16h/8h light/dark cycle. Since our behavioural essays included the reaction to disturbance, we took care to avoid other disturbances to prevent previous habituation to our test.

Parasites

Schistocephalus solidus were bred in an in vitro system in the laboratory (Smyth 1946; Wedekind 1997). We used offspring from parasites dissected from naturally infected fish caught at the "Neustaedter Binnenwasser", northern Germany. Currently, infection rates in sticklebacks are low (below 1%) in this population but had been above 30% some years ago (unpublished data). Because of a considerable time interval between our two experiments, we used different parasite families in each experiment. Eggs were stored in the fridge (4°C) until use. Prior to infection they were incubated for three weeks at 20°C in the dark and then exposed to light overnight to induce the coracidia to hatch (Dubinina 1980).

Infections

Infections took place at two different time points, the day after the copepods had been distributed onto the plates (day 0) and one week later (day 7). For experiment 1, each copepod was exposed to zero, one or two parasites on each of these days in a manner that resulted in six different treatments (Figure III. 1): Unexposed controls (C), singly exposed to one parasite on day 0 (Sing_t0), simultaneously exposed to two parasites on day 0 (Sim_t0), singly exposed to one parasite on day 7 (Sing_t7), simultaneously exposed to two parasites on

day 7 (Sim_t7) and sequentially exposed to two parasites, i.e. exposed to a single parasite each on day 0 and day 7 (Seq) (Figure III. 1). In order to account for the size differences between the parasites from the first and the second infection in sequential infections and potentially resulting differences in how strongly they were able to manipulate, we conducted an additional experiment (experiment 2) in which we infected copepods with one parasite on day 0 and with either zero (Sing_t0), one (Seq) or two (Seq2) additional parasites and day 7. We were unable to measure parasite size within our experiment because that would have exposed the copepods to substantial stress. However, we did so during a preliminary study to confirm that two non-infective parasites could make up about the same volume as one infective one (supporting results III. 2, Figure III. S3). Several copepods were not exposed at all to obtain uninfected controls (C) or only on day 7 to verify the timing of manipulation by a non-infective parasite when alone (Sing_t7) (Figure III. 1).

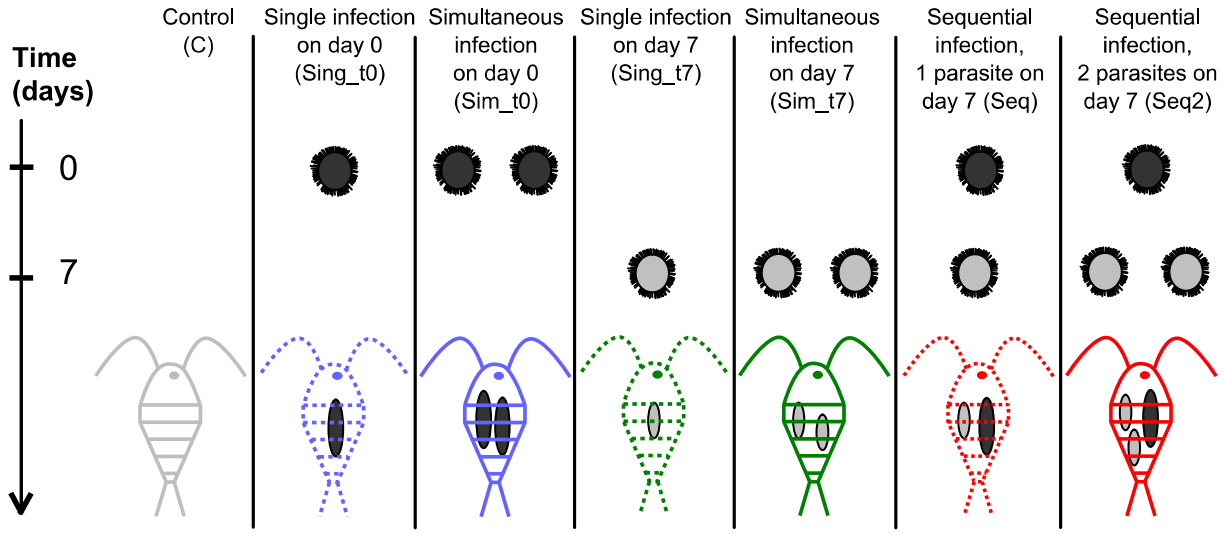

Figure III. 1: Timing of infections with different numbers of parasites to achieve the required treatments. Copepods were exposed to 0, 1 or 2 Schistocephalus solidus on day 0 (t0) and on day 7 (t7) in a manner that resulted in seven different treatments.

We used three (experiment 1) or four (experiment 2) different parasite families to infect copepods and, if a copepod received more than one parasite, they always originated from different families. Treatments were evenly distributed over all plates and randomly arranged on each plate.

To verify that an infection had occurred we placed each copepod under a microscope. Male copepods are transparent making it possible to see any parasite within the living copepod. This took place only after all behavioural recordings had been completed in order not to interfere with copepod behaviour (experiment 1: day 23 and 24, experiment 2: day 21). By this time we expected any parasite to have become infective to the subsequent host. Unfortunately that did not allow us to document the development of individual parasites. However, previous studies found that the rate of development shows little flexibility. Within 11 days post infection, more than 80% of parasites developed a cercomer, which is a good indication that the parasite will soon become infective to the next host (Benesh 2010a,b). Copepods that died before day 24 but after day 13 (experiment 1) or day 19 (experiment 2) were checked in the same manner, though a determination of the infection status was only possible in those copepods that had not yet started to decay. Their behaviour was only used until 3 days prior to their death to exclude the behaviour of dying copepods from the data set. We only included copepods in the subsequent analysis that were correctly infected according to their treatment by

all parasites they had been exposed to. That way we could exclude that differences between treatments were caused by initial differences between copepods, which were also responsible for whether or not a copepod, exposed to a coracidium, was indeed infected by this coracidium (or altered by the effect of a failed infection, which cannot be excluded in mass infections). This resulted in a total of 147 copepods for experiment 1 (C: 41, Sing_t0: 25, Sing_t7: 27, Sim_t0: 11, Sim_t7: 25, Seq: 18) that could be analysed. Of those copepods used for the analysis, 25 died during the experiment. For experiment 2, we could obtain data from a total of 111 copepods, one of which died during the experiment (C: 20, Sing_t0: 25, Sing_t7: 22, Seq: 28, Seq2: 26).

Behavioural recordings and analysis

Copepod behaviour was recorded by carefully placing a 24-well plate with copepods on an apparatus that dropped it by 3 mm in a standardized manner to simulate a failed predator attack (Hammerschmidt et al. 2009). After such a predator attack, the predator is likely to be still present for some time and likely to try attacking the copepod again. Hence the period after the simulated predator attack should be perceived as one of increased predation risk by the copepod. Under this circumstances predation avoidance should be especially crucial and predation enhancement most efficient and we would hence expect them to be strongest. The drop took place after the plate had been on the apparatus for one minute. Starting just before the drop, we video recorded the copepods on the plate for 15 minutes with a camera (Panasonic Super DynamicWV-BP550). Behavioural recordings took place every other day starting on day 9 until day 23 (experiment 1) or day 21 (experiment 2), always on the day when copepods were not fed.

We analysed copepod behaviour (i.e. activity) during one minute right after the simulated predator attack when, following a movement to escape predation, copepods should reduce activity to avoid detection by a potential predator (starting 10 seconds after the simulated predator attack to avoid the initial escape reaction, see Hammerschmidt et al. 2009) and at the end of the recorded period (i.e. between 14 and 15 minutes after the simulated predator attack), when the copepods could be assumed to have recovered from the simulated predator attack. Using the manual tracking plugin within image J (Rasband 2008), we recorded whether or not each copepod moved within each two second interval. All analyses were done blindly with regards to the copepod's treatment.

Statistical analysis

Data were analysed in R (R Development Core Team 2010) using generalized linear mixed models in the lme4 package (Bates et al. 2014). We used copepod identity as random effects including the day after the first infection to account for the presence of intra-individual variation between days and the period in the recording to account for intra-individual variation over time. We fitted a model for the time moved as response variable using binomial distribution to account for the distribution of the data. We further included both the day after the first infection and period in the recording (i.e. after a simulated predator attack vs. after a recovery period). We stepwise added the treatment and all its interactions with day and the period in the recording to the model. Separate models were fitted for experiment 1 and 2 since not all treatments were present in both.

Subsequently, we performed likelihood ratio tests to compare models and find those that gave the best fit. A model was accepted if it was significantly better than a less complex model at explaining the data. The complete outputs of the models are presented in Table III. S1.

For each treatment and period in the recording (i.e. after a simulated predator attack/ after a recovery period) we performed a separate Tukey test using general linear hypotheses within the multcomp package in R (Hothorn et al. 2008) to determine between which consecutive days significant chances in host behaviour took place. The same was done for each day and period in the recording to find out when and between which treatments differences occurred. Only those statistics directly relevant for our question are reported in the results. For a complete overview of the statistical results, refer to Tables III. S2 – III. S5.

Results

The behavior of the copepod hosts was significantly influenced by the three way interaction between the parasite treatment they received, the day *post infection* on which the recording took place, and the period in the recording (i.e. after a simulated predator attack or after a recovery period) ($p<0.001$, See Table III. S1 for further information). Hence, we conducted Tukey's HSD tests for multiple comparisons for each treatment or day and period in the recording to determine when copepod activity changed significantly between days within each treatment and when and between which treatments significant differences occurred. Only p values for the multiple comparisons are reported here. Please refer to Table III. S2 - III. S5 for exact statistical outputs. Here we present only the results we observed directly after a simulated predator attack, because results once the copepods had had time to recover were similar through less pronounced. They are presented in the supporting results III. 1 and Figure III. S1 and III. S2.

Change of copepod host activity over time

We measured the activity of the copepods right after a simulated predator attack. We expected that shortly after infection the parasite would start manipulating its copepod host by decreasing its activity and thus its predation risk (predation suppression) because it would be too early for the parasite to be transmitted to the next host. Once the parasite has reached infectivity for the next host, copepod activity should be increased (predation enhancement) as shown previously (Hammerschmidt et al. 2009). The initial decrease in host activity has been studied before (Hammerschmidt et al. 2009), so that we started recording of host behavior only from day 9 in the experiment, i.e. 9 days after the first infection that took place on day 0, just before the switch in host manipulation is expected to occur in copepods singly infected on day 0 (dashed blue line in Figure III. 2). After the parasites had become infective, copepod activity increased as expected between day 9 and 11, and 11 and 13 ($p<0.001$). Those copepods that were singly infected by one parasite on day 7 (dashed green line in Figure III. 2) displayed the expected delay and showed initial decrease in activity (predation suppression) between day 9 and 11 (i.e. when the parasite was between 2 and 4 days old, $p=0.003$). They also displayed the expected increase in activity after the parasite reached infectivity (predation enhancement,

p<0.001) at the same time *post infection* when copepods singly infected on day 0 showed increased activity (between day 9 and 13 *post infection*, i.e. between day 15 and 19 in the experiment). Note that parasites administered to copepods on day 0 have always been for 7 days longer in the copepod than parasites infecting copepods on day 7 when their behaviour is recorded (Figure III. 1). The control group (unexposed copepods) did not show any significant changes in host activity throughout the course of the experiment (grey line in Figure III. 2, p>0.5).

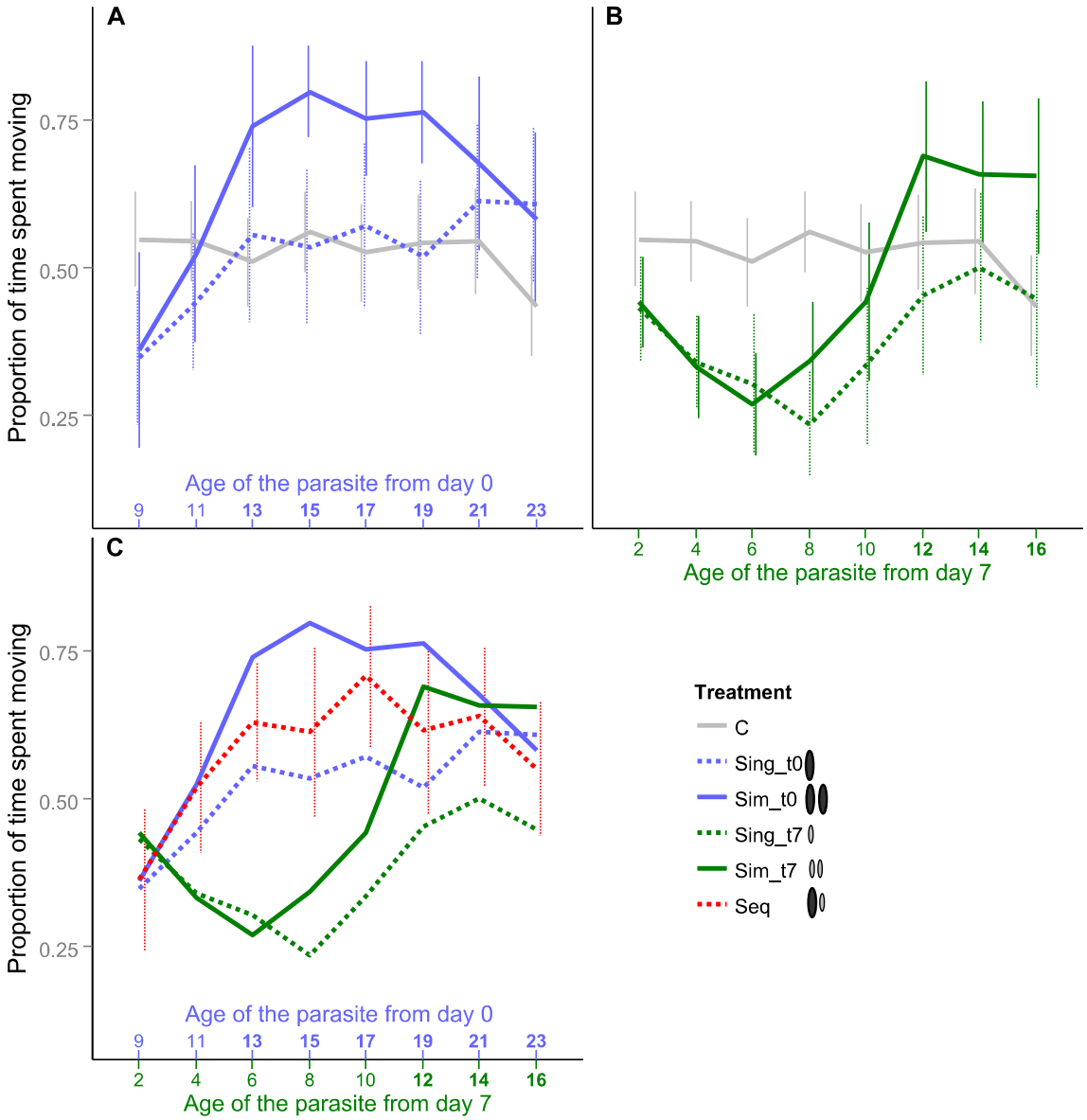

Figure III. 2: Activity of copepods according to treatment, right after a simulated predator attack. Error bars indicate 95% CI. Bold numbers on the X-axis indicated that a parasite of that age was infective. A: Copepods infected on day 0, B: Copepods infected on day 7, C: All treatments. Error bars from the treatments already presented in A and B have been omitted for better readability. C: unexposed control copepods, Sing_t0: copepods singly infected with one parasite on day 0, Sim_t0: copepods simultaneously infected with two parasites on day 0, Sing_t7: copepods singly infected with one parasite on day 7, Sim_t7: copepods simultaneously infected with two parasites on day 7, Seq: copepods sequentially infected with two parasites, one each on day 0 plus day 7.

We found significant differences between the behaviour of control copepods (grey line) and copepods singly infected at either infection time point (day 0 or day 7, dashed blue and green line, respectively, p<0.03) during expected predation suppression (on day 9 for copepods infected on day 0 and between day 11 and day

13 in the experiment for copepods infected on day 7, i.e. between day 4 and 9 *post infection*). After host activity had increased again (i.e. once the parasite was at least 10 days old), no differences between control copepods and singly infected copepods were significant ($p>0.2$). Hammerschmidt et al. (2009) observed both predation suppression before the parasite reached infectivity and predation enhancement after it had reached infectivity. While we can confirm the existence of predation suppression and a switch in host manipulation to enhance predation, we did not observe actual predation enhancement beyond the level of control copepods. However, Hammerschmidt et al. (2009) found predation enhancement especially when measuring the time copepods needed to recover from a simulated predator attack, and much less with regards to the copepods' activity, which is what we focused on in this study. Thus, after generally having confirmed previous findings we can test for synergy and conflict over host manipulation in experimental double infections.

Potential synergy of parasites in simultaneous double infections

We expected that in copepods that harboured two parasites of the same age and hence the same interest, the parasites should strengthen each other's manipulation. Such copepods behaved similarly compared to those infected with just one parasite at the same time point: Copepods infected with two parasites on day 0 (continuous blue line in Figure III. 2) significantly increased their activity between day 9 and 13, i.e. when their parasites became infective ($p<0.001$). In copepods infected with two parasites on day 7 (continuous green line), the onset of manipulation was marked by a significant decrease in host activity between day 9 and 11 ($p<0.001$), which was followed by a significant increase between day 15 and 19 ($p<0.004$), i.e. when also these parasites had reached infectivity. Behaviour of copepods singly or simultaneously infected on day 7 was significantly different from unexposed control copepods during expected predation suppression, i.e. between day 11 and 15 ($p<0.02$), but not on any other day ($p>0.07$). Copepods simultaneously infected on day 0 tended to be more active than controls on day 15 ($p=0.059$), but not on any other day ($p>0.09$).

We found indeed evidence for synergy effects during predation enhancement: simultaneously-infected copepods had a significantly higher activity than singly infected copepods from the same infection time point (copepods infected on day 0, day 15: $p=0.049$, blue lines, copepods infected on day 7, day 19: $p=0.026$, green lines). These differences were significant only after the parasites had reached infectivity, i.e. during predation enhancement, but not before ($p>0.6$), i.e. during predation suppression.

The outcome of a conflict between parasites over host manipulation

If one parasite is infective (and hence should enhance its host's predation risk) and the other one is non-infective (and we therefore expect predation suppression), there is potential for a conflict over the direction of host manipulation between the two parasites. We confirmed that such a conflict exists by comparing copepods singly- or simultaneously infected on day 0 to those infected with the same number of parasites on day 7: Copepods singly infected on day 0 (dashed blue line) were significantly more active than copepods singly infected on day 7 (dashed green line) from day 13 to day 17 ($p<0.03$). The same was true for simultaneously-infected copepods (continuous blue and green line) ($p<0.001$). We did not observe any significant differences

between parasites infected on day 0 or day 7 with the same number of parasites on any other day ($p>0.1$). These results defined the time interval during which parasites that infected the copepod on day 0 were already infective and inducing predation enhancement and parasites that infected the copepod on day 7 and were not infective, yet and induced predation suppression, indicating the time window of conflict. If parasites from either infection time point (day 0 or day 7) have about equal strength of manipulation, we would expect that the behaviour of copepods with one infective (i.e. one parasite that infected the copepod on day 0) plus one non-infective parasite (i.e. one parasite that infected the copepod on day 7), (dashed red line) is intermediate between that of copepods with parasites from only one infection time point (either day 0 or day 7, blue and green lines) during the window of conflict i.e. the dashed red line should be between the blue and the green lines in Figure III. 2.

During the period of conflict over host manipulation, copepods that were sequentially infected with one parasite on day 0 plus one on day 7 (dashed red line in Figure III. 2) differed significantly only from copepods singly- or simultaneously infected on day 7 (green lines, day 13 to 17: $p<0.050$). Throughout the experiment, those copepods sequentially infected on day 0 plus on day 7 (dashed red line) never differed significantly from copepods singly or simultaneously infected on day 0 (blue lines) ($p>0.4$). Thus, the non-infective parasite that infected the copepod on day 7 has no detectable effect in sequential infections with an already infective parasite administered to the copepod on day 0.

Consequently, changes over time in the behaviour of copepods sequentially infected on day 0 and day 7 (dashed red line) mostly resembled copepods infected only on day 0 (blue lines): Copepod activity increased from one day to the next when the parasite administered to the copepod on day 0 became infective to the next host between day 9 and 13 *post infection* (dashed red line, $p<0.05$). However, unlike in copepods only infected on day 0 (blue lines) copepod activity significantly increased further between day 15 and 17 ($p=0.015$). This later increase occurred at the time when the parasite administered on day 7 should be reaching infectivity (between day 8 and 10 *post infection*). At this time the conflict between the two parasites vanishes and synergy may begin. This fits well with the fact that parasites in simultaneously-infected copepods enhance each other's manipulation once both parasites have reached infectivity (see above).

In the most parsimonious mechanistic scenario where both disagreeing parasites continue to manipulate as if alone, we had expected the outcome of this conflict to be somewhat intermediate. This is not the case. Rather, the parasite administered on day 0 wins the conflict, making its host behave indistinguishably from a host infected only on day 0 and not on day 7. Thus the infective parasite that infected the copepod on day 0 suppresses the manipulation by the non-infective parasite that infected the copepod on day 7.

Equal potential strength of parasites that are at a conflict over host manipulation

The missing effect of the non-infective parasite in copepods sequentially infected on day 0 plus day 7 could be due to a size difference between the infective parasite from day 0 and the non-infective parasite from day 7.

Parasites administered on day 0 were always larger than those administered on day 7 (supporting results III. 2, Figure III. S3). This could help the infective parasite from day 0 to overpower the non-infective parasite from day 7. Hence, in a separate experiment, we compared copepods sequentially infected with one parasite on day 0 plus two on day 7 (continuous red line in Figure III. 3) to copepods infected only on day 0 (dashed blue line in Figure III. 3), only on day 7 (dashed green line in Figure III. 3) and copepods sequentially infected with one parasite on day 0 plus one on day 7 (dashed red line in Figure III. 3). Copepods sequentially infected with one parasite on day 0 plus two on day 7 (continuous red line) were never significantly different from copepods sequentially infected with one parasite on day 0 plus one on day 7 (dashed red line, $p>0.1$). So, combined volumes of two non-infective parasites from day 7 did not make a detectable difference to only one non-infective parasite from day 7 in sequentially-infected copepods already infected by a parasite on day 0. Additionally, copepods sequentially infected with one parasite on day 0 plus two on day 7 (continuous red line) never differed significantly from copepods only infected on day 0 (dashed blue line, $p>0.3$). They did, however, differ from copepods infected only on day 7 (dashed green line) between day 13 and 17. This was only a trend on day 13 ($p=0.056$), but significant thereafter ($p<0.01$). Thus, one non-infective parasite from day 7 alone in a copepod has a stronger effect than even two non-infective parasites from day 7 if their host was previously infected by one parasite on day 0 that is now infective.

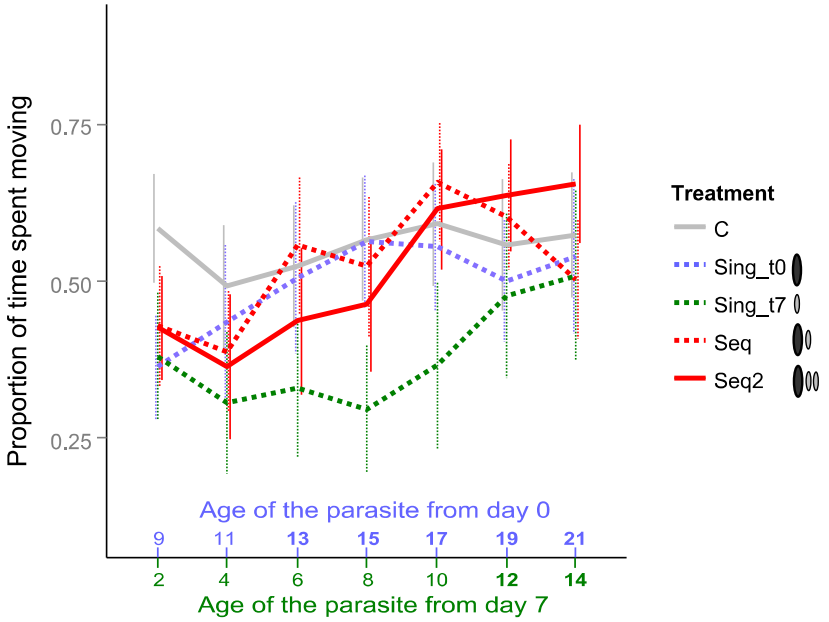

Figure III. 3: Activity of copepods according to treatment, right after a simulated predator attack. Error bars indicate 95% CI. Bold numbers on the X-axis indicated that a parasite of that age was infective. C: unexposed control copepods, Sing_t0: copepods singly infected with one parasite on day 0, Sing_t7: copepods singly infected with one parasite on day 7, Seq: copepods sequentially infected with two parasites, one each on day 0 plus day 7, Seq2: copepods sequentially infected with three parasites, one on day 0 plus two on day 7.

In conclusion, we find no significant effect of the second non-infective parasite from day 7 in copepods sequentially infected on day 0 plus day 7. The two parasites from day 7 have about the same volume as the one parasite from day 0 (supporting results III. 2, Figure III. S3). Thus, if a copepod is infected by two non-infective parasites, both together should have the strength to suppress the manipulated activity of the host to reduce its predation risk to an intermediate level. Instead, it is still the single infective one that wins the conflict.

Discussion

A non-infective *Schistocephalus solidus* parasite should prevent its current copepod host from being eaten by the next host, a stickleback fish – it dies when transmitted too early, whereas an infective parasite should increase its copepod's predation risk - it continues its life cycle when transmitted. This is what both achieve when alone in a copepod (Wedekind and Milinski 1996; Hammerschmidt et al. 2009; Benesh 2010b; Weinreich et al. 2013). When an infective and a non-infective *S. solidus* parasite share the same copepod, they are at a conflict over the direction of host manipulation. The infective parasite clearly wins the conflict while the non-infective one seems to have no effect on host behaviour at all. This is true not only when the predation risk is high but also after it has returned to normal (see supporting results III. 1).

Why does the non-infective parasite fail to reduce manipulation by the infective one, which is potentially disastrous for its fitness? When each parasite is alone, the non-infective one has a large effect on host behaviour while the effect of an infective one was not even measurable in our study. In coinfections, the infective parasite due to its larger volume could have an advantage and be able to produce more host manipulation. We controlled for this by using also two non-infective parasites to allow them to gain about the same volume (and an even larger surface) than the infective parasite. They remained unable to have a distinguishable effect on host behaviour. This seems to be the case even if 3 non-infective parasites are used (supporting results III. 2, Figure III. S4). Parasites can maximize their fitness by being transmitted to the next host at an optimal time point (Hammerschmidt et al. 2009; Parker et al. 2009c). Additionally, by infecting the subsequent stickleback host before the younger co-infecting parasite is ready for transmission, the infective parasite can exclude competition in the subsequent host. Nevertheless, a parasite transmitted later than optimal may still complete its life cycle and reproduce successfully. In contrast, a parasite that is transmitted too early will always fail to infect a fish, achieving a fitness of zero (Hammerschmidt et al. 2009).

Clearly, the non-infective parasite has nothing to gain from facilitating the transmission of the infective one by reducing its own manipulation. In nature it is unlikely, in our experiment excluded that two parasite larvae that are independently consumed by a copepod are close kin. Reallocating energy saved from not manipulating to faster development is no better option. Benesh (2010) found no significant correlation between host manipulation and growth and development of individual parasites. It is plausible that the infective parasite wins the conflict over host manipulation if it actively suppresses the manipulation exerted by the non-infective one: it would be transmitted at an optimal time point. To our knowledge, our findings present the first clear evidence that one parasite successfully sabotages the host manipulating of a co-infecting parasite under strictly experimental conditions.

Any opposition of the non-infective parasite to being suppressed will not be favoured by selection if the mortality of a copepod already harbouring an infective parasite is so high in nature that the second parasite will not reach infectivity. A similar scenario with one side having no fitness gain from opposing to being exploited are the slaves that are stolen as pupae by slave maker ants from foreign nests and brought to their own nest where the slaves raise the slave maker queen's offspring. Usually workers win the conflict with their queen over the sex ratio of the next generation, which is 3:1 in favour of female reproductives in ants (Trivers

and Hare 1976). Having no fitness in the slave maker nest anyway, ant slaves do not gain from opposing to manipulation by the slave maker queen, thus a mutant would not pass on any genes, they produce a 1:1 sex ratio completely in line with the slave maker queen's interests (Nonacs 1986).

If two parasites sharing a host have the same interests, either predation suppression or enhancement, both may profit from amplifying each other's manipulation and/or sharing potential costs. We find that after both parasites reached infectivity they increased host activity more than a single one does. This agrees with findings of an observational study (Urdal et al. 1995). Wedekind and Milinski (1996) found a positive correlation between the activity of both infected and uninfected copepods and predation susceptibility. Thus, an additional increase in host activity through manipulation would lead to predation enhancement. Two non-infective parasites did, however, not amplify each other's manipulation in the present study, nor in a prior observational study (Urdal et al. 1995). Has the parasite an optimal level of manipulation it attempts to reach or does any increase in manipulation convey a fitness benefit? This might well differ before and after reaching infectivity. During predation suppression, decreasing host activity below a certain level might be disadvantageous for the parasite. It might prevent its host from consuming enough energy to allow the parasite to ever reach infectivity, especially when two parasites compete for energy. Two *S. solidus* that share a copepod host grow to a smaller size (Michaud et al. 2006). Accordingly, the number of non-infective parasites has no significant effect on predation susceptibility of copepods infected with non-infective *S. solidus* (Weinreich et al. 2013). However, non-infective parasites may share the potential cost of manipulation.

For a parasite to evolve to either cooperate with a conspecific or to sabotage its manipulation, selection pressures have to be high enough. They will depend largely on the likelihood for a parasite to encounter such a conspecific (Rigaud and Haine 2005). Despite a very low prevalence of *Schistocephalus solidus* in its copepod host, double infections do occur, albeit rarely (Zander et al. 1994). This seems to be a general pattern for cestode-copepod systems (e.g. Zander et al. 1994; Pasternak et al. 1995; Hanzelová and Gerdeaux 2003). Despite those usually low infection rates, there is some evidence that *S. solidus* has evolved strategies to deal with the presence of conspecifics in its copepod host in addition to the present study (Wedekind 1997; Michaud et al. 2006). In both, the second intermediate fish host (Arme and Owen 1967; Heins et al. 2002) and the definite bird host (e.g. Chubb et al. 1995), very high infection intensities can occur. It would hence be plausible that *S. solidus* prevalence in copepods is strongly increased locally, e.g. underneath roosting trees were highly infected birds defecate (Michaud et al. 2006). The frequency of coinfections typically correlates positively with parasite prevalence (Louhi et al. 2013).

Naturally, the results of our study raise questions about the underlying mechanisms. A parsimonious mechanistic explanation would require active manipulation only for one type of manipulation (i.e. predation suppression or predation enhancement) and/or the switch in host manipulation. Predation suppression via decreased activity prior to reaching infectivity could be a stress response to infection (e.g. Poulin 1995, 2010; Thomas et al. 2005; Moore 2013). However, doubled stress by two non-infective parasites had no additional effect. Moreover, such a stress response would have to be switched off precisely when the parasite becomes infective. The subsequent predation enhancement (i.e. increased activity) would not require actual manipulation. It could be due to increased energy drain inevitably caused by growing parasite(s) (Milinski

1990). Animals optimally trade off feeding and avoiding predation, shifting the compromise to the higher need (Milinski and Heller 1978). Higher energy drain would lead to accepting higher predation risk. Naturally, this effect of energy drain would also be caused by non-infective parasites and needs to be suppressed or counterbalanced. Sabotage by the infective parasite of the non-infective one's manipulation could be done with the same mechanism with which the infective parasite switches from predation suppression to predation enhancement. This hypothesis implies changing a hormone to a pheromone and probably producing the substance in higher quantity. Further studies of the mechanisms underlying host manipulation will be necessary in order to understand how one parasite manages to sabotage another parasite's manipulation.

One very common mechanism of host manipulation seems to be the modification of neuromodulatory systems which are closely linked to the immune system with which parasites have to cope in any case. Accordingly, parasites could exploit this link in order to manipulate host behaviour (Adamo 2002; Helluy 2013; Lafferty and Shaw 2013). Especially in sequential coinfections, any effect of the first parasite would be likely to affect the interaction of the second parasite with the immune system. The initial establishment seems to be the crucial part of host-parasite interactions in *S. solidus* infections in copepods (van der Veen et al. 2002). Prior infection with a closely related *S. solidus* reduces susceptibility to a second parasite (Kurtz and Franz 2003), but during simultaneous infections, the chances for a single parasite to establish increase with increasing number of parasites administered (Wedekind 1997). Parasites can be lost for a few days after infections, but this seems to be due to intrinsic mortality rather than the host's immune system or within host competition (van der Veen et al. 2002). Unfortunately we do not know if host manipulation in *S. solidus* in its copepod host is in any way linked to the parasite's interaction with the host's immune system.

Parasites agreeing or disagreeing over whether and how their shared host should be manipulated are expected to be ubiquitous in nature. Even human infectious diseases manipulate their host, e.g. human toxoplasmosis with a worldwide prevalence of about 30% is supposed to permanently manipulate the behavior of infected people (Flegr 2013). Its manipulation could be altered by co-infecting parasites (de Queiroz et al. 2013). Our paper presents a proof of principal that one parasite can impact and even neutralize the manipulation by another parasite.

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Chapter IV

Inter- and intraspecific conflict over host manipulation

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Abstract

Host manipulation is a common strategy by which parasites alter host behavior to enhance their own fitness. In nature, hosts are usually infected by multiple parasites. This can result in a conflict over host manipulation. Studies of such a conflict in experimentally infected hosts are rare. The cestode *Schistocephalus solidus* and the nematode *Camallanus lacustris* use copepods as their first intermediate host. They need to grow for some time inside this host before they are infective and ready to be trophically transmitted to their subsequent fish host. Accordingly, not yet infective parasites manipulate to suppress predation. Infective ones manipulate to enhance predation. We experimentally infected lab-bred copepods in a manner that resulted in copepods harboring (1) an infective *C. lacustris* plus a not yet infective *C. Lacustris* or *S. solidus* or (2) an infective *S. solidus* plus a not yet infective *C. lacustris*. An infective *C. lacustris* completely sabotaged host manipulation by any not yet infective parasite. An infective *S. solidus* partially reduced host manipulation by a not yet infective *C. lacustris*. We hence show experimentally that a parasite can reduce or even sabotage host manipulation exerted by a parasite from a different species.

Introduction

Many parasites possess the ability to modify their host's behavior or appearance to their needs. Such host manipulation has been reported from a large number of host-parasite systems (reviewed by Holmes and Bethel 1972; Poulin and Thomas 1999; Moore 2002, 2013; Poulin 2010) and can have far reaching ecological consequences (Thomas et al. 1998b, 1999, 2005; Lefèvre et al. 2009b; Lafferty and Kuris 2012). In complex life cycle parasites many of the most prominent examples of host manipulation comprise of cases in which parasites enhance the likelihood that their current host is consumed by a suitable subsequent host (reviewed by Holmes and Bethel 1972; Poulin and Thomas 1999; Moore 2002, 2013; Poulin 2010). Normally, these changes do not occur before the parasite is ready for transmission. Premature predation would be fatal. Accordingly, parasites have developed the ability to lower their current's host predation risk prior to becoming infective to the next host (Hammerschmidt et al. 2009; Thomas et al. 2010; Dianne et al. 2011; Weinreich et al. 2013). Most studies of host manipulation, in particular under experimental conditions, focus on hosts

infected with a single parasite species or even one individual. This does not reflect nature where such single infections are extreme exceptions (e.g. Petney and Andrews 1998; Kalbe et al. 2002). Most parasites do encounter several co-infecting parasites, potentially including other manipulating parasites. Even a non-manipulating parasite might have an interest in its host's behavior and might disagree with a co-infecting parasite's manipulation. This can result in a conflict over host manipulation which is likely to alter how hosts are manipulated (Rigaud and Haine 2005; Koella et al. 2006; Thomas et al. 2010, 2011; Mauck et al. 2012; Syller 2012; Cézilly et al. 2014). Nevertheless, only few studies have explicitly investigated any conflict over host manipulation (Cézilly et al. 2000; Thomas et al. 2002a; Sparkes et al. 2004; Haine et al. 2005; Dianne et al. 2010; Hafer and Milinski 2015) and even fewer have used experimental infections (Thomas et al. 2002a; Dianne et al. 2010; Hafer and Milinski 2015). This is, however, crucial to determine any causal relationship (Milinski 1997).

Correlational evidence suggests that in co-infections in which there is potential for an interspecific conflict over host manipulation, both parasites can affect host behavior (Cézilly et al. 2000; Thomas et al. 2002a; Haine et al. 2005). Thomas et al. (2002a) found that hosts naturally infected with nematodes and trematodes are less manipulated than those exclusively infected by the trematodes. Cure and reinfection, however, failed to induce this effect (Thomas et al. 2002a). Other studies using experimentally infected hosts have been restricted to an intraspecific conflict between infective and not yet-infective parasite stages (Dianne et al. 2010; Hafer and Milinski 2015). Hence, to our knowledge no evidence from experimentally infected hosts exists that one parasite can affect host manipulation by a non-conspecific parasite. Such evidence however will be crucial to determine cause and effect if differences in behavior are found between hosts harboring different multi-species assemblages of parasites (Milinski 1997). In a conflict between different developmental stages of the same species, studies on both naturally (Sparkes et al. 2004; Dianne et al. 2010) and experimentally (Dianne et al. 2010; Hafer and Milinski 2015) infected hosts found that the infective parasite always had the stronger effect up to complete sabotage of the effect of the not yet infective parasite (Hafer and Milinski 2015). This raises the question whether, in such a conflict, the infective parasite might have an a priority advantage and thus be generally able to interfere with the manipulation of a not yet infective, conspecific parasite. And, if so, would it be the case also in a conflict between two parasites from different species? They might use different mechanisms to manipulate making interference more difficult. However, parasites would benefit from altering their host in a manner that hinders manipulation by any disagreeing co-infecting parasite.

In this study we use two phylogenetically distinct parasites that use cyclopoid copepods as their first intermediate hosts and fish as second intermediate hosts to investigate intra- and interspecific conflicts over host manipulation under strictly experimental conditions. The cestode, *Schistocephalus solidus* has a three host life cycle with copepods as first and three-spined sticklebacks (*Gasterosteus aculeatus*) as second intermediate hosts and piscivorous birds as definitive hosts (Clarke 1954; Dubinina 1980). Copepods infected by *S. solidus* have decreased activity (Hammerschmidt et al. 2009; Benesh 2010b; Hafer and Milinski 2015) and predation susceptibility (Weinreich et al. 2013) until the parasite reaches infectivity. Once infective, *S. solidus* can increase host activity (Urdal et al. 1995; Wedekind and Milinski 1996; Hammerschmidt et al. 2009) and

predation susceptibility (Wedekind and Milinski 1996). The nematode *Camallanus lacustris* uses perch (*Perca fluviatilis*) as its definitive host. Other fish, including three-spined sticklebacks, can act as paratenic hosts (Moravec 1994). Not yet infective *C. lacustris* reduce the predation susceptibility of their copepod host (Weinreich et al. 2013). Behavior that might be responsible for this change such as activity has not been measured directly in *C. lacustris* infected hosts. We expect a similar pattern of copepod activity as induced by *S. solidus* with an initial phase of decreased activity followed by increased activity. Accordingly, we do not expect an interspecific conflict over host manipulation between *S. solidus* and *C. lacustris* of the same developmental stage but parasites of different stages should disagree when the two parasite species co-occur. Here we study the outcome of an intra- and interspecific conflict over host manipulation. If any such conflict occurs we find that the infective parasite performs better overall and can sabotage manipulation by the not yet infective one, however, the two parasite species differ in the strength with which they sabotage manipulation.

Material and Methods

Hosts

We used lab-bred copepods (*Macrocyclops albidus*) from a stock originating from the "Neustaedter Binnenwasser", Northern Germany. We used adult male copepods to reduce variation between hosts. On the day prior to the first infection, 936 (experiment I) or 768 (experiment II) copepods were filtered from their tank and transferred each to an individual well of a 24-well cell culture plate in about 1 mL of water. Copepods were kept at 18°C in a 16h/8h light/dark cycle. We checked for the presence of dead copepods, cleaned wells when necessary and fed the copepods with five *Artemia* sp. nautili every other day (i.e. the day when no behavioral recordings took place, i.e., 1, 3, 5, 8, 10, 12, 14, 16, 18, 20).

Parasites

Camallanus lacustris was dissected from perch guts obtained from a local fishery and originated from the Grosse Plöner See, Northern Germany. To obtain gravid females, we cut open the blind sacks of perches' guts. Females were cleaned, placed in 0.64% Natrium solution and stored in the fridge (4°C) until use. Gravid females harbor live larvae that are ready to infect copepods (Moravec 1994). To obtain these larvae, we opened up the females with dissection needles allowing the larvae to escape. Larvae were stored in tap water in the fridge overnight prior to copepod infections. A total of 40 (first infection, experiment I), 30 (second infection, experiment I and experiment II) females was used and their offspring mixed.

To obtain *Schistocephalus solidus*, mature *S. solidus* were dissected from fish caught at the "Neustädter Binnenwasser", Northern Germany. They were bred in vitro in the laboratory (Smyth 1946) and eggs were stored in the fridge (4°C) until use. Prior to exposure the eggs were incubated for three weeks at 20 °C and exposed to light over night to induce hatching (Dubinina 1980). *Schistocephalus solidus* stemmed from 2 (experiment I) or 4 (experiment II) families, which were equally distributed between all treatments.

Infections

Infections consisted of adding either one coracidium (*S. solidus*) or one L1-larva (*C. lacustris*) to the well containing the copepod. This took place at two different time points, 7 days apart, i.e. on day 0 and on day 7. We conducted two experiments. In experiment I we investigated either an intraspecific conflict between an infective *C. lacustris* and a not yet infective conspecific or an interspecific conflict between an infective *C. lacustris* and a not yet infective *S. solidus*. Copepods were exposed to *C. lacustris* on day 0 and *S. solidus* or *C. lacustris* on day 7. Including the necessary controls we obtained six different treatments (Figure IV. 1 a-c; e-g): (a) not infected by any parasite (Control), (b) Infected by *C. lacustris* on day 0 (CAM), (c) infected by *C. lacustris* on day 7 (cam), (e) infected by *S. solidus* on day 7 (sch), (f) infected by *C. lacustris* on day 0 plus *C. lacustris* on day 7 (CAM-cam), (g) infected by *C. lacustris* on day 0 plus *S. solidus* on day 7 (CAM-sch) . In experiment II we investigated the potential conflict between an infective *S. solidus* and a not yet infective *C. lacustris*. Hence we used four different treatments (Figure IV. 1 a, c, d, h): (a) not infected by any parasite (Control), (c) infected by *C. lacustris* on day 7 (cam), (d) Infected by *S. solidus* on day 0 (SCH), and (h) infected by *S. solidus* on day 0 plus *C. lacustris* on day 7 (SCH-cam). For each experiment, copepods from each treatment were spread evenly over all plates and distributed randomly.

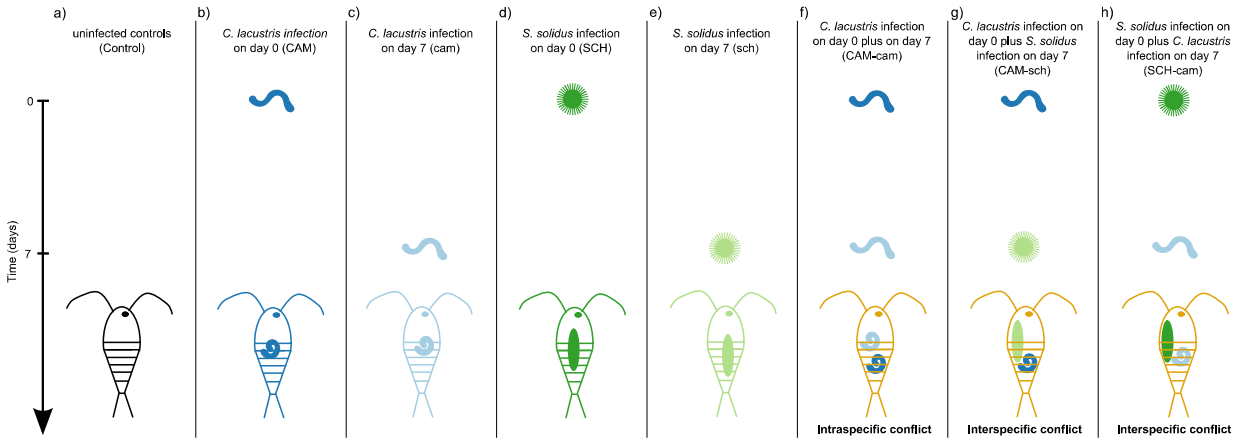

Figure IV. 1: Overview over treatments and timing of infections. Copepods were exposed to *S. solidus*, *C. lacustris* or no parasite at two different time points, day 0 and day 7. This resulted in 6 different treatments for experiment I (Control (a), CAM (b), cam (c), sch (e), CAM-sch (f), CAM-cam (g)) and 4 different treatments for experiment II (Control (a), cam (c), SCH (d), SCH-cam (h)).

At the end of the experiment we checked whether an infection had occurred by placing copepods under a microscope. Since copepods are translucent, parasites within the copepod can be seen that way. We only checked for infection after all behavioral activities have been recorded to avoid stress that could have influenced copepod behavior.

Recording of behavior & analysis

Copepod behavior was recorded by placing a plate with copepods on an apparatus that dropped it by about 3mm (Hammerschmidt et al. 2009). This simulates a failed predator attack after which the copepod should perceive an enhanced predation risk since the predator might still be around. This simulated predator attack was initiated after the plate had been on the apparatus for 1 minute (Hammerschmidt et al. 2009; Hafer and

Milinski 2015). We used a Panasonic Super DynamicWV-BP550 camera (Panasonic Corporation, Osaka, Japan) to record copepod behaviour for 15 minutes after the simulated predator attack.

We analyzed behavior only of copepods that were infected by all parasites they have been exposed to. Copepods that died within 1 day after the last behavioral recordings, i.e. prior to day 22 in the experiment are excluded from the analysis. If more than 40 copepods were available for one treatment, we randomly selected 40 subjects for analysis. This resulted in 240 copepods in experiment I (40 in each treatment) and 150 copepods in experiment II (C: 40, SCH: 30, cam: 40, SCH-cam: 40). Using the manual tracker plugin in image J (Rasband 2008), we recorded the position of every copepod every two seconds for one minute starting 10 seconds after the simulated predator attack to rule out the initial reaction and for one minute at the end of the recording (between 14 and 15 minutes after the simulated predator attack). This was done blindly with regard to treatment. We assume that copepods should have recovered from the simulated predator attack after 14 minutes. From the position data we calculated whether or not a copepod had moved within each 'two second interval' (Hafer and Milinski 2015). We also determined the latency for each copepod to resume moving after the simulated predator attack. If copepods did not move within 15 minutes, we assumed latency to be 15 minutes. This occurred only in 14 out of 2716 behavioral recordings.

Statistical analysis

To investigate the effect of *C. lacustris* and *S. solidus* on host behavior, we used generalized linear mixed models in the lme4 package (Bates et al. 2014) in R (R Development Core Team 2010). To account for variation between individual copepods over time we included copepod identity and the day in the experiment (i.e. after the first infection on day 0) as random effects. To analyse copepod activity (i.e. whether or not a copepod moved within each two second interval) we additionally included the time point in the recording (i.e. after the simulated predator attack or after a recovery period) in the random effect. We fitted two separate models, one with activity as response variable using binomial distribution, the other one with the log transformed latency to resume moving as response variable. We included the day and the time point as fixed effect. We stepwise added treatment and all its interactions with day and time point. We compared the models using likelihood ratio tests. We accepted a model as having a better fit than a less complicated one if it explains the data significantly better. We fitted separate models for experiment I and II since they contained different treatments. Please refer to Tables IV. S1 and IV. S2 in the supplementary information for the complete output of the models.

Since we found significant interactions between treatment, day, and time point (Table IV. S1, Table IV. S2) we conducted post hoc tests. We used Tukey tests using general linear hypotheses within the multcomp package in R (Hothorn et al. 2008). We used separate post hoc tests for each treatment and time point to determine when significant changes occurred between consecutive days. Additionally, we used separate post hoc tests for each day and time point to investigate differences between treatments. In the results section we only report the most relevant statistics to facilitate readability. See Tables IV. S3 - IV. S6 in the supplementary information for all other statistics.

Results

Host manipulation by *Camallanus lacustris*

We expect that prior to reaching infectivity *C. lacustris* will suppress its host's predation risk (i.e. by reducing its activity and increasing its latency to resume moving after a simulated predator attack) because too early predation would be fatal for the parasite (Parker et al. 2009c). *Camallanus lacustris* that had infected their host on day 7 (called 'cam', see Figure IV. 1) caused a drop in host activity during consecutive days: between day 9 and 13, i.e. 2 to 6 *days post infection* ($p<0.001$, Tables IV. S3, IV. S4, Figure IV. 2 a, b). Following these changes in host behavior over time in cam-copepods, cam - copepods were significantly less active than uninfected copepods between day 9 and 17, i.e. 4 to 10 *days post infection* ($p<0.02$, Tables IV. S5, IV. S6, Figure IV. 2 a, b). Before cam reached infectivity, latency was significantly higher in cam – copepods than in uninfected ones. This was clearest from day 11 to day 15, i.e. 2 to 8 *days post infection* ($p<0.001$, Table IV. S5). Copepods that had been infected on day 0 (called 'CAM') were also less active and had a longer latency than uninfected control copepods on day 9, i.e. 9 *days post infection*, i.e. before their parasite reached infectivity ($p<0.001$, Table IV. S5, Figure IV. 2). Thus, we can confirm that copepods infected by not yet infective *C. lacustris* had a reduced activity and increased latency likely to result in reduced predation and hence termed predation suppression (see Parker et al. 2009c).

Once *C. lacustris* has reached infectivity to the next host, it should switch from predation suppression to predation enhancement (see Parker et al. 2009c) which should be detectable by an increase in copepod activity and a decrease in latency. Indeed, in cam-copepods activity increased significantly between consecutive days as the parasite reached infectivity (day 15 to 19, i.e. 8 to 12 *days post infection*, $p<0.001$, Tables IV. S3, IV. S4, Figure IV. 2 a, b). In CAM-copepods activity increased around the same time post infection as in cam, between day 9 and 13, i.e. 9 and 13 *days post infection* ($p<0.02$, Tables S3, S4, Figure IV. 2 a, b). As the parasite became infective (i.e. between 8 and 11 *days post infection*), latency decreased in both cam - copepods (day 15 to 17 Tables IV. S3, IV. S4, Figure IV. 2 c) and CAM – copepods (day 9 to 11, $p<0.001$, S3, Figure IV. 2 c). These changes mostly resulted in slightly higher activity and shorter latency than those of control copepods (Figure IV. 2), although these differences were only partly significant (Tables IV. S5, IV. S6). In conclusion, we found increased activity potentially indicative of predation enhancement (see Parker et al. 2009c) albeit it was much less pronounced than potential predation suppression.

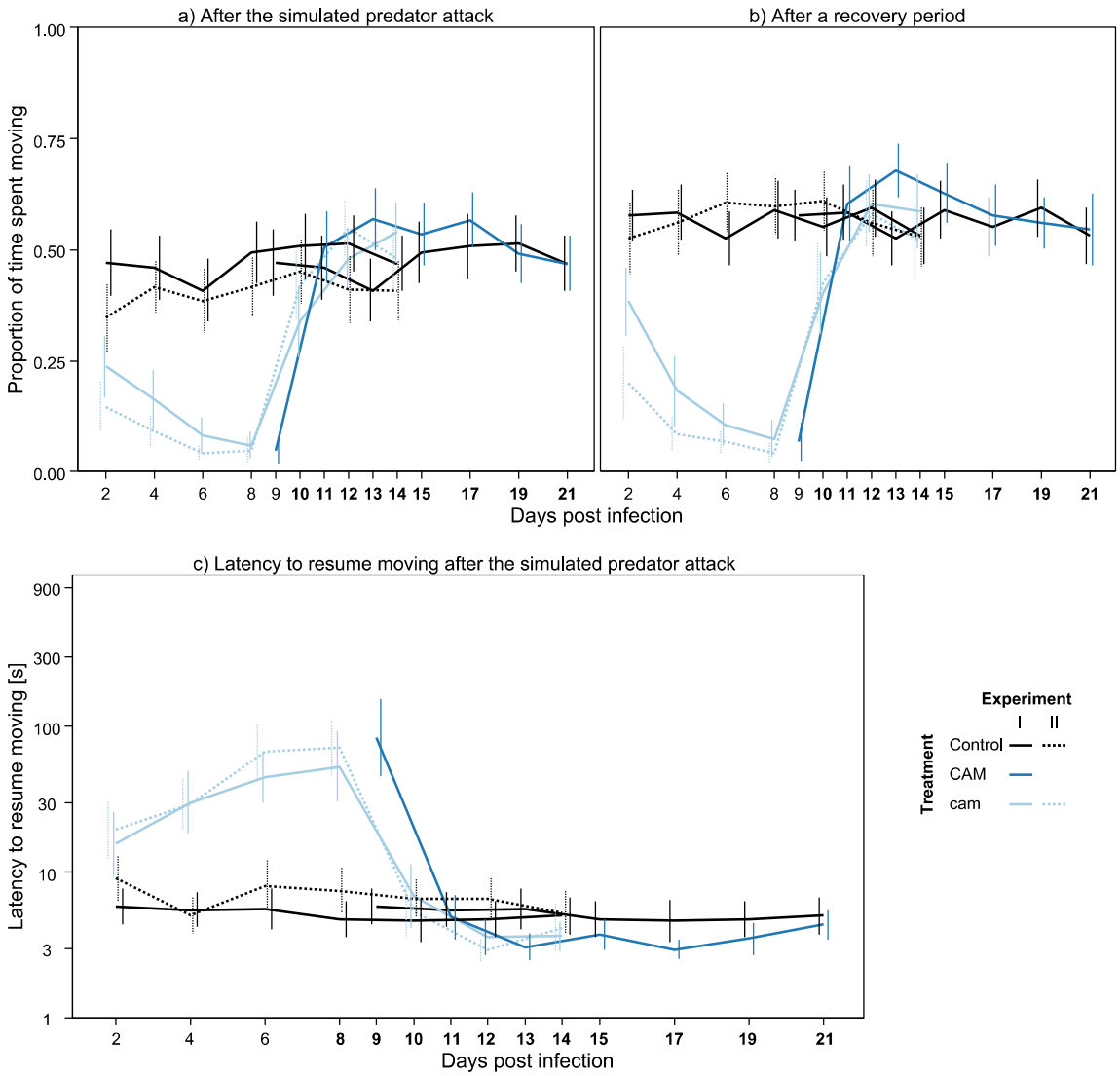

Figure IV. 2: Host manipulation by *C. lacustris*. Error bars indicate 95% CI. a: Activity (proportion of time spent moving) within one minute after a simulated predator attack, b: Activity during one minute after a recovery period, c: Latency to resume moving after a simulated predator attack. Bold letters on the x-axis indicate that a parasite of this age was infective. N: 40 per treatment. Treatments: Control: uninfected control copepods (Figure IV. 1 a), CAM: copepods infected with *C. lacustris* on day 0 (Figure IV. 1 b), cam: copepods infected with *C. lacustris* on day 7 (Figure IV. 1 c). Please note that to plot control lines for experiment I, each control copepod was used twice by assigning it the days post infection of CAM and cam during the appropriate behavioral test.

Intraspecific conflict between two *Camallanus lacustris* parasites

If two parasites manipulate differently, there is potential for a conflict over host manipulation between them. To investigate this potential conflict between different developmental stages of *C. lacustris*, we used copepods infected with *C. lacustris* on day 0 (CAM, Figure IV. 1 b) or day 7 (cam, Figure IV. 1 c) and copepods infected with *C. lacustris* on day 0 plus on day 7 (called 'CAM-cam', Figure IV. 1 f). To establish when such a conflict would occur we compared CAM - copepods to cam - copepods. From day 11 to day 17, i.e. when CAM was already infective but cam was not yet, CAM – copepods were significantly more active and resumed moving sooner than cam - copepods ($p<0.007$, Table IV. S5, Figure IV. 3). Hence, during this time we could expect a conflict between two such parasites if they infected the same host. CAM-cam - copepods were significantly more active and resumed moving sooner than cam - copepods from day 11 to day

15 ($p<0.001$, Table IV. S5, Figure IV. 3). During this time, the behavior of CAM - copepods did not differ significantly from that of CAM-cam - copepods ($p>0.06$, Table IV. S5, Figure IV. 3). Thus the not-yet infective parasite (cam) had no observable effect when together with an infective parasite (CAM). In accordance with this finding, CAM - copepods increased their activity and decreased their latency between day 9 and 11 ($p<0.001$, Table IV. S3, Figure IV. 3) – as CAM reached infectivity. No further significant increases occurred after day 15, when cam should have reached infectivity and hence caused a switch in host behavior (see above). We hence found that this intraspecific conflict was clearly won by the infective parasite.

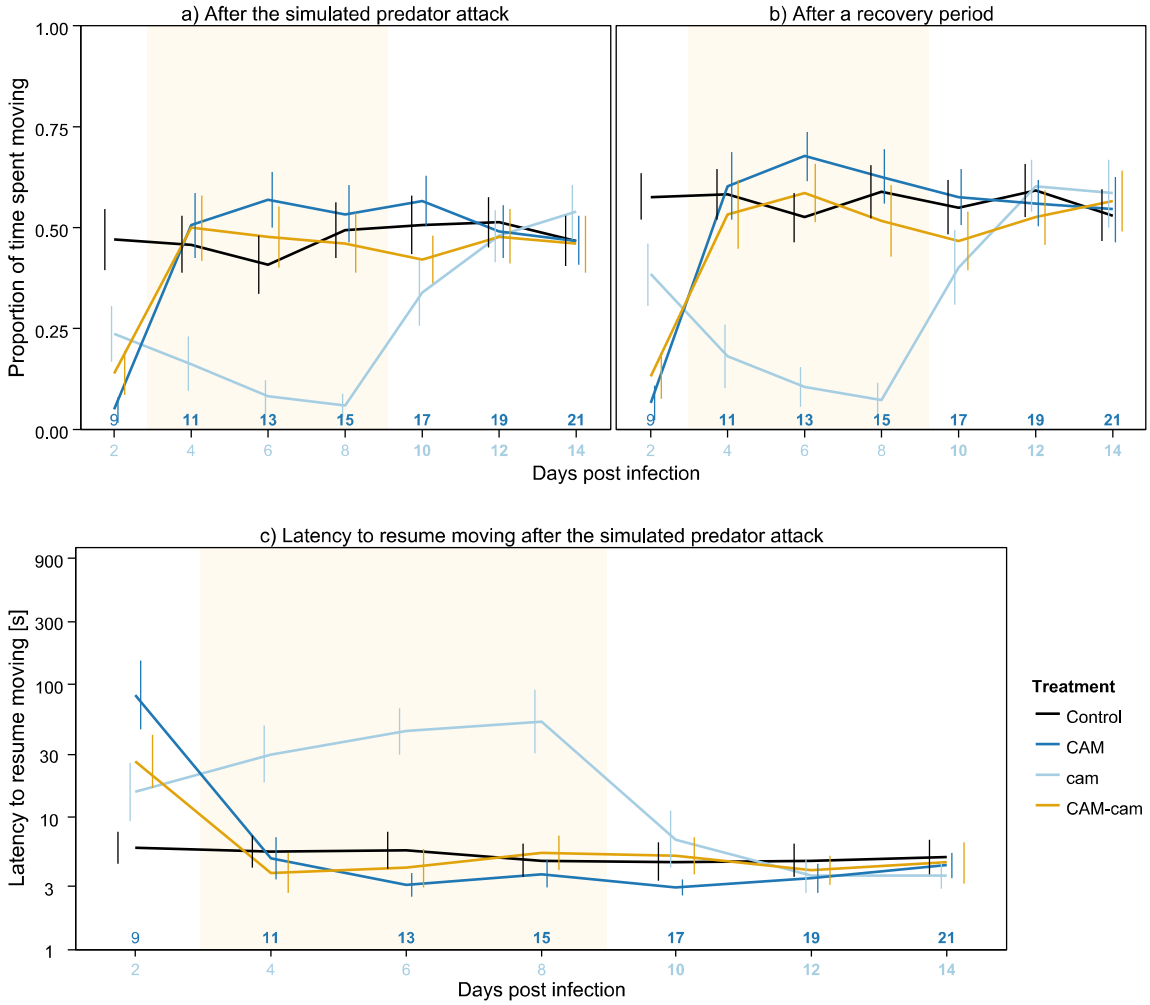

Figure IV. 3: Intraspecific conflict within *Camallanus lacustris.* Error bars indicate 95% CI. a: Activity (proportion of time spent moving) within one minute after a simulated predator attack, b: Activity during one minute after a recovery period, c: Latency to resume moving after a simulated predator attack. The upper labels on the x-axis indicate the age of *C. lacustris* from day 0, the lower labels indicate the age of *C. lacustris* from day 7. Bold letters on the x-axis indicate that a parasite of this age was infective. The colored area indicates when a conflict over host manipulation should occur. N: 40 per treatment. Treatments: Control: uninfected control copepods (Figure IV. 1 a), CAM: copepods infected with *C. lacustris* on day 0 (Figure IV. 1 b), cam: copepods infected with *C. lacustris* on day 7 (Figure IV. 1 c), CAM-cam: copepods infected with one *C. lacustris* on day 0 plus one on day 7 (Figure IV. 1 f).

Interspecific conflict between an infective *Camallanus lacustris* parasite and a not yet infective *Schistocephalus solidus* parasite

To investigate a potential interspecific conflict between an infective *C. lacustris* and a not yet infective *S. solidus*, we used copepods infected with *C. lacustris* on day 0 (CAM, Figure IV. 1 b), copepods infected with *S. solidus* on day 7 (sch, Figure IV. 1 e) and copepods infected with *C. lacustris* on day 0 plus *S. solidus* on day 7 (called 'CAM-sch', Figure IV. 1 g). Again, in order to establish the time window during which a conflict over host manipulation may occur, we first compared the behavior of CAM - copepods to sch – copepods. During day 13 and 15 CAM - copepods were significantly more active and resumed moving sooner than sch - copepods ($p<0.02$, Table IV. S5, Figure IV. 4). During this time CAM was infective and sch was not, so a conflict should have existed between them if they infected the same host. CAM-sch - copepods behaved significantly different from sch – copepods on day 13 and 15 ($p<0.006$, Table IV. S5) but seemed undistinguishable from CAM – copepods during this time ($p>0.8$, Table IV. S5) (Figure IV. 4). Also alike CAM - copepods, CAM-sch – copepods increased their activity and decreased their latency as CAM reached infectivity between day 9 and day 11($p<0.001$, Table IV. S3, Figure IV. 4). Again, the infective parasite, in this case CAM seemed to win the conflict over host manipulation.

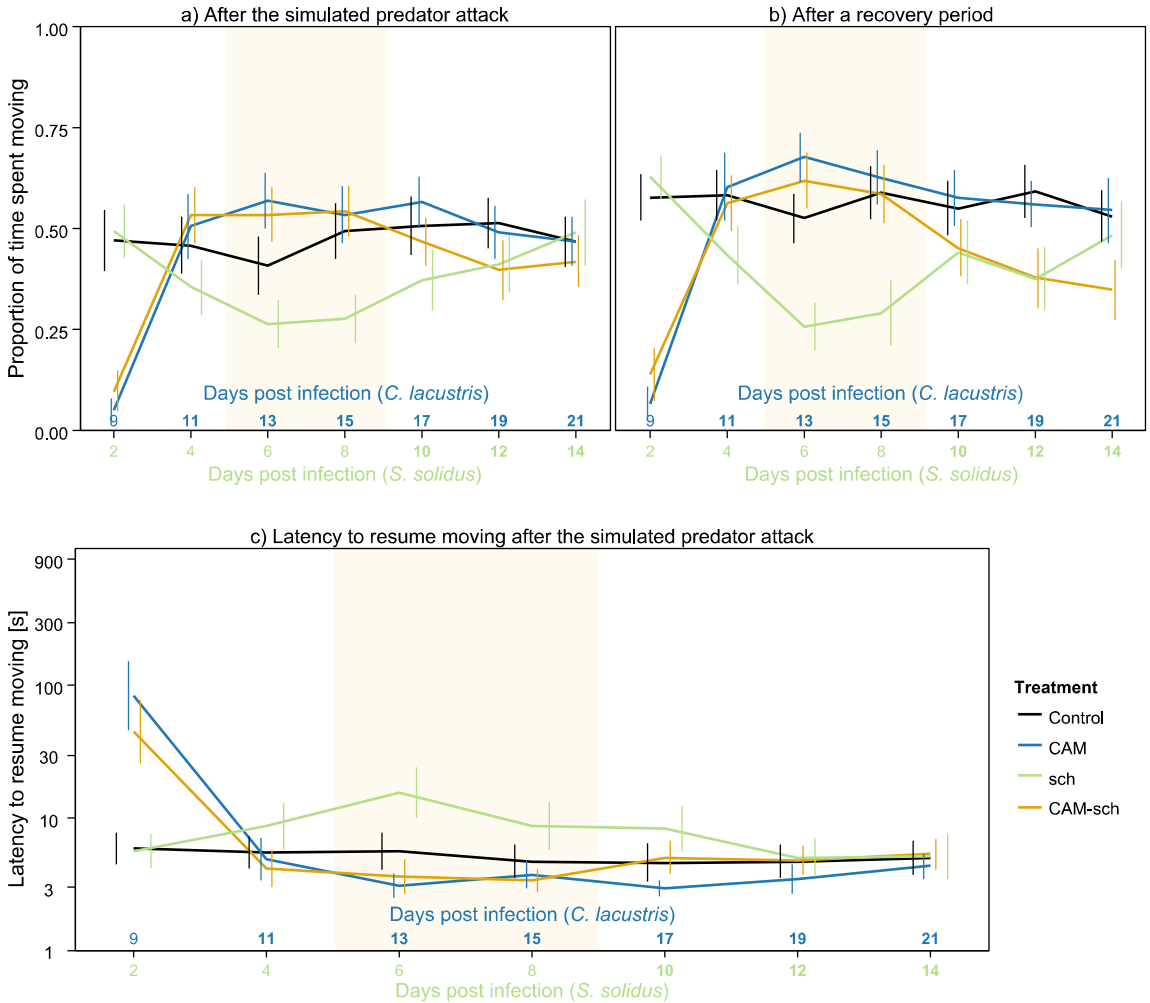

Figure IV. 4: Interspecific conflict between an infective *Camallanus lacustris* and a not yet infective *Schistocephalus solidus*. Error bars indicate 95% CI. A: Activity (proportion of time spent moving) within one minute after a simulated predator attack, B: Activity during one minute after a recovery period, C: Latency to resume moving after a simulated

predator attack. Bold letters on the x-axis indicate that a parasite of this age was infective. The colored area indicates when a conflict over host manipulation should occur. N: 40 per treatment, Control: uninfected control copepods (Figure IV. 1 a), CAM: copepods infected with *C. lacustris* on day 0 (Figure IV. 1 b), sch: copepods infected with *S. solidus* on day 7 (Figure IV. 1 e), CAM-sch: copepods infected with one *C. lacustris* on day 0 plus one *S. solidus* on day 7 (Figure IV. 1 f).

Interspecific conflict between an infective *Schistocephalus solidus* parasite and a not yet-infective *Camallanus lacustris* parasite

To study a conflict over host manipulation between an infective *S. solidus* and a not yet infective *C. lacustris*, we used copepods infected with *S. solidus* on day 0 (SCH, Figure IV. 1 d), copepods infected with *C. lacustris* on day 7 (cam, Figure IV. 1 c) and copepods infected with *S. solidus* on day 0 plus *C. lacustris* on day 7 (called 'SCH-cam', Figure IV. 1 h). We tested for the existence and timing of this conflict by comparing SCH - copepods to cam - copepods. SCH - copepods were significantly more active than cam-copepods and resumed moving sooner between day 11 and 15, i.e. 11 and 15 (*S. solidus*) and 4 and 8 (*C. lacustris*) *days post infection* ($p<0.001$, Table IV. S6, Figure IV. 5). Hence a conflict seemed to exist between day 11 and day 15. However, day 11 should be considered with caution since a significant increase in host activity in SCH occurred only between day 11 and 13 (Table IV. S4). This increase is likely to coincide with when *S. solidus* became infective and hence should increase its host's predation susceptibility, i.e. from day 13 onwards.

The behavior of SCH-cam - copepods in which a conflict over host manipulation occurred is somewhat intermediate between that of SCH – and cam – copepods on day 13 and 15 (i.e. during the time when we expected a conflict). They were more active than cam - copepods, after a simulated predator attack ($p<0.001$, Table IV. S6, Figure IV. 5 a), but not after a recovery period ($p>0.8$, Table IV. S6), but always less active than SCH - copepods ($p<0.001$, Figure IV. 5 a, b, Table IV. S6). Likewise, SCH-cam copepods had a shorter latency than cam - copepods ($p<0.02$, Figure IV. 5 c, Table IV. S6), but a longer latency than SCH – copepods ($p<0.002$, Figure IV. 5 c, Table IV. S6). The behavior of SCH – cam copepods is intermediate between that of cam - and SCH – copepods. A conflict between SCH and cam seemed to result in a compromise when it came to host manipulation.

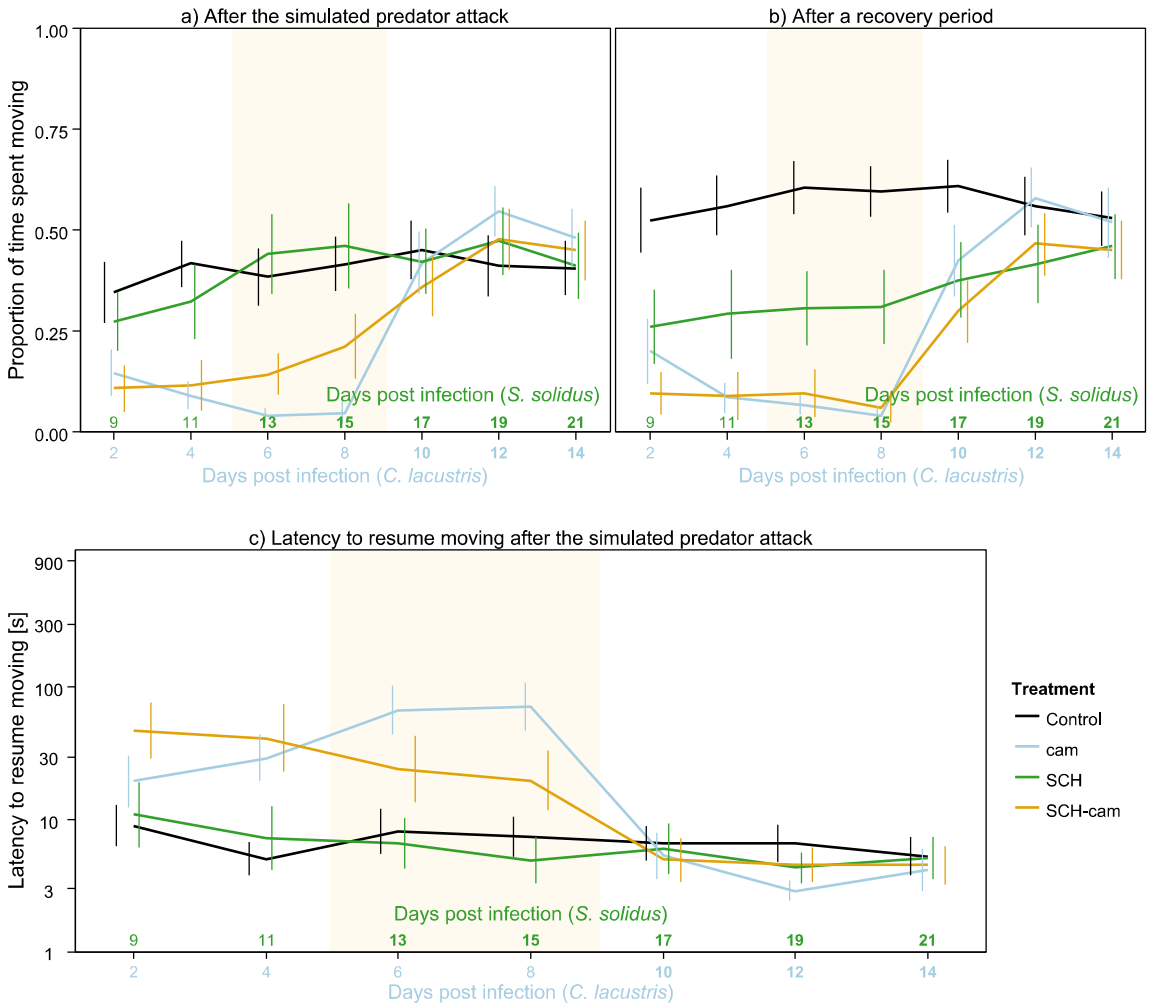

Figure IV. 5: Interspecific conflict between an infective *Schistocephalus solidus* and a not yet infective *Camallanus lacustris*. Error bars indicate 95% CI. A: Activity (proportion of time spent moving) within one minute after a simulated predator attack, B: Activity during one minute after a recovery period, C: Latency to resume moving after a simulated predator attack. Bold letters on the x-axis indicate that a parasite of this age was infective. The colored area indicates when a conflict over host manipulation should occur. N: Control: 40, SCH: 30, cam: 40, SCH-cam: 40. Treatments: Control: uninfected control copepods (Figure IV. 1 a), cam: copepods infected with *C. lacustris* on day 7 (Figure IV. 1 c), SCH: copepods infected with *S. solidus* on day 0 (Figure IV. 1 d), SCH-cam: copepods infected with one *S. solidus* on day 0 plus one *C. lacustris* on day 7 (Figure IV. 1 h).

Discussion

The nematode *Camallanus lacustris* initially decreases its copepod host's activity before it reaches infectivity. Thereafter it increases host activity albeit slightly. This follows a pattern previously predicted (Parker et al. 2009c) and shown in other systems (Koella et al. 2002; Hammerschmidt et al. 2009; Dianne et al. 2011; Hafer and Milinski 2015). The manipulation by *C. lacustris* is similar to that of the cestode *Schistocephalus solidus* in the same copepod host (Hammerschmidt et al. 2009; Hafer and Milinski 2015), but more pronounced than that of *S. solidus*. Nevertheless, host manipulation by *C. lacustris* and *S. solidus* results in a similar reduction of predation susceptibility (Weinreich et al. 2013). Different complex life cycle parasites that exploit the same trophic link also adopt convergent life history strategies (Benesh et al. 2011), suggesting that their host manipulation should also cause similar host behavior.

In any study to date that investigated an intraspecific conflict between different parasite stages, the infective parasite seems to win (Sparkes et al. 2004; Dianne et al. 2010; Hafer and Milinski 2015). This seems to be the case even when the not yet infective parasite manipulates strongly when alone (Hafer and Milinski 2015). We cannot rule out that *C. lacustris* or *S. solidus* also affect host behaviors other than activity and latency to recover after a simulated predator attack, with a modulating effect on what we measure. However, activity of copepods is a predictor of predation susceptibility to sticklebacks (Wedekind and Milinski 1996). Hence we expect that the changes in activity we observed should result in changes in predation susceptibility. At least with regards to changes in host activity and latency, it seems that being the first to infect a host allows the infective parasite to become superior. This presents a puzzle. If the not yet infective parasite does manipulate when alone, it has nothing to gain from ceasing to do so in the presence of an infective conspecific (Hafer and Milinski 2015). In *C. lacustris* a not yet infective parasite is faced with the same problem. Any premature predation would be fatal. Accordingly, a not yet infective *C. lacustris* strongly reduces host activity when alone. Yet, if it shares its host with an infective conspecific, it has no detectable manipulation effect. Competition should not impair the not yet infective parasite's ability to such an extent that it cannot manipulate any more: Up to three *C. lacustris* can grow to normal size in male copepods without increased mortality (Benesh 2011).

In an interspecific conflict between *C. lacustris* and *S. solidus*, *C. lacustris* is always doing better. It is also the stronger manipulator. If there is a conflict over host manipulation between an infective *C. lacustris* and a not yet infective *S. solidus*, the infective *C. lacustris*, just as in an intraspecific conflict, seems to completely sabotage any host manipulation by *S. solidus*. A conflict between an infective *S. solidus* and a not yet infective *C. lacustris* results, however, in a compromise. Thus, a not yet infective *C. lacustris* resists sabotage of its manipulation to some extent. Overall, in both cases the infective parasite performs better in interspecific conflicts. *Camallanus lacustris* completely dominates host behavior when infective but manages to settle for a compromise when not yet infective. An infective *S. solidus* only partly increases host activity when sharing with a not yet infective *C. lacustris*, while the not yet infective *S. solidus* seems to have no effect at all.

Are infective parasites able to manipulate more strongly? They are bigger. Thus, if for instance they manipulate by secreting some substance, they would probably be able to produce larger quantities. We did not test the effect of parasite number, however, in an intraspecific conflict within *S. solidus*, multiple not yet infective *S. solidus* were as unable as a single one to prevent the sabotage of their manipulation by one infective conspecific (Hafer and Milinski 2015). It seems thus unlikely that the better performance of the infective parasite is due to its size. The mechanisms to manipulate and to sabotage manipulation need to be studied in two steps. (i) The mechanism of host manipulation when the parasite is alone in its host has still two steps, to suppress activity before it is infective to the next host and to increase activity thereafter. In *S. solidus* it is most likely (Hafer and Milinski 2015) that decreasing activity is active manipulation, whereas increasing activity is likely to result from the parasite's inevitably draining energy and thus changing the host's trade-off between avoiding predation and feeding towards feeding (Milinski and Heller 1978). This behavioral change would hence be a side effect of the parasite's energy use (Milinski 1990) that achieves exactly what active manipulation would need to do. However, when the parasite becomes infective its prior active manipulation

has to be switched off. Cestodes frequently use hormones and energy drain for host manipulation (Lafferty and Shaw 2013). (ii) If the infective parasite has a non-infective competitor, the active manipulation of the latter parasite is likely to be switched off by the same mechanism of the infective parasite that switches off its own active manipulation – for example a hormone might be excreted as a pheromone (Hafer and Milinski 2015).

In the present study we find a similar situation in *C. lacustris*, when it is alone in its host and when it shares it with a conspecific. Very similar mechanisms as in *S. solidus* can be assumed as the most parsimonious explanation. It has previously been shown that even distantly related parasites seem sometimes to use the same proximate mechanism to manipulate their hosts (Ponton et al. 2006). In interspecific interactions the result again looks similar to intraspecific interaction; the infective parasite sabotages the manipulation of the non-infective other parasite, completely, when *C. lacustris* is the infective parasite, however, only partly when *S. solidus* is the infective parasite. Here is a difference between the two parasite species, which is consistent with our finding that *C. lacustris* is the stronger manipulator. We neither need to postulate that an infective parasite has evolved a specific mechanism to dominate a not yet infective conspecific, it actively stops its own manipulation, and as a side effect that of the conspecific, nor do we need to postulate an evolved mechanism for dominating a not yet infective non-conspecific. Obviously both *S. solidus* and *C. lacustris* use a very similar mode of active manipulation to decrease host activity, which then can be sabotaged also interspecifically. This seems to be the most parsimonious explanation of our complete dataset. Accordingly, it is not important that both intraspecific and interspecific competition between an infective and a not yet infective parasite had occurred often enough so that specific mechanisms could evolve. The necessary mechanism had already been evolved for switching from decreasing to increasing the host's activity in the optimal time window (Parker et al. 2009c). While the effectiveness of this switch against co-infecting parasites could have originated as a side-effect, it nevertheless presents an evolutionary advantage for the infective parasite preventing unsuitable manipulation of the host by other co-infecting parasites.

Once a host is infected by a parasite, this infected and potentially manipulated host will present an environment different from a healthy host (Thomas et al. 1998c; Poulin and Thomas 1999; Lefèvre et al. 2009b). Here we show for the first time using experimental infections that a parasite can influence and even completely sabotage host manipulation by a parasite from a different species. This presents a proof of principle to be followed by studies with other parasites. Host manipulation can have important ecological consequences (Thomas et al. 1998b, 1999, 2005; Lefèvre et al. 2009b; Lafferty and Kuris 2012), for example by altering trophic links in a food web (Lefèvre et al. 2009b; Lafferty and Kuris 2012). Given the abundance of (manipulating) parasites, interactions among a multitude per host are likely to determine host behavior in nature. The host might be a "puppet on the string" moved by its many different parasites.

Acknowledgements

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Chapter V

Growth and ontogeny of the tapeworm *Schistocephalus solidus* in its copepod first host affects performance in its stickleback second intermediate host

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Abstract

Background

For parasites with complex life cycles, size at transmission can impact performance in the next host, thereby coupling parasite phenotypes in the two consecutive hosts. However, a handful of studies with parasites, and numerous studies with free-living, complex-life-cycle animals, have found that larval size correlates poorly with fitness under particular conditions, implying that other traits, such as physiological or ontogenetic variation, may predict fitness more reliably. Using the tapeworm *Schistocephalus solidus*, we evaluated how parasite size, age, and ontogeny in the copepod first host interact to determine performance in the stickleback second host.

Methods

We raised infected copepods under two feeding treatments (to manipulate parasite growth), and then exposed fish to worms of two different ages (to manipulate parasite ontogeny). We assessed how growth and ontogeny in copepods affected three measures of fitness in fish: infection probability, growth rate, and energy storage.

Results

Our main, novel finding is that the increase in fitness (infection probability and growth in fish) with larval size and age observed in previous studies on *S. solidus* seems to be largely mediated by ontogenetic variation. Worms that developed rapidly (had a cercomer after 9 days in copepods) were able to infect fish at an earlier

age, and they grew to larger sizes with larger energy reserves in fish. Infection probability in fish increased with larval size chiefly in young worms, when size and ontogeny are positively correlated, but not in older worms that had essentially completed their larval development in copepods.

Conclusions

Transmission to sticklebacks as a small, not-yet-fully developed larva has clear costs for *S. solidus*, but it remains unclear what prevents the evolution of faster growth and development in this species.

Background

Animals with complex life cycles live in distinct habitats as larvae and adults, and switching from one habitat to the next is a critical life history transition. In many taxa, large larvae have higher survival and fecundity as adults (e.g. Semlitsch et al. 1988; Scott 1994; Paradis et al. 1996; Taylor et al. 1998; Phillips 2002; Altwegg and Reyer 2003; Emlet and Sadro 2006), but, all else equal, it takes longer to grow to a large larval size, increasing the probability of dying before switching. This tradeoff between the benefits of being big and the costs of becoming big is at the heart of many life history models examining optimal switching strategies (Werner and Gilliam 1984; Rowe and Ludwig 1991; Abrams and Rowe 1996; Abrams et al. 1996; Day and Rowe 2002; Berner and Blanckenhorn 2007). In these models, fitness is often a function of size and age at the transition. This may turn out to be too simplistic, because a number of studies have found that size and age at metamorphosis can be poor predictors of fitness under some environmental conditions (Twombly et al. 1998; Rolff et al. 2004; Campero et al. 2008; Dias and Marshall 2010; Van Allen et al. 2010). Other factors that are not necessarily correlated with size and age, such as physiological variables, may couple larval and adult success (Zera and Harshman 2001; Marshall et al. 2003; Pechenik 2006; Marden et al. 2008; Slos et al. 2009). For example, the lifetime mating success of the damselfly *Lestes viridis* is affected not only by size and age at emergence, but also by nutritional and photoperiod treatments whose effects seem mediated by energy stores (De Block and Stoks 2005; Stoks et al. 2006).

Many helminth parasites have complex life cycles in which they are trophically-transmitted between consecutive hosts before reproducing. Trait variation in one host often has carryover effects in the next host (Davies et al. 2001; Gower and Webster 2004; Hammerschmidt and Kurtz 2005a; Walker et al. 2006), and larval size and age at transmission are prime candidates for predicting such carryover effects (Parker et al. 2003b, 2009a,b; Iwasa and Wada 2006; Ball et al. 2008; Chubb et al. 2010). Larvae that grow to a large size in the intermediate host generally have higher infection success or fecundity in the next host (Rosen and Dick 1983; Tierney and Crompton 1992; Scharer et al. 2001; Steinauer and Nickol 2003). However, a few studies suggest that the larval size-fitness correlation may depend on environmental factors like the intensity of infection in the intermediate host (Fredensborg and Poulin 2005; Dörücü et al. 2007) or the size of the intermediate host (Benesh et al. 2012). Older larvae can also have higher fitness in the next host (Mueller

1966; Hammerschmidt et al. 2009). Older larvae are generally bigger, but potentially also more mature, so it is unclear exactly how this effect arises. Whereas free-living animals transition into the next habitat at a comparable developmental stage, parasites have to wait to be eaten by the next host and may thus be transmitted at an underdeveloped stage with negative consequences for fitness. Elucidating which larval traits reliably affect fitness in the next host is necessary to understand the evolution of life history strategies in complex life cycle parasites (Parker et al. 2003a,b, 2009a,b; Gandon 2004; Iwasa and Wada 2006).

Using the tapeworm *Schistocephalus solidus*, we explored the roles of larval size, age, and ontogeny in determining performance in the next host. This tapeworm has a three-host life cycle (Clarke 1954; Dubinina 1980). Adult worms occur in the intestine of fish-eating birds where they mate and release eggs into the environment. Free-swimming larvae hatch from the eggs and are consumed by freshwater copepods, the first host. Tapeworm larvae in copepods, termed procercoids, undergo a period of growth and development before they are infective to three-spined sticklebacks (*Gasterosteus aculeatus*), the second intermediate host. Transmission is trophic, and soon after being consumed by sticklebacks the parasite invades the body cavity (Hammerschmidt and Kurtz 2007). Worms, now dubbed plerocercoids, grow for several weeks in fish before becoming infective to birds (Barber and Scharsack 2010). Here, we focused on the transition from copepods to fish. Fitness in fish (infection probability and growth rate) increases with age at transmission (Hammerschmidt et al. 2009), and when age is kept constant, big procercoids have higher fitness (Benesh et al. 2012). However, the correlation between procercoid size and fitness only holds when copepod size is kept rather constant, i.e. being large relative to the host is beneficial, but not necessarily being large in general. Variation in developmental maturity could explain both the effect of age and the effect of relative size. Morphological changes indicative of infectivity occur as establishment probability increases with procercoid age. Moreover, procercoid size and development are positively correlated within copepod stages (relative size correlates with development), but not between them (copepod-stage-induced size variation is not correlated with development) (Benesh 2010a,b).

We measured three components of worm fitness in fish (infection probability, growth rate, energy storage) and evaluated how they were related to larval traits (size, age, ontogeny). We exposed fish to worms of two different ages (11 or 17 days in copepods). If size affects fitness mainly through its relationship with ontogeny, then we expected a size-fitness correlation to be steeper in the young group (11 days), because there is more developmental variation at this time. We also kept copepods on either a high or low food diet to 1) induce size variation and to 2) assess whether there are nutritionally-determined carryover effects poorly captured by the other measured larval traits.

Methods

Infection protocol and procercoid size measurements

Both the copepods and the tapeworms used in the experiment were raised in the laboratory, but they were originally collected from Lake Skogseidvatnet, Norway (60 °13′ N, 5 °53′ E). Plerocercoids were dissected from the body cavity of sticklebacks that had been reared and infected in the lab. Worms were bred in size-matched pairs in an *in vitro* system that was developed by Smyth (1946) and later modified by Wedekind (1997). Size-matching facilitates outcrossing (Luscher and Milinski 2003). Eggs were collected and stored at 4 °C for 1 week, before being incubated at 20 ° for 3 weeks in the dark. Eggs were exposed to light one day before the copepod exposure to induce hatching.

To produce copepods for the experiment, several tanks (5 L) were set up containing 5–10 egg-bearing female copepods (*Macrocyclops albidus*) (details of the copepod cultures can be found in van der Veen et al. (2002)). After 4 weeks, adult male copepods were collected from these tanks and individually isolated in the wells of a 24-well microtitre plate (~1.5 ml per well). By using only adult male copepods, we eliminated any variation attributable to copepod stage, sex, or growth (adults do not molt further). One day after isolation, each copepod was exposed to a single coracidium. Single-worm infections seem to be the norm for cestode-copepod systems in the field (Zander et al. 1994; Rusinek et al. 1996; Dörücü 1999; Hanzelová and Scholz 2002; Hanzelová and Gerdeaux 2003). Copepods were maintained at 18 °C with an 18:6 L:D cycle, and were fed with either two (low food treatment) or four (high food treatment) *Artemia salina* nauplii every other day. Copepod survival and parasite growth are reduced in the low food treatment (Benesh 2010a), implying these treatments are sufficient to produce variation in the energy available to developing worms.

Copepods are reasonably transparent, permitting worm larvae to be observed *in vivo*. Nine days post exposure (DPE) infected copepods were placed on a slide, and procercoids were recorded as having or not having a cercomer. The cercomer is a round structure that forms on the posterior end of worms, and although its function is not known, its appearance is correlated with the development of infectivity to fish (Smyth and McManus 1989). Thus, cercomer presence/absence 9 DPE dichotomizes worms into groups of fast or slow developers.

The area of larval worms was measured one day prior to exposing fish (either 10 or 16 DPE). Copepods were placed on a microscope slide and photographed two times. Procercoid area was measured using the freeware Image J 1.38x (Rasband, W.S., NIH, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2009) and the two measurements were averaged to give values for individual worms. Area was calculated without the cercomer, because the outline of the cercomer is often difficult to clearly observe *in vivo* and because cercomer size is tightly correlated with procercoid body size (Wedekind et al. 2000). Thus, calculating worm area with or without the cercomer likely gives very similar results.

Fish infection and dissection

Lab-bred sticklebacks (7 to 8 months old, mean length = 4.2 cm (± 4.2 SD)) were randomly assigned to be exposed to procercoids that had been in well- or poorly-fed copepods for either 11 or 17 days. At 17 DPE, nearly all procercoids appear morphologically mature, but at 11 DPE there is substantial developmental variation (Benesh 2010a,b). A few days before exposure, fish were individually isolated in small tanks (18 × 13 × 11 cm), and a dorsal spine was clipped to provide DNA for later identification. Each fish was exposed to one infected copepod. Several days after exposure, fish were weighed, measured, and transferred to larger tanks (30 x 22 x 25 cm) at densities of 15 to 17. Three times per week fish were fed *ad libitum* with frozen chironomids and cladocera. Twenty-five to 28 DPE fish were killed and dissected, and all collected worms were weighed to the nearest 0.1 mg. At this time, worm growth is exponential and apparently unconstrained by fish size (Scharsack et al. 2007), so plerocercoid weight reflects variation in growth rates. We took a tail clip for DNA extraction. By taking fish tissue samples both before exposure and after dissection, we were able to identify individual fish, and thus know to which procercoid it was exposed, without maintaining fish in individual tanks. DNA was extracted from spine and tail clips with the Qiagen DNeasy 96 Blood and Tissue Extraction Kit, following the manufacturer's protocol. Nine microsatellite loci were amplified in two multiplex PCR reactions (conditions given in Kalbe et al. (2009)).

Glycogen assay

Glycogen is the most important macronutrient for energy storage in tapeworms (Smyth and McManus 1989). We quantified the glycogen content of the young plerocercoids for two reasons: 1) to use as an additional fitness component and 2) to check whether growth rate impacts energy reserves and thus to critically evaluate plerocercoid size as a fitness component. Glycogen content was assayed based on the protocol described by Gómez-Lechón *et al*. (Gomez-Lechon et al. 1996). Plerocercoids were homogenized in a cell mill (Qiagen TissueLyser II, Retsch GmbH). Glycogen standards of known concentration were prepared and run simultaneously (Sigma G0885, concentrations: 900, 700, 500, 300, 200, 100, 50, 0 µg). Samples were diluted to concentrations of ~0.1 to 1.8 µg μl^{-1}, and 40 µl per sample were pipetted into the wells of a 96-well microtitre plate. 60 µl of a glucoamylase solution (250 mU/well enzyme [Sigma A1602] in 0.2 M sodium acetate buffer, pH 4.8) were added to each well, and samples were incubated for 2 hr at 40°C with shaking. Plates were then spun at 2500 rpm for 5 min and 10 µl of 0.25 M NaOH were added to stop the enzymatic reaction. To quantify the freed glucose, a Glucose Oxidase/Peroxidase coloring reagent was prepared following the manufacturer's instructions (Sigma G3660) with 1 mg/ml ABTS (Merck 194430) in 100 mM phosphate buffer, pH 7, included. This coloring reagent was added to each well (200 µl), samples were incubated in the dark for 30 min, and absorbance was recorded at 405 nm with a PowerWave Microplate Spectrophotometer (Bio-Tek Instruments). Samples were run in triplicate. Glycogen values were repeatable within individuals (Intraclass correlation coefficient = 0.995, $P < 0.001$), so they were averaged. Glycogen was expressed as a density (µg per mg plerocercoid fresh weight).

Data analyses

We separately analyzed three fitness components: infection rate in fish, growth in fish (plerocercoid weight), and the energy reserves of plerocercoids (µg glycogen per mg fresh weight). Infection was analyzed with logistic regression, while general linear models (ANOVA) were used to assess growth and glycogen. We included four predictors in all statistical models: procercoid age at transmission (11 or 17 days, AGE), procercoid size at transmission (PROC), cercomer presence/absence day 9 (fast and slow development, DEVO), and feeding treatment (high and low, FEED). For the analysis of plerocercoid weight and glycogen content, we also included as a factor the number of days worms spent in fish (25, 26, 27, or 28). All main effects were tested as well as the following potentially interesting interactions AGE x DEVO (is developmental variation measured 9 DPE particularly important at a young age?), AGE x PROC (does size only matter when there is developmental variation early on?), and AGE x FEED (does time spent in the feeding treatments matter?). Preliminary analyses and previous studies (Benesh et al. 2012) indicated that characteristics of the fish host, such as its size, sex, and condition (hepatosomatic index), did not influence the measured fitness components, so they were not considered.

Statistical analyses were conducted with SPSS 18.0 (SPSS Inc., Chicago, Ill.) and R 2.14.1 (R Development Core Team, Vienna, Austria). The dataset is available as an additional file. We also re-visited some of the results of Benesh et al. (2012). They studied how *S. solidus* procercoid size 14 DPE affects infection probability and growth in fish. This is between our two age groups (11 and 17 DPE), so we present their results for comparative purposes. Note that due to the different experimental conditions we did not jointly analyze data from Benesh et al. (2012) and the current experiment. Cercomer presence/absence 9 DPE had been recorded in the previous study, but its importance was not evaluated. Plerocercoid size measurements in the two studies are not easily comparable (fully-developed worms vs the young, growing plerocercoids studied here), so we only show infection rate results. The results from their experiments using large copepods are presented, as that is most comparable to this study.

Results

Determinants of infection

At 11 DPE, only 9.5 % (11/116) of procercoids successfully infected fish, whereas 82 % (82/100) were successful at 17 DPE. Given the low variation in infection success within the two age groups, there was relatively low power to detect interactions between AGE and the other predictors, so non-significant effects need to be interpreted cautiously. There was, however, a significant AGE x DEVO interaction (Wald $\chi_1^2 = 5.92$, $P = 0.015$). Fast developers had a higher infection probability 11 DPE, but not 17 DPE, and the results of Benesh et al. suggest an intermediate effect 14 DPE (Figure V. 1). Surprisingly, neither PROC nor its interaction with AGE was significant (Wald $\chi_1^2 = 0.054$, $P = 0.82$ and Wald $\chi_1^2 = 0.026$, $P = 0.87$,

respectively), even though bigger worms seemed more successful at day 11 (and day 14) (Figure V. 2). Similarly, FEED seems important when considered in isolation, with procercoids from the low food treatment having lower infection rates (Figure V. 3), but the effect was not significant in the full model (Wald $\chi_1^2 = 2.23$, $P = 0.135$). The PROC and FEED main effects remained non-significant when their interactions with AGE were removed from the model ($P = 0.84$ and $P = 0.09$, respectively). The absence of significant PROC or FEED effects could reflect collinearity, i.e. the variation in infection attributable to these variables is better captured by AGE or DEVO.

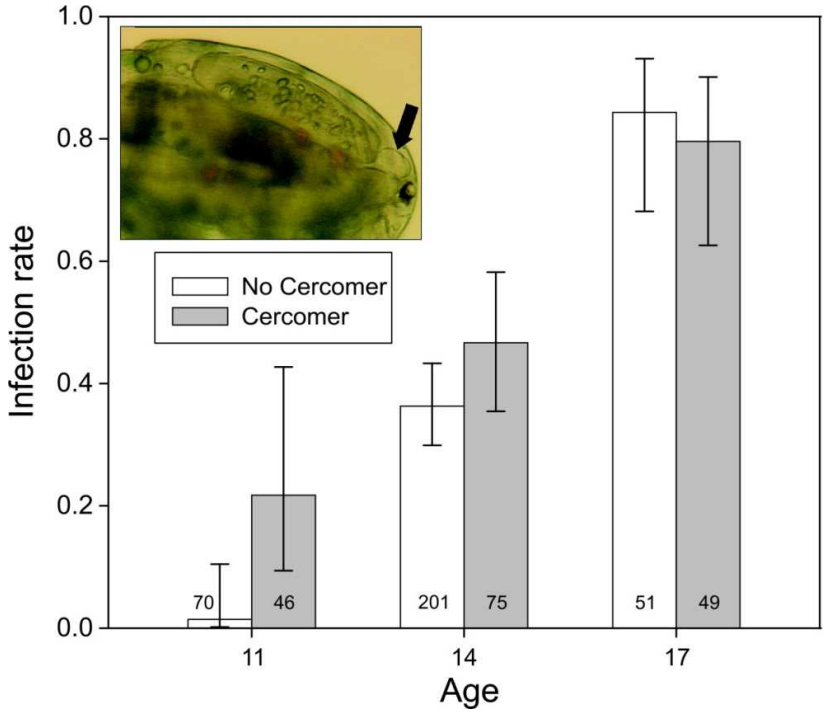

Figure V. 1: Infection rate of procercoids after 11, 14, or 17 days in copepods. Procercoids were recorded as having or not having a cercomer on day 9. Inset photograph shows a procercoid with a well-developed cercomer *in vivo* (arrow). Error bars represent the 95 % CI and numbers within columns are sample sizes. Data from day 14 were from Benesh et al. (2012).

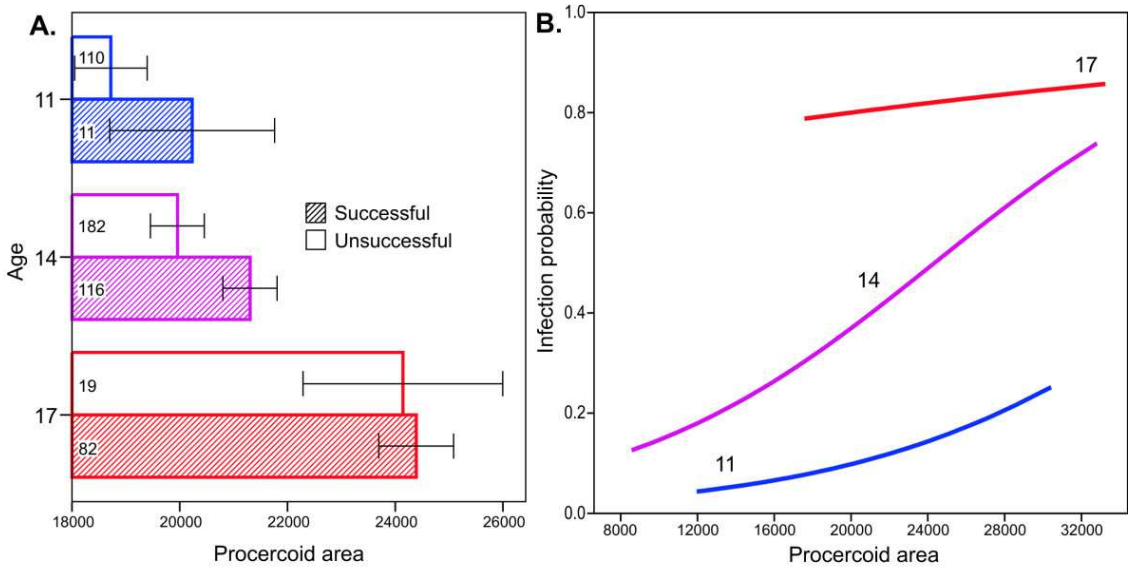

Figure V. 2: (A) The mean size of procercoids (um^2) that did (hatched bars) and did not (open bars) successfully infect sticklebacks after 11, 14, or 17 days in copepods. Error bars represent the 95 % CI and numbers within columns are sample sizes. Data from day 14 were from Benesh et al. (2012). (B) The relationship between infection probability and procercoid area predicted by logistic regressions performed separately for each of the three age groups. Only the regression at day 14 was statistically significant

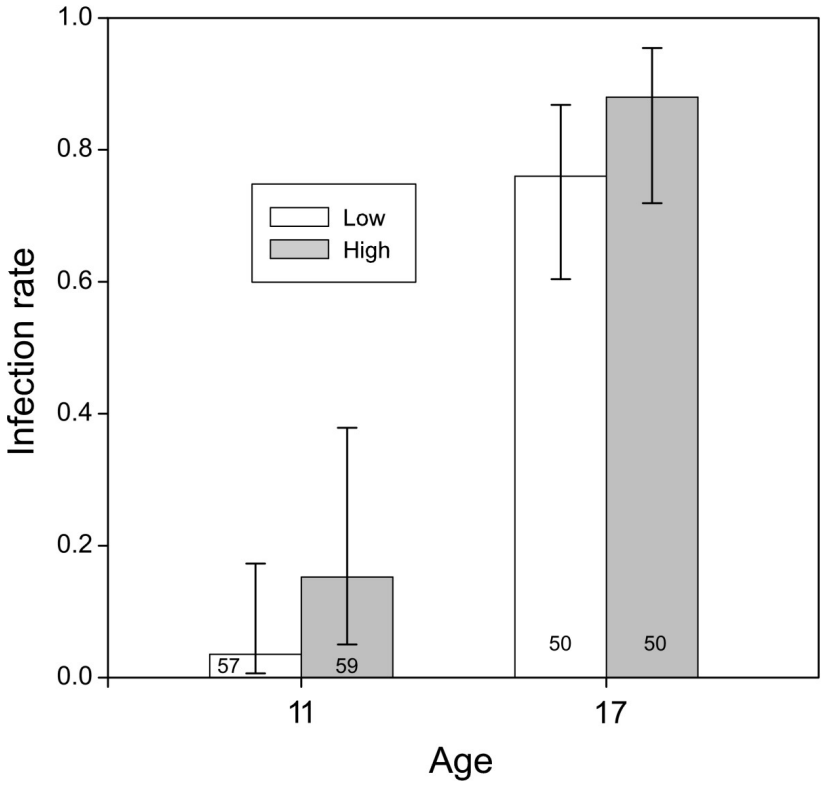

Figure V. 3: Infection rate of procercoids in the high and low feeding treatments after 11 and 17 days in copepods. Error bars represent the 95 % CI and numbers within columns are sample sizes

Determinants of plerocercoid size

No terms significantly affected plerocercoid weight in the full model (all $P > 0.05$), with the exception of days in fish ($P = 0.038$). However, this model was significantly better than an intercept-only model ($R^2 = 0.306$, $F_{8, 84} = 3.62$, $P < 0.001$), suggesting additional variables had explanatory value. Because 88 % (82/93) of the worms recovered from fish were from 17 DPE, the interactions between AGE and the other predictors were estimated with large standard errors. Removal of the interaction terms one-by-one did not significantly reduce explanatory power and result in a worse model (all $F_{1, 84} < 0.692$, all $P > 0.41$, R^2 dropped from 0.306 to 0.294 with all interactions removed). A model with only the five main effects indicated that plerocercoid size increased with days in fish ($F_{3, 85} = 2.89$, $P = 0.04$), that it increased with procercoid size ($F_{1, 85} = 12.92$, $P = 0.001$), and that worms with a cercomer 9 DPE grew to be larger plerocercoids ($F_{1, 85} = 9.68$, $P = 0.003$) (Figure V. 4). AGE and FEED were not significant ($F_{1, 85} = 0.18$, $P = 0.67$ and $F_{1, 85} = 0.14$, $P = 0.71$, respectively).

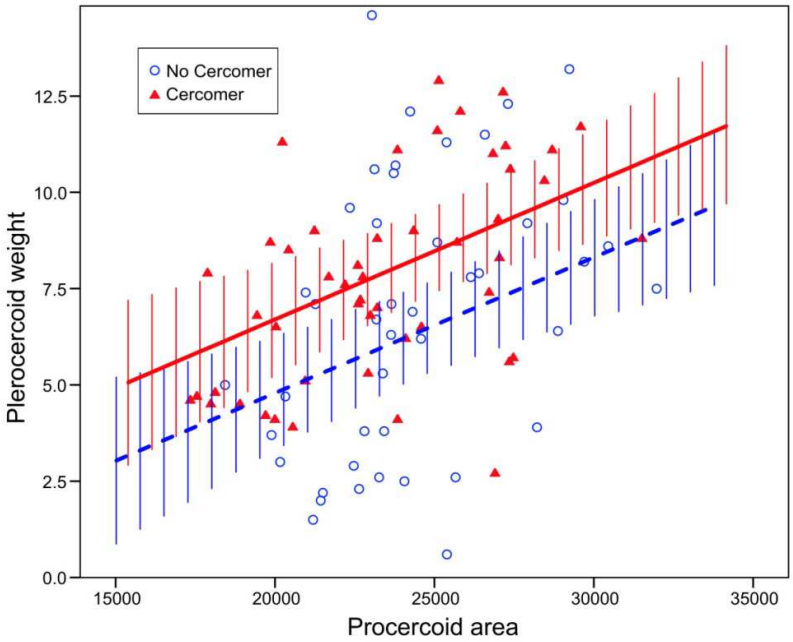

Figure V. 4: The relationship between procercoid area (um^2) in copepods and plerocercoid weight (mg) in fish. The best fit regression, estimated by the general linear model with just main-effects, was plotted separately for procercoids that did (filled triangles, solid line) and did not (open circles, dashed line) have a cercomer after 9 days in copepods. Bars around regression lines are the 95 % CI.

Determinants of energy content

None of the two-way interactions had a significant effect on glycogen content (all $F_{1, 80} < 2.84$, all $P > 0.096$), and their removal did not significantly decrease explanatory power (R^2 dropped from 0.191 to 0.158, $F_{3, 80} = 1.09$, $P = 0.36$). A main-effects-only model indicated that plerocercoids had higher glycogen content if they had a cercomer 9 DPE ($F_{1, 83} = 9.61$, $P = 0.003$) (Figure V. 5). There was also a non-significant

trend for worms from the high feeding treatment to have more glycogen ($F_{1, 83} = 3.17$, $P = 0.079$), but all other effects were not significant (all $F < 2.12$, all $P > 0.15$).

Figure V. 5: Mean glycogen content (ug mg^{-1} fresh weight) of plerocercoids from fish that had developed fast (cercomer after 9 days in copepods) or slow (no cercomer on day 9) in copepods

It should also be noted that plerocercoid size and glycogen content were positively correlated ($R^2 = 0.259$, $F_{1, 90} = 31.2$, $P < 0.001$) (Figure V. 6).

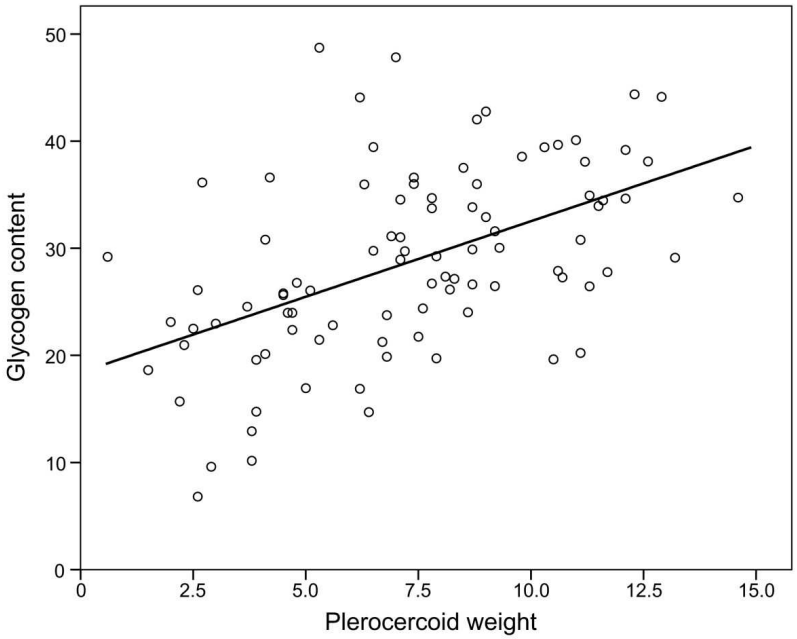

Figure V. 6: Scatterplot of plerocercoid weight (mg) versus glycogen content (ug mg^{-1} fresh weight) with the least squares regression line

Discussion

How long (Hammerschmidt et al. 2009) and how fast (Benesh et al. 2012) *S. solidus* grow in copepods is known to influence infection and growth in sticklebacks. Our results complement those studies and suggest that larval ontogeny is very important for the coupling of performance in the two hosts. Procercoids that develop faster (have a cercomer 9 DPE) are able to infect fish sooner, and they tend to grow to larger sizes with larger energy reserves in fish. Moreover, the previously documented association between procercoid size and fitness seems partially attributable to ontogeny. Fast-growing procercoids tended to have higher infection rates at 14 DPE, and perhaps 11 DPE. Ontogeny is positively correlated with procercoid size at this time, at least when environmental conditions (copepods stage) are constant (Benesh 2010a,b), suggesting that developmental variation may underlie the increase in infection probability with procercoid size. At 17 DPE worms are essentially fully developed, and at this time point there was no influence of procercoid size or ontogeny on infection probability. On the other hand, worms that were bigger when entering fish were also bigger when recovered, suggesting that procercoid size may influence fitness independently of ontogeny.

Growth and development are interwoven processes, so their individual contributions to fitness are difficult to completely disentangle. It is nonetheless clear that worms that grow and develop rapidly have the highest fitness under experimental settings. For example, let us compare a fast-growing, fast-developing worm (cercomer 9 DPE and one SD larger than the sample mean) with a slow-growing, slow-developing worm (no cercomer 9 DPE and one SD smaller than the mean). The fast-growing worm is predicted to have up to 20 % higher infection probability (at 11 DPE), and to be ~85 % bigger with ~25 % higher glycogen content after 3.5 weeks in sticklebacks. Hammerschmidt et al. (2009) suggested that the optimal switching time for *S. solidus* balances increasing establishment probability in fish and decreasing survival probability in copepods. This is similar to the size-age tradeoffs thought to shape switching times in free-living animals with complex life cycles. Just as the tradeoff between size and age depends on growth rate (Roff and Fairbairn 2007), the tradeoff between establishment probability and mortality is mediated by developmental rate; worms that rapidly develop may switch earlier to fish, avoiding age-related mortality in copepods. Thus, the advantages to rapid growth and development appear pronounced: earlier infectivity and the resulting avoidance of mortality in copepods as well as faster and more efficient growth in fish.

Although there seems to be selection for rapid growth and development in copepods, long-term phenotypic change is unlikely. Parasite species from divergent taxa with similar life cycles (e.g. transmission from a copepod to a fish) tend to exhibit characteristic rates of larval growth and development, strongly suggesting life history strategies converge to universal adaptive peaks for a given type of life cycle (Benesh et al. 2011). Thus, there are presumably important tradeoffs that make extremely rapid growth or development suboptimal. Several hypotheses exist: 1) rapid growth and ontogeny requires over-consumption of host nutrients reducing host survival (i.e. the virulence tradeoff (Frank 1996; Parker et al. 2003b)), 2) acquiring the resources for rapid growth and ontogeny requires host specialization and reduced generality (Combes 1991), 3) rapid growth or ontogeny is less efficient, resulting in higher susceptibility to environmental stressors, such as starvation

(Metcalfe and Monaghan 2001; Stoks et al. 2006; Crean et al. 2011), 4) maturation, and the cell differentiation it entails, reduces growth potential (Arendt 2000). Benesh (2011) argued that there is relatively little evidence for hypothesis one for the larval stages of trophically-transmitted helminths, including *S. solidus* in copepods (Wedekind 1997; van der Veen et al. 2002; Michaud et al. 2006). Hypothesis two cannot be discounted, because host specificity seems important for the larval life history of some tapeworms (Dupont and Gabrion 1987), although *S. solidus* is a generalist in copepods (Orr and Hopkins 1969). Hypotheses three and four are allocation tradeoffs (somatic growth vs energy storage, maturation vs growth potential). Such tradeoffs can be masked by variation in resource acquisition (Van Noordwijk and de Jong 1986; Reznick et al. 2000). For instance, in opposition to hypothesis four, fast-growing *S. solidus* procercoids also develop quicker (Benesh 2010b), perhaps because they have more resources available to them. Certainly there is more work to do to identify the tradeoffs shaping larval life history strategies in parasites.

The feeding treatment had only moderate, non-significant effects on infection rate and glycogen content, and no effect on plerocercoid size. A possible explanation for this is that, given their stronger effects, procercoid size and cercomer presence/absence explain the variation in fitness induced by the feeding treatment. In any case, carryover effects attributable to unmeasured condition variables do not appear to be pronounced. Some of the covariance between larval traits (growth and ontogeny) and the fitness components was surely induced by the feeding treatment and thus environmentally-determined. Because genetic variation is a prerequisite for trait evolution, it will be interesting to see if there is genetic covariance between larval traits and fitness, i.e. do parasite genotypes that rapidly grow and develop also have higher infection probability?

Glycogen makes up approximately 16 % of the weight of fully-developed plerocercoids taken from fish (>100 mg) (Hopkins 1950). In the young plerocercoids studied here (~7 mg on average), glycogen constituted 2.9 % of worm wet weight, and in medium-sized plerocercoids (~75 mg) it is about 10 % (Benesh and Kalbe, unpublished data). Thus, worms appear to steadily increase their glycogen reserves as they grow in fish. We observed that the fastest-growing worms in fish had the highest glycogen content, suggesting rapid growth is not inefficient and contradicting hypothesis three above. This may be another case in which variation in resource acquisition masks an allocation tradeoff, i.e. worms in good condition can invest in both somatic growth and energy storage.

Conclusions

Transmission up the food web into bigger, ‘better’ hosts does not imply a new start for parasites. Analogous to free-living organisms with complex life cycles, phenotypic variation in the intermediate host can have carryover effects in the next host, though additional studies are needed to generalize this. For *S. solidus* procercoids, transmission to sticklebacks as a small, not-yet-fully developed larva has clear costs in terms of lower infection probability and stunted, inefficient growth. Given the seemingly strong selection for rapid

growth and development in copepods, more work is needed to identify what prevents change in the ontogenetic schedule of *S. solidus* (ecological tradeoffs? genetic constraints? developmental thresholds?).

Acknowledgments

Thanks to R. Leipnitz and M. Schwarz for help with the copepods, to T. Arlt for help with the glycogen assays, and to I. Samonte-Padilla for providing fish.

Chapter VI

Does resource availability affect host manipulation? - An experimental test with *Schistocephalus solidus*.

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Abstract

Host manipulation is a common strategy parasites employ to increase their fitness by changing the behavior of their hosts. Whether host manipulation might be affected by environmental factors such as resource availability has received little attention. We experimentally infected lab-bred copepods with the cestode *Schistocephalus solidus*, submitted infected and uninfected copepods to either a high or a low food treatment, and measured their behavior. Uninfected, but not infected, copepods moved slower under low food conditions, and consequently the difference between infected and uninfected copepods, i.e. host manipulation, depended on the feeding regime. These differences could be mediated by the physical condition of the host rather than changes in host manipulation by parasites. Additionally, we measured three fitness-relevant traits of the parasite (growth, development, infection rate in the next host) to identify potential trade-offs with host manipulation. The largest parasites in copepods appeared the least manipulative, but this may again reflect variation in copepod condition, rather than parasite life history trade-offs. Our results raise the possibility that parasite transmission depends on environmental conditions.

Introduction

The environment organisms experience is rarely uniform; it varies over time and space. This applies to both free-living organisms and their parasites. Environmental stressors can reshape host-parasite interactions in various ways depending on the species and stressors involved (Lafferty and Kuris 1999). Many parasites enhance their fitness by changing the behavior of their host (reviewed by Holmes and Bethel 1972; Poulin and Thomas 1999; Moore 2002, 2013; Poulin 2010). Such host manipulation could also be influenced by the host's environment (Thomas et al. 2012). Biotic factors such as predator cues (e.g. Jakobsen and Wedekind 1998; Baldauf et al. 2007; Durieux et al. 2012; Dianne et al. 2014) or the presence of other parasites (Cézilly et al. 2000, 2014; Haine et al. 2005; Dianne et al. 2010; Hafer and Milinski 2015) can influence host manipulation. Differences in abiotic factors might also play a role. In isopods infected by an acanthocephalan parasite, host manipulation changes between seasons but this does not seem to be caused by either temperature

or lighting conditions but might rather be related to host or parasite age (Benesh et al. 2009a). Resource availability, too, could affect host manipulation. Hungry and satiated hosts can differ in the extend of host manipulation they show (e.g. Giles, 1987; Barber *et al.*, 1995; Jakobsen and Wedekind, 1998). Depending on how changes in resource availability affect host-parasite interactions and what mechanisms parasites employ to manipulate their hosts, different outcomes with regards to host manipulation will ensue.

Host manipulation can result in energetically costly behavior such as increased activity (reviewed by Poulin, 1994a). Resource limitation will reduce a host's ability to perform such behaviors (Thomas et al. 2011, 2012). Limited resources could also reduce host manipulation, if parasites need to use energy to manipulate and are limited by the resources their host has available. Energetic costs of host manipulation have often been assumed, e.g. because parasites might use some substance they have to produce and emit to manipulate their hosts (Poulin 1994b, 2010; Biron et al. 2005b; Thomas et al. 2005, 2011; Vickery and Poulin 2009), but have yet to be convincingly demonstrated. Potential trade-offs between the level of host manipulation and other important parasite traits could hint at such a cost (Franceschi et al. 2010a; Maure et al. 2011). Alternatively, host manipulation could increase under resource limitation. For example, a lack of resources could impair a host's defenses against manipulation (Roitberg 2012). Another way for manipulation to increase under resource limitation is if parasites affect host behavior by draining energy from the host (reviewed by Adamo 2012; Lafferty and Shaw 2013). Energy drain shifts the trade-off between predator avoidance and feeding towards feeding and might hence resemble host manipulation that serve to increase a host's predation susceptibility (Milinski 1990).

Schistocephalus solidus has a three host life cycle consisting of a cyclopoid copepod, a three-spined stickleback and a piscivorous bird (Clarke 1954; Dubinina 1980). At 18°C it spends about two weeks in copepods before it is ready for transmission to the next host, which takes place when the copepod is consumed by a stickleback. During this time, it reduces the activity (Hammerschmidt et al. 2009; Benesh 2010b; Hafer and Milinski 2015) and predation susceptibility (Weinreich et al. 2013) of its host. In a previous study (Benesh and Hafer 2012), we investigated the effect of the performance of *S. solidus* in its copepod host (i.e. growth and development) on performance in the fish host (e.g. infection success and growth). To create variation in copepod quality, we used two different feeding treatments. Here, we report the impact of these feeding treatments on the behavior of infected and uninfected copepods. In addition, we explored potential trade-offs between host manipulation and other life history traits that could hint at an energetic cost to host manipulation.

Methods

Schistocephalus solidus and infection

Schistocephalus solidus originated from Lake Skogseidvatnet, Norway. They were obtained by dissecting wild caught three-spined sticklebacks (*Gasterosteus aculeatus*) and breeding the adult parasites in pairs in an

in vitro system (Smyth 1946). Tapeworm eggs were stored in the fridge (4°C) until use, incubated for three weeks at 20 °C, and then exposed to light over night to induce hatching (Dubinina 1980). Infection took place by exposing copepods to one coracidium each. Copepods that were used as uninfected controls received no coracidia but were otherwise treated the same.

Copepod maintenance

We used copepods (*Macrocyclops albidus*) from a laboratory stock that originated from the same population as *S. solidus* (Lake Skogseidvatnet, Norway). On the day prior to infection they were distributed on 24-well microtitre plates with about 1mL of water. Copepods were maintained at 18°C in a 16h/8h light/dark cycle and fed with *Artemia* sp. naupili every other day. Copepods in the high food treatment (H) received four *Artemia* at each feeding, while copepods in the low food treatment (L) received two (Figure VI. 1). These treatments are sufficient to affect copepod mortality and parasite growth (Benesh 2010a). In our experiment, the high food treatment led to faster parasite development, faster growth and higher infection success in one experimental group (day -11 copepods). In a second experimental group (day 17 copepods), the high food treatment only significantly improved parasite growth but not development or infection success (See supplementary information VI).

Measurements of parasite performance

Copepods are transparent making it possible to view and measure a parasite in vivo (Wedekind et al. 2000; Benesh et al. 2012). We checked copepods for infection 6-8 days post infection (dpi). Parasites were additionally checked inside their hosts for the presence or absence of a cercomer 9 dpi. While the function of the cercomer is unknown, it is a good indicator for parasite development and their ability to infect fish (Smyth and McManus 1989). On the day prior to exposure to fish (10 or 16 dpi), we measured the size of the parasite. This took place by photographing each parasite twice within its host under a microscope. From these photos we measured the area of the parasite (without cercomer) using image J (Rasband 2008) and took the average from these two measurements. Three-spined sticklebacks (*Gasterousteus aculeatus*) were individually exposed to a single copepod, either 11 (day-11 copepods) or 17 (day-17 copepods) dpi. On day 11, any potential trade-off between manipulation and the ability to infect fish should be especially crucial, since at this time only the fastest developing *S. solidus* might be ready to infect the next host (Hammerschmidt et al. 2009; Benesh and Hafer 2012). By day 17 nearly all *S. solidus* are ready to infect fish (Benesh and Hafer 2012). Approximately four weeks after exposure, we dissected the fish to determine infection success (see Benesh and Hafer 2012). Fish experiments were conducted with permission of the 'Ministry of Energy, Agriculture, the Environment and Rural Areas' of the state of Schleswig-Holstein, Germany (reference number: V 313-72241.123-34).

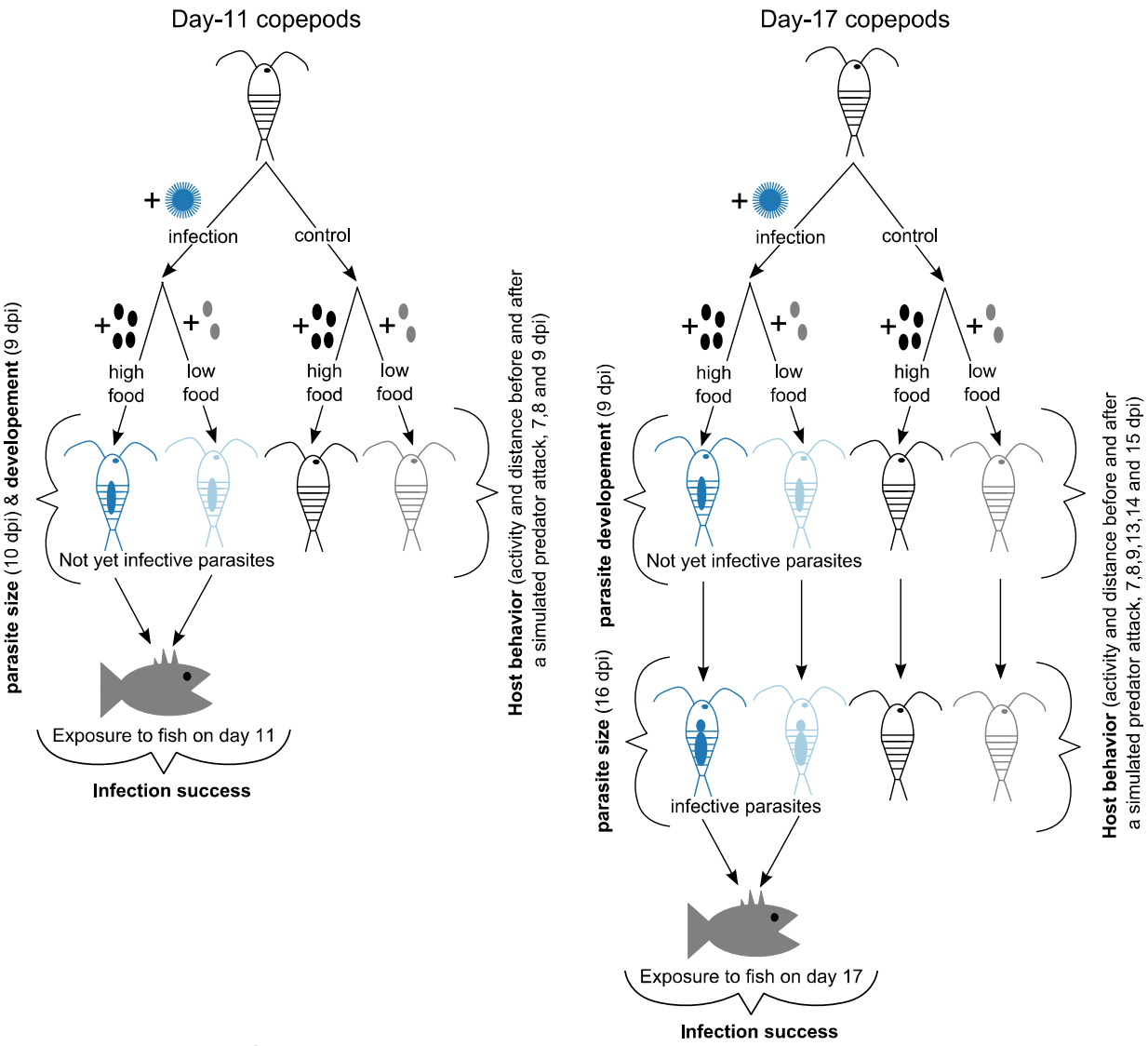

Figure VI. 1: overview of the experimental set up. Copepods were either exposed to *S. solidus* coracidia or not, subjected to a high or a low food treatment, and exposed to fish at two different time points, day 11 (day-11 copepods) or day 17 (day-17 copepods). We measured three aspects of parasite performance: development and size in copepods and infection success in fish. We measured two aspects of copepod behavior: activity (i.e. proportion of time copepods spent moving) and, when moving, the distance moved.

Copepod behavioral measurements

Schistocephalus solidus has to spend between 10 days and two weeks at 18°C in its copepod host before it becomes infective to sticklebacks, at which time host manipulation switches from predation suppression to predation enhancement (Hammerschmidt et al. 2009; Hafer and Milinski 2015). This switch is not obvious in all populations and some populations show no clear predation enhancement, including the population used in this study (Benesh 2010b). We measured behavior on 7, 8 and 9 dpi (not yet infective parasite) and 13, 14 and 15 dpi (infective parasite, only for the day-17 copepods). Multiple observations help to more accurately quantify an individual's typical behavior and make correlations between host manipulation and other parasite traits more robust (Benesh et al. 2008).

Each well plate with copepods was gently placed on an apparatus that dropped it by 3 mm in a standardized way to simulate a predator attack (see Hammerschmidt et al. 2009). Once the plate had been on the apparatus for a 1 minute acclimation period, we started recording copepod behavior using a video camera (Panasonic Super DynamicWV-BP550, Panasonic Corporation, Osaka, Japan). Copepods were recorded for 90 seconds, then the plate was dropped and copepods were recorded for an additional 90 seconds. We split the recordings into 2-second intervals (90 observations per copepod per recording event). Preliminary analyses found that little extra movement was recorded with shorter intervals. Using the manual tracking plugin within image J (Rasband 2008), we recorded whether or not each copepod moved within each of these two second intervals (activity) and, if so, how far it moved (distance). We only analyzed the behavior of exposed and infected and unexposed and uninfected copepods that survived until the day that its treatment group was used to infect fish (day 11 or day 17). In total we recorded the behavior of 382 copepods (day-11 copepods: infected: H: 65, L: 68, uninfected: H: 45, L: 44, day-17 copepods: infected: H: 51, L: 51, uninfected: H: 30, L: 28).

Statistical analysis

We analyzed copepod behavior at a fine scale, i.e. each 2-second observation. Thus, we had repeated observations on the same copepod at two levels: within a recording event (i.e. each copepod on a given day) and across recording events on different days. To analyze copepod activity (proportion of time spent moving), we used generalized linear mixed models with binomial error family in the lme4 package (Bates et al. 2014) in R (R Development Core Team 2010). To analyze the distance copepods moved, we included only data from when movement had occurred. We used linear mixed models (lme4 package, Bates et al. 2014) in R (R Development Core Team 2010) after log transforming the distance. To account for variation between individual copepods over days we included copepod identity into the random effects. Additionally, we included the recording event to account for variation within days during the recording event together with the time interval in the recording (before vs. after the simulated predator attack) to account for differences before and after the simulated predator attack. For day-17 copepods we additionally included the infectivity status in the random effects with the copepod identity to account for differences before and after parasites reached infectivity. Both, time interval and infectivity status (only day-17 copepods) were also included as fixed effects. We stepwise added infection, feeding treatment and their pairwise interactions with each other and time interval and infectivity status (only day-17 copepods). We compared models using likelihood ratio tests. We accepted a model as having a better fit than a less complicated one if it explained the data significantly better as judged by likelihood ratio tests. See Table VI. 1 for the details and outputs of the models. We fitted separate models for day-11 and day-17 copepods since only the later included the behavior of copepods harboring already infective parasites. If we found any significant interactions between infection and feeding, we conducted Tukey post hoc tests. We used the same models described above but combined infection and feeding into a single factor whose levels comprised all possible combinations of these two factors and removed all other interactions involving feeding or infection from the model. On these models we applied

general linear hypotheses within the multcomp package in R (Hothorn et al. 2008). For all other statistics and more detailed information on the models used please refer to Table VI. S1.

To investigate a potential association between copepod behavior and parasite size, development (i.e. presence or absence of a cercomer on day 9) and infection success in fish, we used the same mixed models described above except we limited them to infected copepods. We added the parasite performance traits (size, development, and infection success) to the models, as well as their interactions with the other fixed factors, and performed likelihood ratio tests (see Table VI. S2). Since this involved multiple comparisons we took adjusted p-values (after bonferroni) into account when we found any significant differences.

Results

Confirmation of the effect of parasite infection

Schistocephalus solidus reduces the predation susceptibility of its copepod host before reaching infectivity (Weinreich et al. 2013). This is marked by a reduction in activity (Hammerschmidt et al. 2009; Benesh 2010b; Hafer and Milinski 2015) which we also found in our study (Figure VI. 2 A, B, $p<0.0002$, Table VI. 1). In day-17 copepods there was an interaction between infection and infectivity status (i.e. whether or not the parasite was ready to infect the next host, $p=0.0141$, Table VI. 1). Differences between infected and uninfected copepods were more pronounced before the parasites reached infectivity (Figure VI. 2 B). This is in line with previous predictions (Parker et al. 2009c) and findings that any predation suppression at least decreases as *S. solidus* becomes infective (Hammerschmidt et al. 2009; Benesh 2010b; Hafer and Milinski 2015). The distance which copepods moved was also significantly affected by an infection with *S. solidus* (Figure VI. 2 C, D, $p<0.0001$, Table VI. 1); infected copepods moved shorter distances. Infectivity status did not significantly interact with how far copepods moved (Table VI. 1). There was some interaction between infection and how copepods responded to the predator attack for activity in day – 11 copepods ($p=0.0062$, Table VI. 1) and the distance copepods moved in day – 17 copepods ($p=0.0325$, Table VI. 1). In day – 11 copepods infected copepods reacted more strongly while in day 17 copepods infected copepods reacted less strongly than uninfected ones. However, both uninfected copepods and infected ones always showed the same, clear reaction to the simulated predator attack; they reduced their activity and the distance they moved. Accordingly, our results do not seem highly sensitive to the time interval in the recording. Overall we can confirm that *S. solidus* alters the behavior of its copepod host by decreasing its activity and the distance it moves.

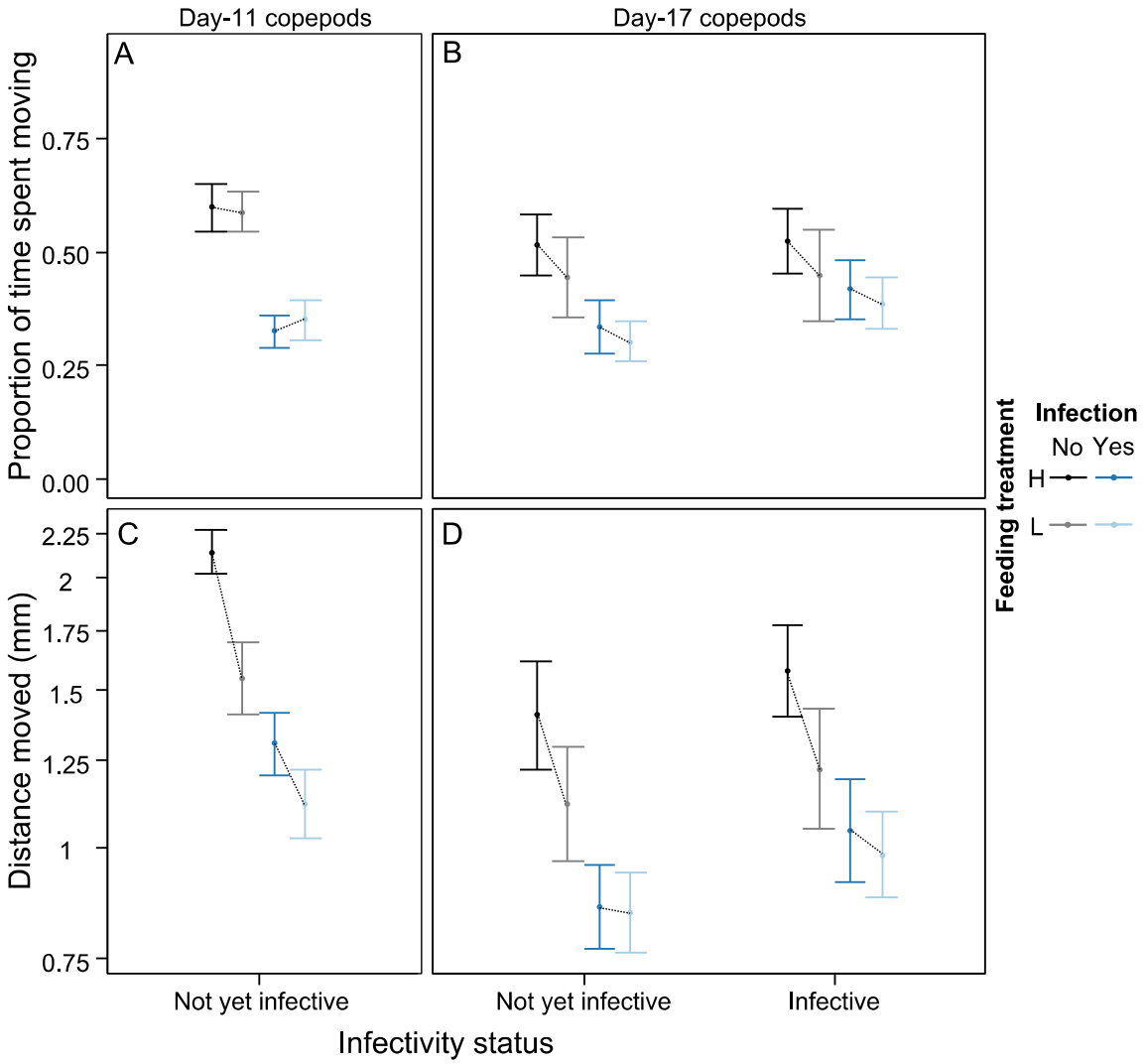

Figure VI. 2: Activity (A, B) and Distance (C, D) as response to infection and feeding treatment. Error bars present means +/- 95 % CI. Feeding treatment: H: High food treatment, L: Low food treatment. N: Day-11 copepods: not infected: H: 45, L: 44, infected: H: 65, L: 68, day-17 copepods: Not yet infective: not infected: H: 30, L: 28, infected: H: 51, L: 49, day-17 copepods: Infective: not infected: H: 30, L: 27, infected: H: 51, L: 51. Dotted lines connecting groups are to aid comparison of feeding treatments, whereas the bar colors differentiate infection status.

The effect of feeding on host behavior and host manipulation

If feeding treatment affects host manipulation, a significant interaction between feeding treatment and infection should occur. Neither this interaction nor feeding alone had any significant effect on copepod activity (Figure VI 2 A, B, Table VI. 1). However, distance was affected by both, feeding treatment (Figure VI 2 C, D, $p<0.03$, Table VI. 1) and its interaction with infection (Figure VI. 2 C, D, $p<0.05$, Table VI. 1). Feeding treatment affected uninfected copepods more strongly than infected ones (Figure VI. 2 C, D). Post hoc tests reveal that uninfected copepods moved significantly slower if they were in the low food treatment than if they were in the high food treatment ($p<0.02$, Table VI. S1). In infected copepods distance did not differ between feeding treatments (Table VI. S1, Figure VI. 2 C, D). Consequently, differences between

infected and uninfected copepods, i.e. host manipulation, were larger in the high food treatment than in the low food treatment (Table VI. S2, Figure VI. 2 C, D).

Table VI. 1: Outcome of likelihood ratio tests on the effect for copepod activity (i.e., proportion of time spent moving) and the distance copepods moved when moving. Whether or not a copepod moved within a two second interval (Activity) or, if it moved, how far it moved (distance, log transformed) were our response variables. Copepod identity (ID),the recording event (RE, i.e. a combination of copepod identity and the day of the recording), and the time interval in the recording (i.e. before vs. after the simulated predator attack, INTERVAL) were used to construct the random effects. INTERVAL was additionally included as a fixed effect. For day 17-copepods we additionally included whether or not parasites were infective for fish (INFECTIVITY) as both fixed and random effect (together with ID). Subsequently, we added whether or not copepods were infected (INFECTION) and which feeding treatment they received (FEED) and all their pairwise interactions with TIME and INFECTIVITY (day-17 copepods). Test statistics and MCMC-estimated p-values are for the comparison with the preceding model. Null model: Day-11 copepods: INTERVAL + (INTERVAL |RE) + (1|ID), Day-17 copepods: INTERVAL + INFECTIVITY + INTERVAL : INFECTIVITY + (INTERVAL|RE) + (INFECTIVITY | ID). Significant p-values have been marked in bold.

	Factors	Activity			Distance		
		DF	Chisq	p	DF	Chisq	p
Day-11 copepods	+INFECTION	1,7	87.180	**<0.0001**	1,8	73.990	**<0.0001**
	+INFECTION:INTERVAL	1,8	8.651	**0.0033**	1,9	0.820	0.365
	+FEED	1,9	0.059	0.8082	1,10	20.952	**<0.0001**
	+FEED:INTERVAL	1,10	7.497	**0.0062**	1,11	5.253	0.0219
	+FEED:INFECTION	1,11	0.680	0.4094	1,12	5.164	0.0231
		56232 observations on 639 RE and 222 copepods			24769 observations on 635 RE and 222 copepods		
	Factors	Activity			Distance		
		DF	Chisq	p	DF	Chisq	p
Day-17 copepods	+INFECTION	1,11	16.155	**0.0001**	1,12	42.062	**<0.0001**
	+INFECTION: INTERVAL	1,12	0.099	0.7533	1,13	4.572	**0.0325**
	+INFECTION: INFECTIVITY	1,13	6.020	**0.0141**	1,14	0.212	0.2118
	+FEED	1,14	2.121	0.1453	1,15	4.859	**0.0275**
	+FEED: INTERVAL	1,15	0.003	0.9605	1,16	0.013	0.9103
	+FEED:INFECTIVITY	1,16	0.001	0.9716	1,17	1.476	0.2244
	+FEED:INFECTION	1,17	1.118	0.2903	1,18	3.868	**0.0492**
		79376 observations on 906 RE and 160 copepods			32668 observations on 879 RE and 160 copepods		

Correlations between host manipulation and other fitness related traits

If host manipulation is costly and those costs lead to trade-offs with other fitness related trades, parasites that manipulate less (i.e. behave more similar to uninfected hosts) should do better.

Parasite size and development

Parasite size on day 10 in day-11 copepods did not correlate with copepod activity (Table VI. S2, Figure VI. 3 A). However, there was a positive correlation between size and distance in day-11 copepods (p=0.0017, adjusted p value = 0.0204, Table VI. S1, Figure VI. 3 C). Parasite size on day 16 in day-17 copepods correlated positively with activity and distance (p<0.003, adjusted p value < 0.04, Figure VI. 3 B, D, Table VI. S1). Copepods with larger parasites moved more often and further (Figure VI. 3 B – D).

Development (presence or absence of a cercomer 9 dpi) correlated positively with activity in day-11 copepods (more active, i.e. less manipulated, copepods are more likely to have a cercomer 9 dpi, p=0.0103,

Table VI. S1), but this association disappeared when correcting for multiple testing (p=0.1236). Neither distance in day 11 copepods nor activity nor distance in day 17 copepods showed any correlation with parasite development (Table VI. S2). Overall, developmental rate did not seem to be associated with host behavior.

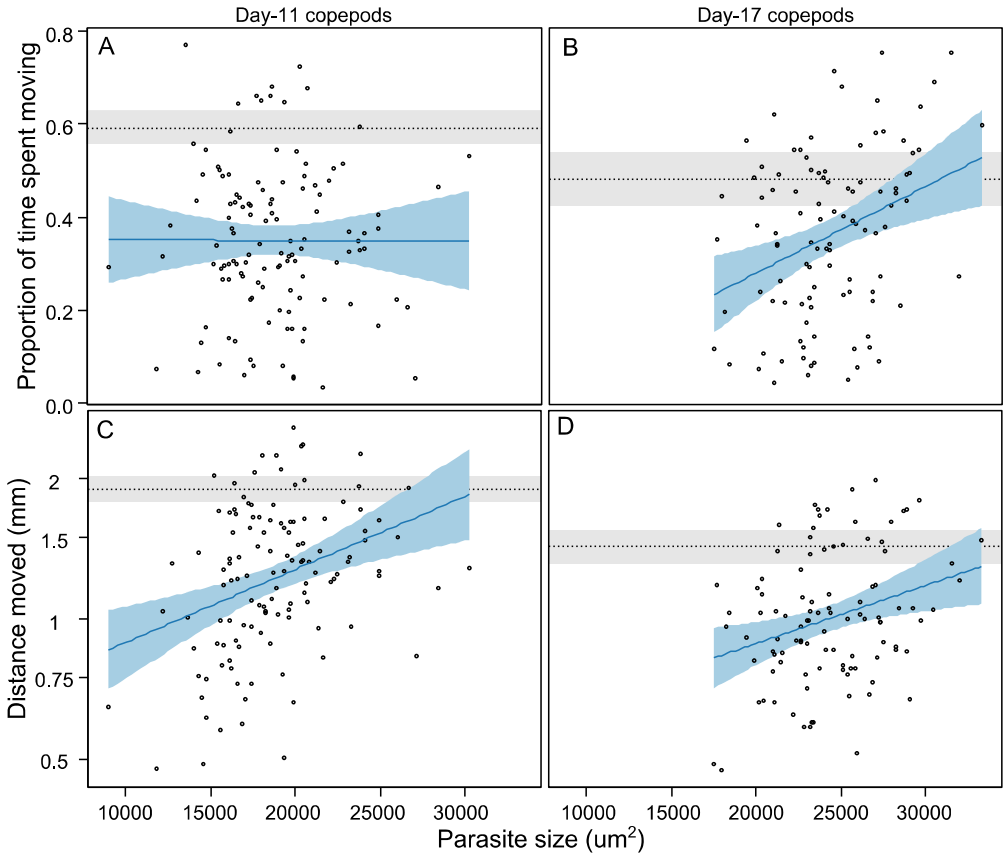

Figure VI. 3: The effect of parasite size on host activity (A, B) and distance hosts moved (C, D). Solid lines indicate the trend line. Dotted horizontal lines indicate mean behavior of uninfected copepods. The shaded areas around the lines indicate 95% CI. N: day-11 copepods: 122, day-17 copepods: 102.

Infection probability in fish

Parasites in day-11 copepods that moved further had a higher probability to establish themselves in fish on day 11 (p=0.0049, Table VI. S2, Figure VI. 4 B), albeit with marginal significance after adjusting for multiple testing (p=0.0588). However, their host's activity did not correlate with the probability to infect fish (Table VI. S2, Figure VI. 4 A). In day-17 copepods, we found no significant correlation between infection probability in fish and host behavior (Table VI. S2, Figure VI. 4). It appears that parasites in hosts that move larger distances – and hence are possibly less manipulated – might be better at infecting fish, but only at the earlier time point (day 11).

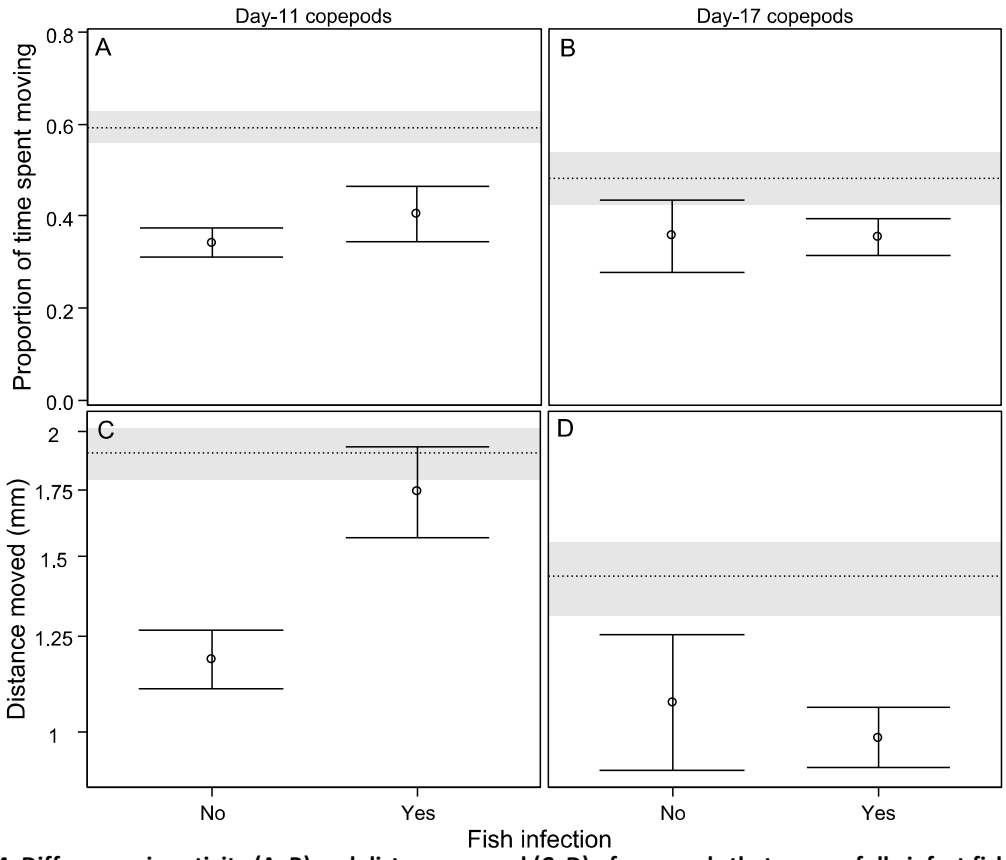

Figure VI. 4: Differences in activity (A, B) and distance moved (C, D) of copepods that successfully infect fish and those that do not. Error bars indicate 95 % CI. Dotted horizontal lines indicate mean behavior of uninfected copepods. The shaded areas around the lines indicate 95% CI. N (Fish infection successful/failed): day-11 copepods: 110/11, day-17 copepods: 18/81).

Discussion

Copepods infected by *S. solidus* seem unaffected by resource availability. In a low food environment they show the same altered behavior as their counterparts in a high food environment. In uninfected copepods, activity (i.e. proportion of time spent moving) is not affected by resource availability either, but they move less far in a low food than in a high food environment. This results in differences in host manipulation; parasites appear to manipulate less in a low food environment. Rather than changes in parasite-induced manipulation, such an effect could be produced by differences in copepod condition between feeding treatments. Copepods that are in worse condition (e.g. because they have been limited in their resources) should be able to invest less in muscle tissue, limiting the distance they are able to move. Copepods that die sooner have less muscle tissue (Franz and Kurtz 2002). Infected copepods already move less than uninfected ones, so it might not be practical for them to reduce their movement even further in a low food environment, since they still have to be able to e.g. forage.

If reduced physical condition is responsible for the reduced distance travelled by uninfected copepods under food constraints, could the modified behavior of infected copepods also be caused by a decline in host condition, in this case due to parasite infection (see McElroy and de Buron, 2014)? Infection with *S. solidus*

has no significant effect on either lipid storage or the amount of muscle tissue in the host (Franz and Kurtz 2002). Other parasites have even been found to increase the energy reservoirs of their host (Amat et al. 1991; Plaistow et al. 2001; Ponton et al. 2005). *Schistocephalus solidus* not only reduces how far but also how often its host moves. The latter is not affected by feeding regime. Furthermore, an adverse effect of a parasite infection should increase with infection intensity. Host activity is not affected by the number of not yet infective *S. solidus* and even increases with the number of infective *S. solidus* (Hafer and Milinski 2015).

We find no clear correlation between cercomer presence 9 dpi (development) and host behavior. However, host activity in day-17 copepods and distance in day-11 and day-17 copepods correlates positively with size on day 10 (day-11 copepods) or day 16 (day-17 copepods). This is puzzling since Benesh (2010b) found no such correlations. On a family level, there was even a negative correlation between parasite size on day 11 and development and activity before reaching infectivity (Benesh 2010b). Possibly our food treatments introduced more variation in parasite size and/ or copepod behavior that enhanced co-variation between them. A positive correlation between size and activity after reaching infectivity is in line with the finding that two infective *S. solidus* enhance the activity of their host more than one (Hafer and Milinski 2015). Maybe this is not so much an effect of number as of size. However, there need not be any causal relationship between parasite size and host activity. Copepods in better physical condition might at the same time be able to move further (see above) and allow their parasites to grow to a larger size. Indeed, parasites whose hosts are in a high food treatment do grow larger (Benesh 2010a, supplementary information VI).

We found a trend for a correlation between infection rates in fish on day 11 and the distance copepods with not yet infective parasites moved. Copepods that moved further (i.e. are manipulated less) are more likely to infect fish. Previous studies have also reported a negative correlation between parasite fitness and host manipulation, suggestive of trade-offs (Franceschi et al. 2010a; Maure et al. 2011). Since these studies are correlational, alternative explanations cannot be ruled out. In our case, a trade-off may not be the best explanation. As discussed above, the distance which copepods move might be affected by their physical condition. It seems reasonable to assume that copepods that are in better physical condition can move further and can harbor rapidly growing parasites that are better at infecting fish. This will be especially crucial for parasites that are transmitted to fish early (i.e. 11 dpi) when there is still variation in development and only the fastest developing parasites succeed in infecting fish. By day 17 even parasites in copepods in worse condition should have been able to catch up in their development and hence be able to infect fish (Benesh and Hafer 2012).

Host manipulation can have important ecological consequences (Thomas et al. 1998b, 1999, 2005; Lefèvre et al. 2009b; Lafferty and Kuris 2012), such as changes in food webs (Lefèvre et al. 2009b; Lafferty and Kuris 2012). Differences between infected and uninfected copepods seem more pronounced in a high food environment. Infected copepods are less likely to be consumed by sticklebacks before reaching infectivity, i.e. while *S. solidus* reduces activity (Weinreich et al. 2013), and more likely to be consumed thereafter, i.e. while *S. solidus* enhances activity (Wedekind and Milinski 1996). While we do not know if the differences between

feeding treatments are large enough to influence predation susceptibility, our results nonetheless indicate that behavioral differences between infected and uninfected copepods can depend on resource availability. This raises the possibility that *S. solidus* transmission to fish might be environmentally-dependent.

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Conclusion

The conclusion has partly been altered from: Hafer, N., and M. Milinski (submitted). Cooperation or Conflict: Host manipulation in multiple infections in H. Mehlhorn, eds. Parasites and Behavioural Changes. Parasitology Research Monographs. Springer, Heidelberg.

Not everything that appears to be adaptive host manipulation has to be caused by mechanisms that have evolved for this specific purpose. Rather, such behavioral alterations can be caused by side-effects from energy drain imposed by the parasite shifting the trade-off between predation avoidance and feeding towards the later making the host more prone to predation (Chapter II). This adds to solving a long standing debate on whether or not behavioral alterations in *S. solidus* infected sticklebacks are caused by true host manipulation or are side-effects of increased energy drain (reviewed by Milinski 1990; Barber and Huntingford 1995; Barber et al. 2000, 2007; Barber and Scharsack 2010). Nevertheless, in nature, behavioral changes caused by side-effects could still be adaptive and result in enhanced transmission. This could then preclude the evolution of true and potentially costly host manipulation.

When two manipulating parasites infect the same host, there is potential for conflict. I show clearly for the first time using experimental infections that one parasite can change or even completely sabotage host manipulation of both a conspecific (Chapter III, IV) and a phylogenetically distinct parasite (Chapter IV). While there are some differences in how strongly *C. lacustris* and *S. solidus* manipulate (Chapter IV), the infective parasite performs better overall. This is in agreement with previous studies (Sparkes et al. 2004; Dianne et al. 2010). Even when alone, upon reaching infectivity a parasite switches from predation suppression to predation enhancement (Hammerschmidt et al. 2009; Parker et al. 2009c; Dianne et al. 2011, Chapter III, IV). If it does so by switching off its previous predation suppression, this switch could also act against any co-infecting parasite as long as both parasites use similar enough mechanisms to manipulate their host. This is obvious if there is an intraspecific conflict, but my findings that an intra and an interspecific conflict show a similar outcome (Chapter IV) indicate that this could also be the case between different parasite species. Such sabotage need not have been evolved but could present a side-effect. Nevertheless, it should increase the fitness of the infective parasite if it prevents its host from becoming unsuitably manipulated.

An exact understanding of the mechanisms underlying host manipulation is still largely missing, though much progress has been made in recent years (Adamo 2012; Helluy 2013; Houte et al. 2013; Hughes 2013; Lafferty and Shaw 2013; Perrot-Minnot and Cézilly 2013). Understanding host manipulation by an individual parasite will be crucial to understand how parasites interact at a mechanistic level in multiple infections where there is potential for either cooperation or conflict. It comes down to a question that has driven studies of host manipulation for decades: Did host manipulation specifically evolve for that purpose or is it, at least originally, a side-effect of infection e.g. caused by inevitable energy drain (Milinski 1990; Poulin 1995,

2010; Cézilly and Perrot-Minnot 2005; Moore 2013, Chapter II). When two or more parasites co-occur in the same host, do they specifically interact with each other's manipulation, do their manipulations simply add up or are they predisposed to interact as my results (Chapter III, VI) suggest?

Parasites with different developmental stages are not the only ones that might be at a conflict over host manipulation. Parasites with different transmission strategies or definitive hosts might be at a conflict, too. We need more studies using experimental infections with different parasite species between which a clear conflict over host manipulation exists. Investigating such conflict over host manipulation requires the availability of at least two suitable species and their host that can be handled in the laboratory. We should further widen the scope of the contestants we use and not only consider (manipulating) parasites but also commensals and symbionts as agents that could be interested in altering host behavior or at least sabotaging host manipulation. Indeed, symbiotic microbes do seem to affect host behavior (reviewed by Feldhaar 2011; Ezenwa et al. 2012).

Whether parasites will evolve specific responses to the presence of other, potentially manipulating parasites will largely depend on the underlying selection pressures which will be strongly influenced by the likelihood of such interactions to occur (Rigaud and Haine 2005). We do not know what proportion of hosts is actually manipulated by a parasite. Only a limited number of systems has been investigated and studies are biased towards traits that are easily accessible by human perception, while those that are not might be overlooked (Moore 2013). Parasites may not be able to adapt to encounter a particular parasite but every parasite will encounter some other parasite, commensal or symbiont. Manipulating parasites can probably expect to encounter organisms which have an interest in a normally behaving host. Parasites would benefit from evolving general strategies to suppress any parasite with manipulation unsuitable to them – or counter manipulation. Are they likely to have the ability to do so? In chapter IV I show that a parasite can sabotage host manipulation by a phylogenetically distinct parasite. We need to test the interaction of one parasite species with multiple other ones. Of course doing so, especially using experimental infections, will be a challenge. If such a generalist mechanism was possible it might put the manipulator at an advantage – it will always encounter other, non-manipulating organisms, but not every organism within a host might encounter a manipulating parasite.

Parasites in nature are not only faced with other co-infecting and potentially also manipulating parasites but also with differences in their host's environment. Differences in resource availability seem to have little influence on host manipulation by *S. solidus* in its copepod host (Chapter VI). They do however affect how far healthy but not infected copepods can move resulting in larger differences between infected and uninfected copepods in a high food treatment (Chapter VI). In *S. solidus* infected sticklebacks, host behavior can be affected by the hosts hunger status which will of course depend on resource availability (Giles 1987b; Barber and Huntingford 1995; Barber et al. 1995). This finding might partly depend on host and parasite age and size (Chapter II). Such differences, whether or not they are due to actual changes in host manipulation, could make host manipulation more successful in certain environments. More studies on how resource availability and other environmental differences might affect host manipulation are clearly needed.

Studying the interaction between different parasites or between parasites and their environment when it comes to host manipulation might be more than just an academic enterprise. Humans too are subject to manipulating parasites. About 30% of humans worldwide are infected by *Toxoplasma gondii* known to change various personality traits (e.g. da Silva and Langoni 2009; Flegr 2013). Are there also other parasites, pathogens, commensals or symbionts that can impact the behavior of infected human hosts? Humans are not only (accidental) intermediate hosts to manipulating parasites, they may also encounter manipulating parasites as definitive hosts. Malaria presents a severe health issue in many countries. Its transmission is aided by host manipulation that alters the behavior of its insect vectors and the attractiveness of its human host to enhance transmission (e.g. Koella et al. 2002; Koella 2005; Lacroix et al. 2005; Cator et al. 2012). Could other parasites or symbionts alter the extent of that manipulation and thereby also possibly infection probabilities?

Host manipulation should not be viewed in isolation as the effect of one parasite on one host in one static environment. Hosts are infected by multiple parasites, symbionts and commensals all of whom might affect host behavior and living in a complex, variable environment. Doing this thesis has answered a number of questions but it has also raised new ones, possibly even more than I had when I started. How do parasites interact on a mechanistic level? Do they interact with each other directly or do they just continue to do what they are doing and thereby interact indirectly? Is there a qualitative difference between inter- and intraspecifc conflict? What happens to host manipulation if other players such as symbionts or commensals enter the game? To what extend do differences in the environment affect host manipulation in nature? What are the ecological impacts of this variability in host manipulation? We are, at best, only beginning to understand how various factors might interact to shape host manipulation and the resulting host behavior in nature. The host, that much at least seems certain, is not solely in control of its behavior.

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Appendices

Appendix for chapter II

Supplementary information II. 1: Reaction to the simulated heron attack

To confirm that the fish reacted to the simulated heron attack, we compared the time between feeding successive food items for the first 5 food items a fish consumed. We only used fish that had consumed at least 5 food items. The simulated heron attack occurred after two food items had been consumed. We used a paired Wilcox test in R with bonferroni corrections to account for multiple testing (R Development Core Team 2010). We did this for each of the four time points separately.

The fish's latency to resume feeding after the simulated heron attack was significantly greater than either the latency to consume the second food item prior to the simulated heron attack or the latency to consume additional food items once feeding had been resumed after the simulated heron attack during all time points ($p<0.0001$, Figure II. S1, Table II. S1). Clearly, fish perceived the simulated heron attack and reacted to it as a frightening event throughout the entire experiment

Table II. S1: Pairwise comparison for the time taken to feed successive food items. Significant p-values ($\alpha<0.05$) have been marked in bold. The simulated heron attack occurred once two food items had been consumed.

Time point	Number of food items already consumed		N	Test statistics[1]		
				v	p	p adjusted[2]
1	1	2	56	0	**<0.0001**	**<0.0001**
1	2	3	56	1537	**<0.0001**	**<0.0001**
1	3	4	56	747	0.9725	1
2	1	2	65	0	**<0.0001**	**<0.0001**
2	2	3	65	2100	**<0.0001**	**<0.0001**
2	3	4	65	616	**0.0180**	0.0540
3	1	2	65	0	**<0.0001**	**<0.0001**
3	2	3	65	2076.	**<0.0001**	**<0.0001**
3	3	4	65	1007	0.3607	1
4	1	2	62	0	**<0.0001**	**<0.0001**
4	2	3	62	1816	**<0.0001**	**<0.0001**
4	3	4	62	750	0.2255	0.6765

1: Values represent V statistic and p values from a paired Wilcox test

2: Bonferroni correction for multiple testing

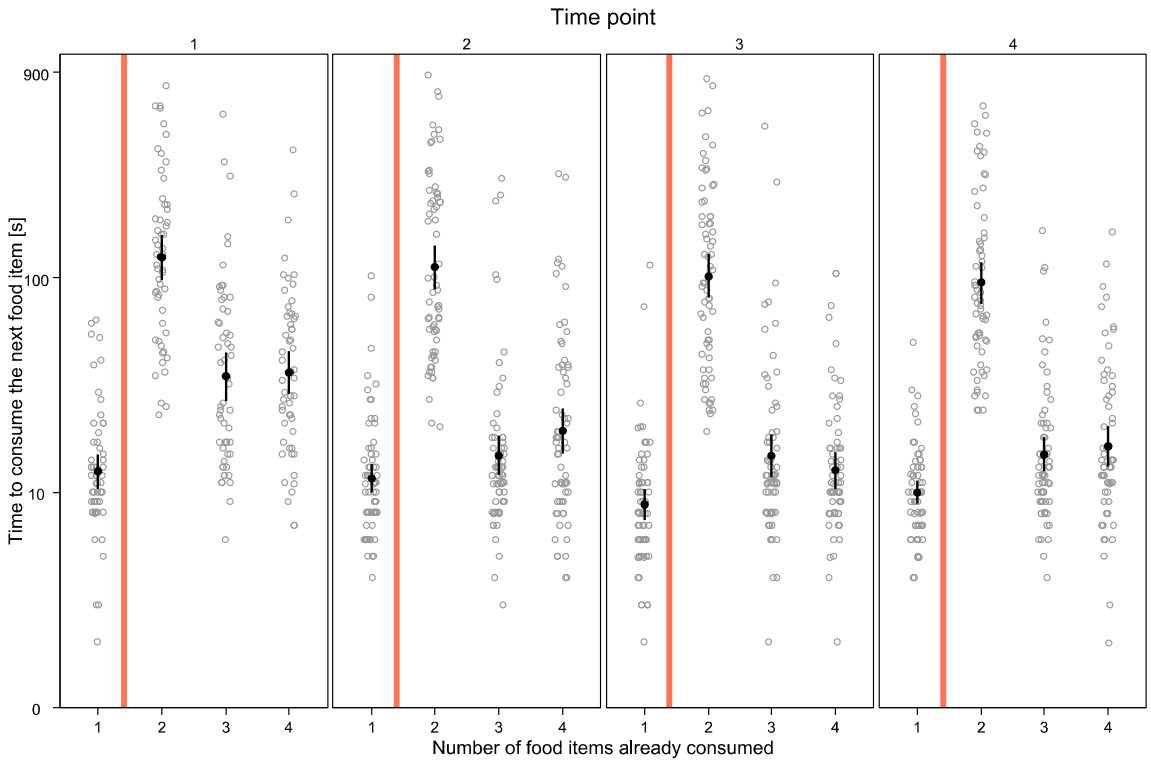

Figure II. S1: Confirmation of the initial reaction to the simulated heron attack: Latency to consume a subsequent food item. Note that the latency to consume subsequent food items increases after the simulated heron attack (marked by the vertical red line). Error bars present 95% CI. N: Time point 1:56, Time point 2:65, Time point 3:65, Time point 4:62.

Supplementary information II. 2: Differences in the reaction to the simulated heron attack

To test the fish's reaction to the simulated heron attack, used Fisher's Exact Test in R (R Development Core Team 2010). We categorised each fish's reaction into 5 different categories (None: Fish showed no reaction to the simulated heron attack, Slow movement: Fish moved slowly; Fast, erratic movement: Fish darted into any direction without reaching hiding or jumped around without obvious direction, Freezing: Fish remained motionless in its current position for at least several seconds; Fleeing to hiding: Fished moved into hiding within a few seconds after the simulated heron attack.) and analysed the number of fish in each treatment that performed each reaction for each time point. Fish that were hiding during the simulated heron attack were excluded from the analysis. If we found significant differences, we conducted post-hoc tests by repeatedly conducting the same models on only two treatments for each possible treatment combination and used bonferroni corrections to correct for multiple testing.

In experiment I, treatment did not affect host fish reacted to the simulated heron attack during the first three time points (Fisher's exact test: Time point 1: p=0.3882, Time point 2: p=0.1599, p=0.06143. During the third time point sequentially infected fish tended to show less strong reactions such as no reactions at all or slow movements than uninfected fish that froze or hid more often (p adjusted (bonferroni correction) = 0.087. During the fourth time point, treatment did have a significant effect on host behaviour. Post hoc tests with bonferroni corrections revealed that uninfected fish whoa arguably more risk averse reactions than fish infected on day 0 (p adjusted = 0.045) and fish sequentially infected on day 0 plus day 31 (p adjusted = 0.006). Sequentially infected fish also behaved significantly different, arguably less risk averse from those infected only on day 31 (p adjusted = 0.025, Figure II. S2).

In experiment II, nearly all fish reacted to the simulated heron attack by fleeing to hiding. Accordingly, we found no significant differences between infected and uninfected fish (Fisher's exact test: Early fish: p=0.6342, Late fish 2: p=0.7860, Figure II. S2).

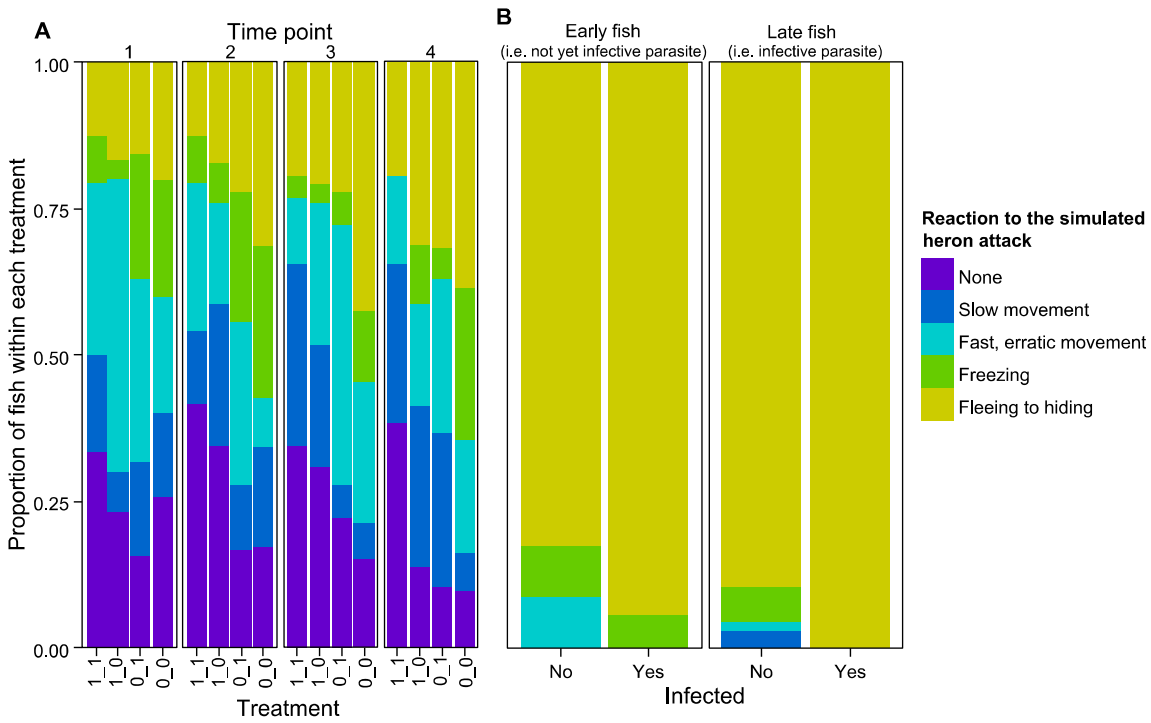

Figure II. S2: Reaction to the simulated heron attack by treatment. A: Experiment I, B: Experiment II. None: Fish showed no reaction to the simulated heron attack, Slow movement: Fish moved slowly; Fast, erratic movement: Fish darted into any direction without reaching hiding or jumped around without obvious direction, Freezing: Fish remained motionless in its current position for at least several seconds; Fleeing to hiding: Fished moved into hiding within a few seconds after the simulated heron attack. Treatment: 1_1 fish sequentially infected by one S. solidus on day 0 plus one on day 31, 1_0 fish only infected by one S. solidus on day 0, 0_1 fish only infected by one S. solidus on day 31, 0_0 uninfected fish. N: Time point1: 1_1: 24 , 1_0: 30, 0_1: 19, 0_0: 35; Time point2: 1_1: 24 , 1_0: 29, 0_1: 18, 0_0: 35; Time point3: 1_1: 26, 1_0: 29, 0_1: 18, 0_0: 33; Time point4: 1_1: 26, 1_0: 29, 0_1: 19, 0_0: 31.

Supplementary information II. 3: Parasite growth & parasite index

Parasite growth during experiment I

Since we wanted to measure the behaviour of each fish repeatedly, measuring directly when parasites would become infective was impossible in our study. This seems to vary between studies (Barber and Svensson 2003; Scharsack et al. 2004, 2007). We made use of the fact that *S. solidus* is usually assumed to reach 50 mg before it is able to reproduce inside its bird host and that the growth curves of S. solidus have previously been estimated (Barber and Svensson 2003; Scharsack et al. 2004, 2007) to calculate at what age parasites in each of our treatment would reach 50 mg and become infective in our study.

To obtain growth estimate for each parasite throughout the experiment we used the Nonlinear Least Squares function in the stats package in R (R Development Core Team 2010). Following know estimates of the growth of *S. solidus* (Barber and Svensson 2003; Scharsack et al. 2004, 2007), we assumed it to roughly follow a logistic growth and hence used the formula for logistic growth: $y = \frac{a}{1+e^{b*(x-c)}}$. We obtained parasite weight and appropriate parasite age from the literature (Barber and Svensson 2003; Scharsack et al. 2004, 2007) and used this data to obtain values for *a* and *b* as well as a starting value for *c*. We then inserted our known parasite weight and age at dissection and a parasite weight of 0 at infection into the formula to obtain an individual value for c for each parasite. The formulas we thereby obtained could be used to obtain an estimate of the weight of each parasite during each time point since we knew exactly how old parasites were at that time. Since we found no appropriate data on growth in *S. solidus* sharing their host with a conspecific, we had to assume that it resembled that of individual fish even so this probably represents a simplification.

To compare parasite weight between different treatments, we only used the weight at dissection. We fitted an analysis of variance model (ANOVA) in R (R Development Core Team 2010) using parasite weight as the response and a factor that combined treatment and parasite age as fixed effect. Since the ANOVA showed a significant effect of our treatment/ parasite age factor, we subsequently conducted a Tukey's post hoc test.

Treatment and parasite age combined had a significant effect on parasite weight during dissection ($F_{96,3}$=60.56, p<0.0001). A post hoc test revealed that whether sharing their host with a conspecific or not, parasites that infected their host on day 31 were always smaller than those from day 0 (Figure II. S3, p<0.0001). Parasites that infected their host on day 31 grew significantly smaller when they had to share their host with an older conspecific than when they were alone (Figure II. S3, p<0.0001). By contrast, parasites that infected their host on day 0, did not significantly differ in their weight whether they had to share their host or not (Figure II. S3, p=0.8167).

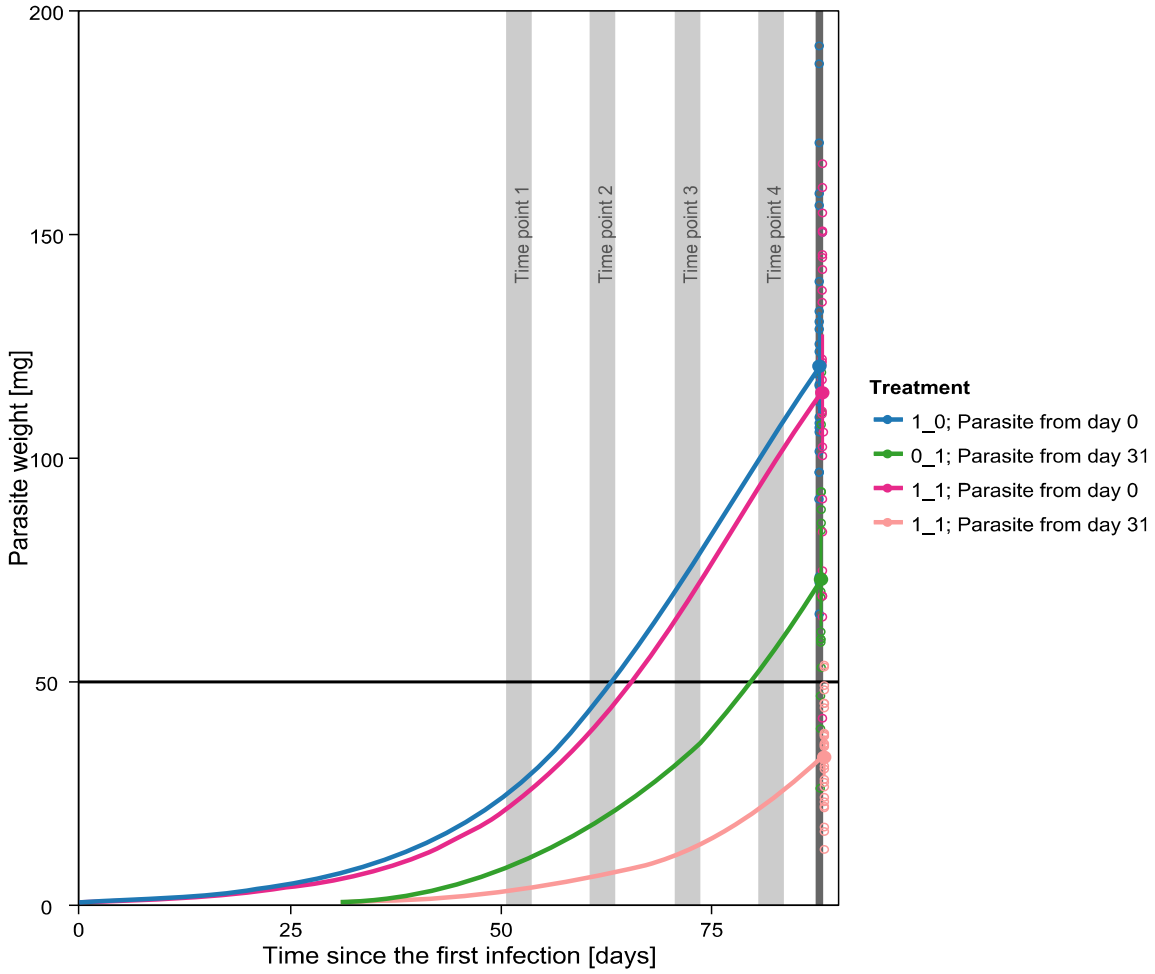

Figure II. S3: Estimated parasite growth curves. Shape of the growth curves were obtained from the literature (Barber and Svensson 2003; Scharsack et al. 2004, 2007) and in combination with parasite weight and age during dissection used to estimate the average growth curved for each treatment. Actual parasite weight was only measured at dissection (dark grey bar). Error bars indicate 95% CI. The X-axis indicates the age of the parasite from day 0. Parasites that infected their host on day 31 are always 31 days younger. The horizontal black line indicates the 50 mg threshold below which little reproduction inside the definite bird host is possible (Tierney and Crompton 1992). The light grey bars indicate the time during which the behavioural measurements for the corresponding time point took place. 1_0; Parasite from day 0: Parasites that infected their host on day 0, 0_1; Parasite from day 31: Parasites that infected their host on day 31, 1_1; Parasite from day 0: Parasites that infected their host on day 0 and had to share with another parasite, 1_1; Parasite from day 31: Parasites that infected their host on day 31 and had to share with another parasite. Parasites from day 0 and day 31 from 1_1 steamed from the same sequentially infected fish. N: 1_0: 29, 0_1:19, 1_1:26.

Reproduction in the definitive bird host is usually assumed to be possibly only after *S. solidus* has reached a weight of at least 50 mg in the fish (Tierney and Crompton 1992). Adaptive host manipulation enhancing the fish's predation susceptibility should only occur thereafter. Our estimated growth curved allowed rough estimates of when we expect parasites to have reached the 50 mg threshold and host manipulation to set in (Figure II. S3). Parasites in fish only infected on day 0 should have reached the 50 mg threshold just over 60 days post infection (i.e. around time point 2) and should hence have manipulated their host from time point 2 or 3 onwards. Parasites in fish only infected on day 31 should have reached the 50 mg threshold around day 50 post infection (i.e. around day 80 after the first infection, between time point 3 and 4). Accordingly, parasites from day 31 should only manipulate during the fourth time point. For sequentially infected fish, manipulation should depend on the outcome of a conflict over when manipulation should set in between the parasite from

day 0 and from day 31 sharing a host. The parasite from day 0 in sequential infections reached 50 mg around 60 days post infection, around the same as parasites from day 0 that did not have to share their host. If it dominates, manipulation should set in during time point 2 or 3. By contrast, parasites that infected hosts already infected on day 0 on day 31 (sequential infections) failed to reach the 50 mg threshold before the end of the experiment. If the parasite from day 31 dominated host behaviour, fish sequentially infected by one parasite on day 0 plus one on day 31 should have never have been manipulated throughout the experiment. Of course, any intermediate behaviour indicative of a compromise might also be possible. In either case, if true host manipulation is at work, sequentially infected fish should not be manipulated sooner or more strongly than fish only infected on day 0. Any such host manipulation would indicate an effect of energy drain rather than true host manipulation.

Surprisingly, fish infected on day 31 start displaying more risk prone behaviour already from the second time point onwards when their parasite is just over 30 days old and not yet infective and thus should not induce risk prone behaviour. During the same time also fish infected on day 0 (whose parasites accordingly are 31 days older) increase their risky behaviour. However, their parasites are infective. When fish experience the heron during the second time point, they have already experienced it once before and might be aware that it does not occur a second time during the trial. This could have reduced the frightening level of this stimulus. We do see no evidence of any habituation in uninfected fish. However, infected fish are faced with an additional energy drain, which could cause them to habituate more readily to a frightening stimulus; they might learn more quickly to adjust their behaviour because they have to eat more. This effect could be independent of parasite size, if energy drain rather than actual host manipulation is responsible for the behavioural changes we observed. Further similarities between fish infected on day 0 and on day 31 could be facilitated by differences in parasite growth. Parasites from day 31 grow faster (they become infective at about 50 days after infection while parasites from day 0 only become infective just over 60 days post infection, see above, Figure II. S3) and hence they do drain more energy. Both of these explanations are consistent with behavioural changes caused by enhanced energy drain but not with behavioural changes caused by actual host manipulation. Actual host manipulation will not enhance the risk taking of fish infected on day 31 during the second time point when the parasites are not yet infective.

Parasite weight and parasite index after dissection

For both experiments we measured fish and parasite weight (Figure II. S4 A, B) at dissection. From these we calculated the parasite index (parasite weight/combined fish and parasite weight, Figure II. S4 C, D) as a measure of relative parasite burden. We combined data of infected fish from experiment I and II and used an analysis of variance model (aov, R (R Development Core Team 2010)) with parasite index or parasite weight as response and the treatment (experiment I) or the time (experiment II) as independent variable followed by a Tukey's post hoc test. Our treatment/ time significantly affected parasite index $F_{4,108}=128$, $p<0.0001$) and weight ($F_{4,109}=57$, $p<0.0001$). While late fish, i.e. fish with infective parasites from experiment II harboured about the same total parasite weight as fish infected on day 0 and fish infected on day 0 plus on day 31 in

experiment I, they had a significantly lower parasite index (Table II. S2, Figure II. S4) because they were much larger. This probably contributed to differences in stickleback's risk averseness between experiment I and II. Some parasites in experiment I, but none in experiment II, might have grown large enough in relation to their host to compress the gut of their host thereby increasing their hunger. Energy drain, too, is probably more efficient in smaller fish. True host manipulation should not be switches off completely just because fish are too large, but with side-effects the size of both host and parasite could play a crucial role.

Table II. S2: Results of the Tukey post hoc tests for parasite weight and parasite index. 1_1 fish sequentially infected by one S. solidus on day 0 plus one on day 31, 1_0 fish only infected by one S. solidus on day 0, 0_1 fish only infected by one S. solidus on day 31, early: fish harbouring infective parasites, late: fish harbouring not yet infective parasites.

Parasite index \ Parasite weight			Experiment				
			I			II	
			1_1	1_0	0_1	early	late
Experiment	I	1_1		0.012	<0.001	<0.001	0.303
		1_0	0.004		<0.001	<0.001	0.853
		0_1	<0.001	<0.001		<0.001	<0.001
	II	early	<0.001	<0.001	<0.001		<0.001
		late	<0.001	<0.001	0.522	<0.001	

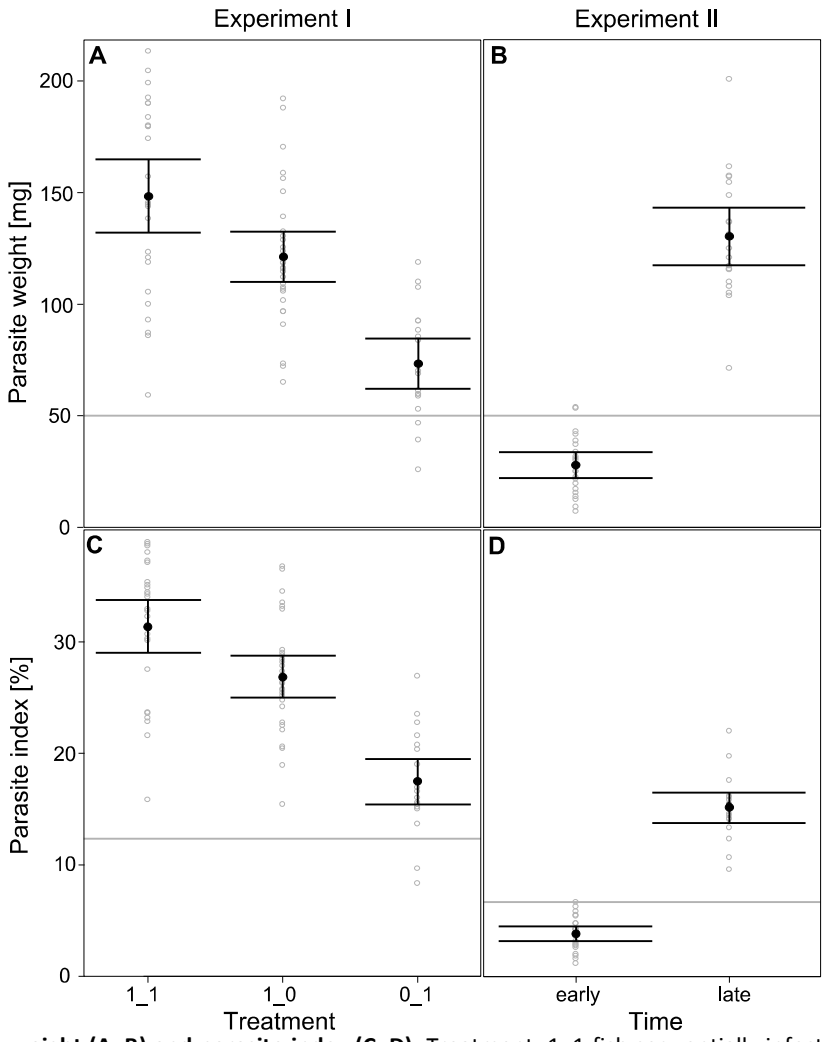

Figure II. S4: Worm weight (A, B) and parasite index (C, D). Treatment: 1_1 fish sequentially infected by one S. solidus on day 0 plus one on day 31, 1_0 fish only infected by one S. solidus on day 0, 0_1 fish only infected by one S. solidus on day 31. Time: early: fish harbouring infective parasites, late: fish harbouring not yet infective parasites. Horizontal grey lines indicate the critical size to reach infectivity (A, B) or the parasite index at which this critical size should have been reached given the average size of fish in that experiment (C, D).

Table II. S3

Table II. S3: Outcome of likelihood ratio tests. For the number of food items consumed and the feeding position we used generalized linear mixed models with Poisson error family. For fish activity we used linear mixed models after log transforming the data. We included time point as fixed factor and used fish identity as random effects including the repeat to account for the presence of intra-individual variation between repeats. For the feeding position we additionally included the time interval in the recording (i.e. before vs. after the simulated heron attack) both in the random effects and as a fixed effect. Subsequently, we added the treatment and its interactions with repeat. Test statistics and MCMC-estimated p-values are for the comparison with the preceding model. Significant p-values have been marked in bold.

	Total amount of food consumed within 5 minutes			Fish activity (average distance moved within 2 seconds)			Average position where the first two food items before and after the simulated heron attack were consumed		
Factors	Df	Chisq	p	Df	Chisq	p	Df	Chisq	p
+Treatment	8,3	17.968	**0.0005**	9,3	2.279	0.5165	23,3	1.891	0.5953
+Treatment : Time point	11,3	15.221	**0.0016**	12,3	2.763	0.4296	32,9	12.196	0.2025
+Treatment : Time interval							35,3	1.7218	0.6321
+Treatment : Time point: Time interval							47,12	13.258	0.3505
	422 observations on 110 fish			405 observations on 110 fish			518 observations 89 on fish		

Table II. S4

Table II. S4: Outcome of multiple comparisons for each time point for the latency to resume feeding. We conducted survival analysis including only two treatments at once. Significant p-values are highlighted in bold. 0_0: Fish not infected by *S. solidus*, 1_0: Fish singly infected on day 0, 0_1: Fish singly infected on day 31, 1_1: Fish sequentially infected on day 0 plus on day 31. If there were differences between treatments, the first treatment is the one with the longer time to resume feeding within each comparison, i.e. the more risk averse fish.

Comparison		Time point 1			Time point 2			Time point 3			Time point 4		
		ChiSq	p	p adj[1]	ChiSq	p	p adj[1]	ChiSq	p	p adj[1]	ChiSq	p	p adj[1]
0_0	1_0	0.48	0.9242	1	22.50	**0.0001**	**0.0003**	14.17	**0.0027**	**0.0161**	11.30	**0.0102**	0.0612
0_0	0_1	0.01	0.9997	1	12.26	**0.0065**	**0.0392**	9.68	**0.0215**	0.1292	4.63	0.2009	1.2051
0_0	1_1	13.11	**0.0044**	**0.0265**	30.80	**<0.0001**	**<0.0001**	42.95	**<0.0001**	**<0.0001**	54.11	**<0.0001**	**<0.0001**
0_1	1_0	0.45	0.9292	1	0.34	0.9515	1	0.01	0.9997	5.9984	0.73	0.8652	5.1911
1_0	1_1	8.60	**0.0351**	0.2103	1.68	0.6410	1	10.01	**0.0185**	0.1108	19.45	**0.0002**	**0.0013**
0_1	1_1	10.54	**0.0145**	0.0868	2.97	0.3961	1	8.73	**0.0330**	0.1983	23.97	**<0.0001**	**0.0002**

1: adjusted p-values represent Bonferroni correction for multiple testing

Table II. S5

Table II. S5: Post hoc comparison between treatments for the amount of food consumed within 5 minutes after the simulated heron attack. We used Tukey test using general linear hypotheses. Fish identity and repeat were included as random effect. Significant p-values have been marked in bold. 0_0: Fish not infected by *S. solidus*, 1_0: Fish singly infected on day 0, 0_1: Fish singly infected on day 31, 1_1: Fish sequentially infected on day 0 plus on day 31. If there were differences between treatments, the first treatment is the one with the lower number of food items consumed within each comparison, i.e. the more risk averse fish.

Comparison		Time point							
		1		2		3		4	
		t	p	t	p	t	p	t	p
0_0	1_0	0.940	0.7808	-4.054	**0.0002**	-7.598	**<0.0001**	-6.031	**<0.0001**
0_0	0_1	0.871	0.8176	-2.095	0.1526	-5.691	**<0.0001**	-3.482	**0.0030**
0_0	1_1	-3.831	**0.0006**	-7.298	**<0.0001**	-9.111	**<0.0001**	-7.985	**<0.0001**
0_1	1_0	0.050	1	1.421	0.4829	1.297	0.5606	2.049	0.1674
1_0	1_1	-4.425	**0.0001**	-3.419	**0.0032**	-1.778	0.2804	-2.292	0.0980
0_1	1_1	-3.869	**0.0006**	-4.237	**0.0002**	-2.809	**0.0250**	-3.946	**0.0005**

Table II. S6

Table II. S6: Analysis of the time spent hiding before and after a simulated heron attack. The Table presents the outcome of likelihood ratio tests. We used linear mixed models after log transforming the data. We included fish identity as random effects. Subsequently, we added the time interval in the recording (before vs. after the simulated heron attack), the feeding treatment, the infection treatment and all their interactions. Test statistics and MCMC-estimated p-values are for the comparison with the preceding model. Significant p-values have been marked in bold.

	Early (6 weeks *post infection*)			Late (10 weeks *post infection*)		
Factors	Df	Chisq	p	Df	Chisq	p
+Time interval	4,1	81.225	**<0.0001**	4,1	48.537	**<0.0001**
+Infection	5,1	0.002	0.9661	5,1	0.001	0.9811
+Time interval : Infection	6,1	0.015	0.9026	6,1	0.529	0.4671
+Feeding	7,1	19.751	**<0.0001**	7,1	29.357	**<0.0001**
+Feeding : Time interval	8,1	38.144	**<0.0001**	8,1	28.876	**<0.0001**
+Feeding : Infection	9,1	0.047	0.8292	9,1	0.116	0.7337
+Feeding : Infection : Time interval	10,1	0.156	0.6932	10,1	0.393	0.5309
	174 observations on 87 fish			168 observations on 84 fish		

Table II. S7

Table II. S7: Post hoc comparison between treatments and time interval (before vs. after heron) for the time fish spend hiding. We used Tukey test using general linear hypotheses. Fish identity was included as random effect. Significant p-values have been marked in bold.

	Early (6 weeks *post infection*)		Late (10 weeks *post infection*)	
Comparison	t	p	t	p
Before the simulated heron attack: starved – satiated	-7.698	**<0.0001**	-8.142	**<0.0001**
After the simulated heron attack: starved – satiated	0.031	1	-1.206	0.6184
Hungry: before the simulated heron attack – after the simulated heron attack	-14.778	**<0.0001**	-10.973	**<0.0001**
Full: before the simulated heron attack – after the simulated heron attack	-4.562	**<0.0001**	-2.212	0.1176
before the simulated heron attack, starved – after the simulated heron attack, satiated	-11.453	**<0.0001**	-10.059	**<0.0001**
before the simulated heron attack, satiated – after the simulated heron attack, starved	-3.785	**0.0009**	-0.710	0.8913

Appendix for chapter III

Supplementary results III. 1: Results after a recovery period

Change of copepod host activity over time

After copepods had had time to recover, copepods singly infected on day 0 (Figure III. S1, dashed blue line), increased their activity between day 11 and 13 ($p=0.009$), but not before ($p>0.5$). Copepods singly infected on day 7 (Figure III. S1, dashed green line) initially decreased their activity as host manipulation set in between day 9 and 11 in the experiment ($p<0.001$). They increased their activity again at the same time *post infection* as copepods infected on fay 0, i.e. between 11 and 13 days *post infection*, between day 17 and 19 in the experiment ($p<0.001$). Unexposed control copepods, however, also increased their activity between day 11 and 13 ($p<0.001$) but decreased it between day 17 and 19 ($p=0.043$). Before the parasite reached infectivity (predation suppression) singly-infected copepods were always significantly different from control copepods ($p<0.03$). Copepods singly on day 7 continued to be significantly less active than control copepods throughout the experiment ($P<0.001$) except on day 19 ($p=0.331$).

Potential synergy of parasites in simultaneous double infections

Like copepods singly infected on day 0, copepods infected with two parasites on day 0 (Figure III. S1, continuous blue line) significantly increased their activity between day 11 and 13 ($p<0.001$). In those infected with two parasites on day 7 (Figure III. S1, continuous green line), the onset of manipulation was marked by a significant decrease in host activity between day 9 and 11 ($p<0.001$) and the switch from predation suppression to predation enhancement by a significant increase between day 17 and 19 ($p<0.002$). Copepods simultaneously infected on day 0 never significantly differed from controls ($p>0.3$). Copepods simultaneously on day 7 were significantly less active than controls during predation suppression, i.e. between day 11 and 17 ($p<0.2$). We never observed any significant differences between singly-infected copepods and simultaneously-infected copepods from the same infection time point ($p>0.07$).

The outcome of a conflict between parasites over host manipulation

The conflict we observed immediately after a simulated predation attack was also observable after a recovery period. On day 15, copepods singly infected on day 0 were significantly more active than those singly infected on day 7 ($p=0.003$). The same was true when comparing simultaneously-infected copepods on day 15 and 17 ($p<0.04$). No other significant differences occurred for those comparisons ($p>0.06$). Sequentially-infected copepods (i.e. infected with one parasite each on day 0 plus on day 7, Figure S1 dashed red line), however, never differed from simultaneously-infected copepods ($p>0.1$). They were never significantly different from copepods singly infected on day 0, either ($p>0.7$), but did differ from those singly infected on day 7 on day 17 and day 21 ($p<0.03$), though not on any other day ($p>0.1$). Hence, there is less

evidence for a conflict over host manipulation after a recovery period, but if anything it still seems to be won by the infective parasite from day 0.

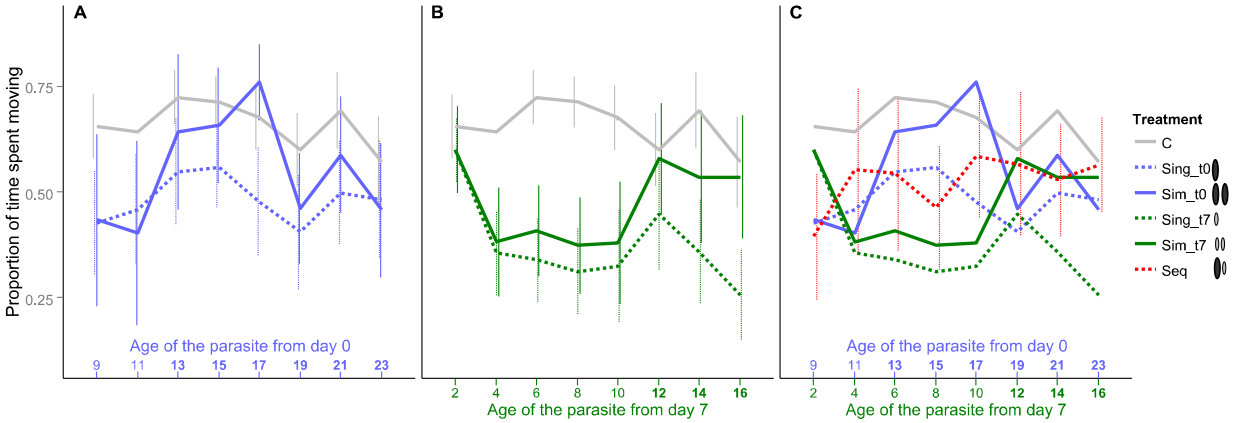

Figure III. S1: Activity (i.e. proportion of time spent moving) of copepods according to treatment, after a recovery period. Error bars indicate 95% CI. Bold numbers on the X-axis indicated that a parasite of that age was infective. A: Copepods infected on day 0, B: Copepods infected on day 7, C: All treatments. Error bars from the treatments already presented in A and B have been omitted for better readability. C: uninfected control copepods (*n* = 41), Sing_t0: copepods singly infected with one parasite on day 0 (*n* = 25), Sim_t0: copepods simultaneously infected with two parasites on day 0 (*n* = 11), Sing_t7: copepods singly infected with one parasite on day 7 (*n* = 27), Sim_t7: copepods simultaneously infected with two parasites on day 7 (*n* = 25), Seq: copepods sequentially infected with two parasites, one each on day 0 plus day 7 (*n* = 18).

Equal potential strength of parasites that are at a conflict over host manipulation

We never observed any significant differences between copepods singly infected on day 0 (Figure III. S2 dashed blue line) and copepods sequentially infected with one parasite on day 0 and one (Figure III. S2, dashed red line) or two (Figure III. S2, continuous red line) on day 7 ($p>0.2$). Copepods sequentially infected with one parasite on day 0 plus two on day 7 did differ significantly from those only infected on day 7 (Figure III. S2, dashed green line) on day 13 ($p=0.007$), but on no other day ($p>0.08$). Just like after a simulated predation attack, we find no clear evidence that increasing parasite number changes the outcome of the conflict over host manipulation.

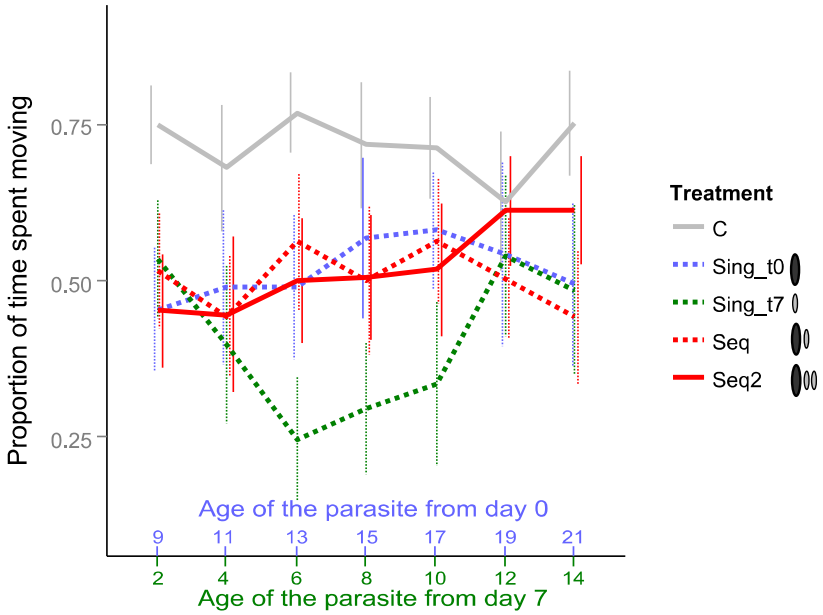

Figure III. S2: Activity (i.e. proportion of time spent moving) of copepods according to treatment, after a recovery period. Error bars indicate 95% CI. Bold numbers on the X-axis indicated that a parasite of that age was infective. C: uninfected control copepods (*n* = 20), Sing_t0: copepods singly infected with one parasite on day 0 (*n* = 25), Sing_t7: copepods singly infected with one parasite on day 7 (*n* = 22), Seq: copepods sequentially infected with two parasites, one each on day 0 plus day 7 (*n* = 28), Seq2: copepods sequentially infected with three parasites, one on day 0 plus two on day 7 (*n* = 26).

Supplementary results III. 2: Additional confirmation of the effect of the number of non-infective parasites from day 7

Material and methods

Copepods were exposed to one *S. solidus* on day 0 and 1 or 5 on day 7 in the same manner described in the main paper. The parasite administered on day 0 always originated from a different parasite family than the parasite(s) administered on day 7, but if 5 parasites were used on day 7, they resulted from the same family. Unlike in the main experiment we used any copepod infected by one parasite on day 0 and any number of day 7 parasites, resulting in 103 copepods in 4 different treatments infected with one parasite on day 0 and a variable number of parasites on day 7 (0 parasites on day 7: 25, 1 parasite on day 7: 39, 2 parasites on day 7: 30, 3 parasites on day 7: 9). Only one copepod was infected by more than 3 parasites on day 7 and excluded from analysis.

Parasites and copepods stemmed from the same population and were maintained in the same manner described in the main paper. We checked copepods for infection on day 15, i.e. when parasites from day 0 were 15 and parasites from day 7 were 8 days old and non-infective. We measured parasite size on day 16 (parasites from day 7 were 9 days old and hence still non-infective) in copepods infected with one parasite on day 0 and at least one on day 7. It is possible to do so in the living copepod (e.g. Wedekind et al. 2000; Michaud et al. 2006; Benesh and Hafer 2012). Shortly, we took a photo of each parasite within its host and

measured the area the parasite occupied using image J (Rasband 2008). From this we calculated the proportion of the non-infective parasites from day 7 among the total parasite area.

Results

Parasite size

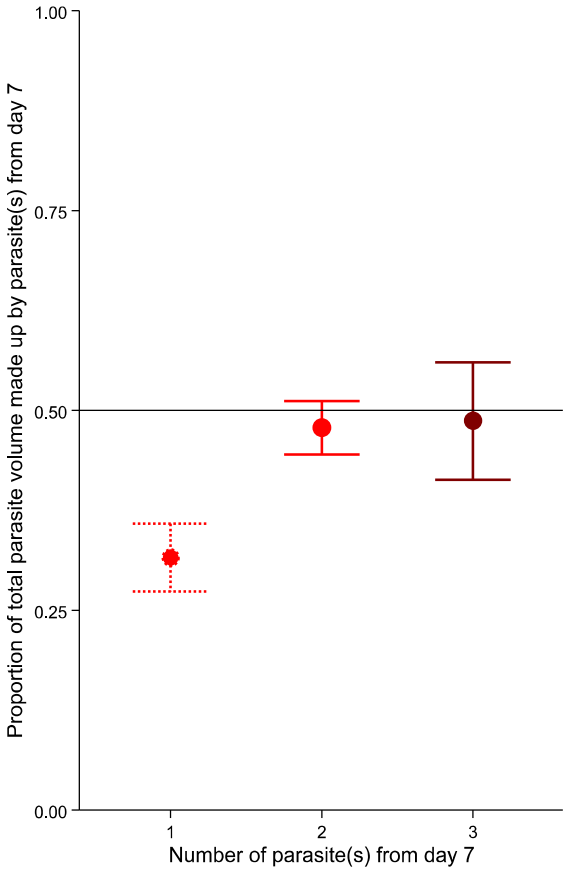

Figure III. S3: Relative combined size of parasites from day 7 depending on the number of parasites from day 7 per copepod. Error bars indicate 95% CI. The horizontal black line indicates equal size of the parasite from day 0 and all parasites from day 7 within that copepod. Each copepod was infected by one parasite on day 0 and 1 (n=19), 2 (n=16) or 3 (n=5) parasites on day 7.

Size could be measured for a total of 38 copepods with one parasite from day 0 and a varying number of parasites from day 7 (19 with 1 parasite from day 7, 16 with 2 parasites from day 7, 5 with 3 parasites from day 7). In copepods with one parasite from day 0 and one parasite from day 7, the parasite from day 7 made up 23 +/- 2%, significantly less than 50%, of the total area parasites occupied within these copepods ($t=-11.7$, $df=18$, $p<0.0001$, Figure III. S3). In copepods harboring more than 1 non-infective parasite from day 7, the area these parasites occupied out of the total parasite area was not significantly different from 50% (2 parasites from day 7: 48 +/- 4 %, $t=-1.3087$, $df=15$, $p=0.2103$, 3 parasites from day 7: 49 +/- 8 %, $t=-0.3561$, $df=4$, $p=0.7397$, Figure III. S3).

Behavioral experiment

Neither the number of parasites a copepod was infected by on day 7 nor its interaction with day had any effect of how often it moved (Figure III. S3). Additionally including the interaction between parasite number and time point in the recording (i.e. before vs. after a simulated predation attack) and the three way interaction including also the day significantly improved the model (Table III. S6). Post-hoc tests however, revealed no differences between pairwise comparisons between the activity of copepods with different numbers of parasites from day 7 (Table III. S7).

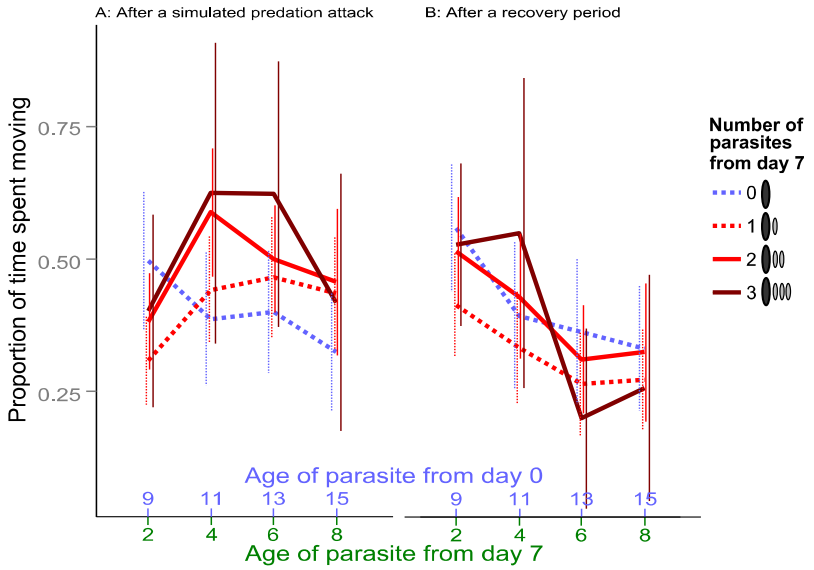

Figure III. S4: Activity (i.e. proportion of time spent moving) of copepods according to treatment, after a simulated predation attack (A) and after a recovery period (B). Error bars indicate 95% CI. Each copepod was infected by one parasite on day 0 (day-0 parasite) and 0 (n=22-22), 1(n=33-39), 2(n=23-30) or 3 (n=7-9) parasites on day 7.

Table III. S1

Table III. S1: Outcome of likelihood ratio tests. All comparisons were significant. The initial model used whether or not a copepod moved within a two second interval as response and the day after the first infection (DAY), the Period in the recording (PERIOD), i.e. after a simulated predation attack vs. after a recovery period and the interaction between DAY and TIME as fixed effects. We used the copepod identity as a random factor and included DAY and PERIOD. Subsequently, we added the treatment (TREAT) and all its interactions with DAY and PERIOD. Test statistics and MCMC-estimated p-values are for the comparison with the preceding model.

Experiment 1				Experiment 2			
Factors	DF	Chisq	p	Factors	DF	Chisq	p
+ TREAT	15,5	29.381	**<0.0001**	+ TREAT	14,4	24.650	**<0.0001**
+ DAY : TREAT	20,5	15.459	**0.0086**	+ DAY : TREAT	18,4	11.083	**0.0257**
+ PERIOD : TREAT	25,5	138.240	**<0.0001**	+ PERIOD : TREAT	22,4	71.088	**<0.0001**
+ PERIOD : DAY : TREAT	30,5	119.914	**<0.0001**	+ PERIOD : DAY : TREAT	26,4	13.233	**0.0102**
63060 observations on 147 copepods				49980 observations on 121 copepods			

Table III. S2

Table III. S2: Outcome of multiple comparisons between days for each treatment and period in the recording (i.e. after a simulated predation attack vs. after a recovery period). Results from experiment 1. Significant p-values are highlighted in bold. C: uninfected control copepods, Sing_t0: copepods singly infected with one parasite on day 0, Sim_t0: copepods simultaneously infected with two parasites on day 0, Sing_t7: copepods singly infected with one parasite on day 7, Sim_t7: copepods simultaneously infected with two parasites on day 7, Seq: copepods sequentially infected with two parasites, one each on day 0 plus day 7.

After simulated predation attack												
Treatment	C		Sing_t0		Sing_t7		Sim_t0		Sim_t7		Seq	
Comparison	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
day9 - day11	-0.48	1.000	4.53	**<0.001**	-3.87	**0.003**	4.31	**<0.001**	-4.42	**<0.001**	5.37	**<0.001**
day11 - day13	-1.89	0.556	4.60	**<0.001**	-2.87	0.078	5.79	**<0.001**	-2.56	0.170	3.07	**0.045**
day13 - day15	1.89	0.554	0.43	1.000	-2.18	0.365	1.78	0.632	2.82	0.089	-0.49	1.000
day15 - day17	-1.86	0.575	0.95	0.981	4.56	**<0.001**	-1.42	0.848	3.82	**0.003**	3.40	**0.015**
day17 - day19	1.27	0.912	-2.24	0.327	4.40	**<0.001**	0.65	0.998	9.60	**<0.001**	-2.52	0.188
day19 - day21	-0.14	1.000	3.88	**0.003**	1.94	0.521	-2.78	0.098	-1.08	0.960	0.08	1.000
day21 - day23	-5.25	**<0.001**	-0.21	1.000	-1.79	0.627	-2.55	0.174	-0.22	1.000	-2.82	0.091
Observations	8610		5370		5760		2610		5280		3900	
Copepods	41		25		27		11		25		18	

After a recovery period												
Treatment	C		Sing_t0		Sing_t7		Sim_t0		Sim_t7		Seq	
Comparison	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
day9-day11	-0.63	0.998	1.93	0.529	-9.90	**<0.001**	-0.80	0.993	-8.74	**<0.001**	5.91	**<0.001**
day11-day13	4.30	**<0.001**	3.57	**0.009**	-1.22	0.927	6.17	**<0.001**	1.73	0.670	-1.45	0.835
day13-day15	-0.87	0.989	1.25	0.918	-0.96	0.980	0.41	1.000	-1.80	0.617	-2.51	0.192
day15-day17	-2.53	0.184	-3.66	**0.006**	0.70	0.997	2.93	0.065	0.00	1.000	4.36	**<0.001**
day17-day19	-3.08	**0.043**	-2.84	0.086	4.97	**<0.001**	-7.49	**<0.001**	8.37	**<0.001**	0.81	0.993
day19-day21	4.81	**<0.001**	3.40	**0.015**	-4.03	**0.002**	3.05	**0.047**	-1.46	0.831	-3.31	**0.020**
day21-day23	-6.27	**<0.001**	-0.71	0.997	-4.21	**0.001**	-3.38	**0.016**	-0.33	1.000	2.34	0.271
Observations	8610		5370		5760		2610		5280		3900	
Copepods	41		25		27		11		25		18	

Table III. S3

Table III. S3: Outcome of multiple comparisons between treatments for each day and period in the recording (i.e. after a simulated predation attack vs. after a recovery period). Results from experiment 1. Significant p-values are highlighted in bold. C: uninfected control copepods, Sing_t0: copepods singly infected with one parasite on day 0, Sim_t0: copepods simultaneously infected with two parasites on day 0, Sing_t7: copepods singly infected with one parasite on day 7, Sim_t7: copepods simultaneously infected with two parasites on day 7, Seq: copepods sequentially infected with two parasites, one each on day 0 plus day 7.

After simulated predation attack																
Day	9		11		13		15		17		19		21		23	
Comparison	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
C-Sing_t0	-3.29	**0.013**	-1.38	0.733	0.43	0.998	-0.37	0.999	0.70	0.982	-0.27	1.000	1.02	0.908	2.18	0.243
C-Sing_t7	-1.70	0.526	-3.15	**0.020**	-3.13	**0.021**	-5.08	**<0.001**	-2.65	0.083	-1.04	0.901	-0.54	0.994	-0.04	1.000
C-Sim_t0	-2.24	0.216	0.01	1.000	2.61	0.091	2.78	*0.059*	2.00	0.340	2.08	0.292	1.27	0.798	1.47	0.681
C-Sim_t7	-1.42	0.712	-3.36	**0.010**	-3.42	**0.008**	-3.26	**0.014**	-1.07	0.890	2.30	0.188	1.56	0.620	2.69	0.076
C-Seq	-2.41	0.148	-0.11	1.000	1.41	0.714	0.96	0.929	2.10	0.285	0.88	0.950	1.18	0.843	1.38	0.733
Sing_t0-Sing_t7	1.51	0.654	-1.54	0.633	-3.13	**0.021**	-4.13	0.001	-3.03	**0.028**	-0.67	0.984	-1.45	0.690	-1.99	0.346
Sing_t0-Sim_t0	0.23	1.000	0.99	0.921	2.11	0.277	2.85	0.049	1.35	0.754	2.14	0.260	0.43	0.998	-0.30	1.000
Sing_t0-Sim_t7	1.67	0.547	-1.77	0.475	-3.38	**0.009**	-2.55	0.108	-1.61	0.588	2.31	0.185	0.55	0.994	0.52	0.995
Sing_t0-Seq	0.46	0.997	1.00	0.915	0.90	0.945	1.17	0.846	1.35	0.753	1.03	0.905	0.20	1.000	-0.53	0.995
Sing_t7-Sim_t0	-0.94	0.933	2.21	0.226	4.63	**<0.001**	6.20	**<0.001**	3.85	**0.002**	2.72	0.070	1.62	0.580	1.39	0.731
Sing_t7-Sim_t7	0.21	1.000	-0.28	1.000	-0.20	1.000	1.56	0.618	1.46	0.684	3.06	**0.026**	1.94	0.373	2.45	0.136
Sing_t7-Seq	-0.89	0.948	2.40	0.153	3.87	**0.001**	5.03	**<0.001**	4.14	**<0.001**	1.65	0.556	1.58	0.605	1.29	0.785
Sim_t0-Sim_t7	1.09	0.884	-2.40	0.153	-4.84	**<0.001**	-4.92	**<0.001**	-2.68	0.077	-0.31	1.000	0.04	1.000	0.74	0.977
Sim_t0-Seq	0.16	1.000	-0.09	1.000	-1.30	0.779	-1.75	0.495	-0.13	1.000	-1.13	0.865	-0.25	1.000	-0.18	1.000
Sim_t7-Seq	-1.05	0.898	2.60	0.095	4.12	**<0.001**	3.56	**0.005**	2.84	*0.050*	-1.02	0.908	-0.33	0.999	-0.99	0.918
Observations	4200		4200		4170		4140		3840		3780		3750		3450	
Copepods	140		140		139		138		128		126		125		115	

After a recovery period																
Day	9		11		13		15		17		19		21		23	
Comparison	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
C-Sing_t0	-3.05	**0.027**	-1.89	0.401	-2.35	0.172	-2.16	0.250	-2.34	0.177	-2.27	0.200	-2.52	0.115	-0.90	0.947
C-Sing_t7	-0.86	0.955	-3.22	**0.016**	-4.93	**<0.001**	-6.36	**<0.001**	-4.68	**<0.001**	-2.01	0.331	-4.27	**<0.001**	-3.90	**0.001**
C-Sim_t0	-2.05	0.310	-1.91	0.387	-0.79	0.968	-0.78	0.970	0.80	0.966	-1.15	0.857	-1.07	0.890	-1.30	0.784
C-Sim_t7	-0.88	0.951	-3.22	**0.016**	-3.96	**0.001**	-5.11	**<0.001**	-3.47	**0.007**	-0.10	1.000	-1.86	0.421	-0.78	0.970
C-Seq	-3.33	**0.011**	-0.81	0.965	-1.63	0.574	-3.10	**0.023**	-0.98	0.923	-0.58	0.992	-2.08	0.295	0.03	1.000
Sing_t0-Sing_t7	2.05	0.308	-1.15	0.855	-2.28	0.196	-3.65	**0.003**	-2.15	0.259	0.32	1.000	-1.56	0.620	-2.73	0.068
Sing_t0-Sim_t0	0.23	1.000	-0.46	0.997	0.93	0.937	0.85	0.956	2.49	0.124	0.59	0.992	0.90	0.946	-0.53	0.995
Sing_t0-Sim_t7	1.93	0.374	-1.22	0.821	-1.38	0.733	-2.61	0.092	-1.01	0.911	1.99	0.339	0.47	0.997	0.07	1.000
Sing_t0-Seq	-0.59	0.991	0.75	0.975	0.45	0.998	-1.03	0.906	1.05	0.898	1.29	0.788	0.28	1.000	0.77	0.972
Sing_t7-Sim_t0	-1.36	0.748	0.44	0.998	2.77	0.061	3.81	**0.002**	4.24	**<0.001**	0.34	0.999	2.19	0.240	1.78	0.472
Sing_t7-Sim_t7	-0.05	1.000	-0.10	1.000	0.94	0.935	0.99	0.918	1.15	0.859	1.74	0.500	1.95	0.369	2.72	0.070
Sing_t7-Seq	-2.44	0.138	1.77	0.475	2.57	0.103	2.31	0.187	3.03	**0.029**	1.04	0.903	1.76	0.485	3.26	**0.014**
Sim_t0-Sim_t7	1.29	0.786	-0.51	0.996	-2.04	0.312	-2.98	**0.034**	-3.33	**0.011**	1.01	0.911	-0.47	0.997	0.58	0.992
Sim_t0-Seq	-0.70	0.982	1.04	0.901	-0.51	0.996	-1.67	0.547	-1.47	0.677	0.54	0.994	-0.63	0.988	1.16	0.855
Sim_t7-Seq	-2.33	0.176	1.83	0.442	1.73	0.502	1.38	0.737	1.99	0.343	-0.46	0.997	-0.18	1.000	0.68	0.984
Observations	4200		4200		4170		4140		3840		3780		3750		3450	
Copepods	140		140		139		138		128		126		125		115	

Table III. S4

Table III. S4: Outcome of multiple comparisons between days for each treatment and period in the recording (i.e. after a simulated predation attack vs. after a recovery period). Results from experiment 2. Significant p-values are highlighted in bold. C: uninfected control copepods, Sing_t0: copepods singly infected with one parasite on day 0, Sing_t7: copepods singly infected with one parasite on day 7, Seq: copepods sequentially infected with two parasites, one each on day 0 plus day 7, Seq2: copepods sequentially infected with three parasites, one on day 0 plus two on day 7.

After simulated predation attack										
Treatment	C		Sing_t0		Sing_t7		Seq		Seq2	
Comparison	Z	p	Z	p	Z	p	Z	p	Z	p
day9-day11	-3.37	**0.013**	2.99	**0.045**	-2.98	**0.045**	-1.86	0.505	-2.82	0.071
day11-day13	1.55	0.715	3.01	**0.043**	0.93	0.968	7.27	**<0.001**	3.30	**0.017**
day13-day15	1.12	0.921	2.46	0.174	-1.35	0.829	-1.42	0.792	1.07	0.937
day15-day17	0.61	0.997	-0.70	0.993	2.78	0.080	5.76	**<0.001**	6.32	**<0.001**
day17-day19	-0.90	0.972	-1.97	0.435	4.28	**<0.001**	-2.79	0.077	0.87	0.976
day19-day21	0.55	0.998	1.52	0.736	1.28	0.863	-3.87	**0.002**	0.80	0.985
Observations	4140		5130		4590		5790		5340	
Copepods	20		25		22		28		26	
After a recovery period										
Treatment	C		Sing_t0		Sing_t7		Seq		Seq2	
Comparison	Z	p	Z	p	Z	p	Z	p	Z	p
day9-day11	-2.81	0.074	1.51	0.739	-5.43	**<0.001**	-3.56	**0.007**	-0.35	1.000
day11-day13	3.62	**0.006**	0.00	1.000	-6.22	**<0.001**	5.17	**<0.001**	2.32	0.233
day13-day15	-2.21	0.291	3.30	**0.017**	2.17	0.309	-2.44	0.180	0.16	1.000
day15-day17	-0.36	1.000	0.35	1.000	1.54	0.722	2.50	0.161	0.56	0.998
day17-day19	-3.05	**0.037**	-1.42	0.791	8.05	**<0.001**	-2.82	0.071	3.72	**0.004**
day19-day21	4.87	**<0.001**	-1.75	0.584	-2.16	0.319	-2.42	0.191	0.07	1.000
Observations	4140		5130		4590		5790		5340	
Copepods	20		25		22		28		26	

Table III. S5

Table III. S5: Outcome of multiple comparisons between treatments for each day and period in the recording (i.e. after a simulated predation attack vs. after a recovery period). Results from experiment 2. Significant p-values are highlighted in bold. C: uninfected control copepods, Sing_t0: copepods singly infected with one parasite on day 0, Sing_t7: copepods singly infected with one parasite on day 7, Seq: copepods sequentially infected with two parasites, one each on day 0 plus day 7, Seq2: copepods sequentially infected with three parasites, one on day 0 plus two on day 7.

After simulated predation attack														
Day	9		11		13		15		17		19		21	
Comparison	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
C-Sing_t0	-3.18	**0.013**	-0.75	0.945	-0.44	0.992	0.14	1.000	-0.46	0.991	-0.50	0.987	-0.41	0.994
C-Sing_t7	-3.09	**0.017**	-2.44	0.104	-2.23	0.167	-3.49	**0.004**	-3.04	**0.020**	-1.07	0.821	-0.85	0.916
C-Seq	-2.40	0.116	-1.21	0.745	0.23	0.999	-0.46	0.991	0.99	0.859	0.77	0.938	-0.89	0.901
C-Seq2	-2.23	0.169	-1.69	0.438	-1.14	0.783	-1.02	0.845	0.27	0.999	1.17	0.770	1.16	0.772
Sing_t0-Sing_t7	-0.01	1.000	-1.80	0.372	-1.92	0.306	-3.81	**0.001**	-2.75	**0.047**	-0.61	0.973	-0.45	0.992
Sing_t0-Seq	0.88	0.903	-0.46	0.990	0.74	0.948	-0.64	0.968	1.56	0.522	1.35	0.656	-0.47	0.990
Sing_t0-Seq2	1.05	0.832	-0.99	0.858	-0.75	0.946	-1.23	0.733	0.78	0.937	1.76	0.399	1.64	0.473
Sing_t7-Seq	0.86	0.911	1.41	0.621	2.68	*0.056*	3.26	**0.010**	4.33	**<0.001**	1.94	0.296	0.01	1.000
Sing_t7-Seq2	1.02	0.846	0.84	0.917	1.22	0.741	2.68	*0.057*	3.52	**0.004**	2.32	0.139	2.07	0.231
Seq-Seq2	0.17	1.000	-0.56	0.981	-1.51	0.554	-0.61	0.974	-0.77	0.938	0.44	0.992	2.21	0.177
Observations	3600		3600		3600		3570		3540		3540		3540	
Copepods	120		120		120		119		118		118		118	

After a recovery period														
Day	9		11		13		15		17		19		21	
Comparison	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
C-Sing_t0	-4.42	**<0.001**	-2.26	0.158	-3.39	**0.006**	-1.37	0.649	-1.66	0.456	-0.94	0.883	-3.21	**0.012**
C-Sing_t7	-3.28	**0.009**	-3.14	**0.014**	-6.27	**<0.001**	-4.56	**<0.001**	-4.76	**<0.001**	-0.77	0.940	-3.36	**0.007**
C-Seq	-3.65	**0.002**	-2.50	0.089	-2.50	0.090	-2.47	0.097	-2.00	0.267	-1.32	0.676	-3.88	**0.001**
C-Seq2	-4.54	**<0.001**	-2.76	**0.045**	-3.33	**0.008**	-2.31	0.142	-2.45	0.102	-0.03	1.000	-1.93	0.300
Sing_t0-Sing_t7	1.09	0.813	-1.01	0.852	-3.24	**0.011**	-3.40	**0.006**	-3.35	**0.007**	0.16	1.000	-0.20	1.000
Sing_t0-Seq	0.91	0.894	-0.19	1.000	1.05	0.831	-1.13	0.790	-0.30	0.998	-0.37	0.996	-0.55	0.981
Sing_t0-Seq2	-0.09	1.000	-0.54	0.984	0.10	1.000	-0.96	0.871	-0.83	0.922	0.96	0.871	1.42	0.617
Sing_t7-Seq	-0.23	0.999	0.86	0.913	4.32	**<0.001**	2.40	0.115	3.18	**0.013**	-0.53	0.984	-0.34	0.997
Sing_t7-Seq2	-1.18	0.760	0.49	0.989	3.37	**0.007**	2.55	0.080	2.58	0.073	0.78	0.935	1.60	0.497
Seq-Seq2	-1.01	0.852	-0.37	0.996	-0.96	0.873	0.17	1.000	-0.56	0.981	1.38	0.640	2.06	0.236
Observations	3600		3600		3600		3570		3540		3540		3540	
Copepods	120		120		120		119		118		118		118	

Table III. S6

Table III. S6: Outcome of likelihood ratio tests. Significant p-values are marked in bold. The initial model used whether or not a copepod moved within a two second interval as response and the day after the first infection (DAY), the Period in the recording (PERIOD), i.e. after a simulated predation attack vs. after a recovery period and the interaction between DAY and TIME as fixed effects. We used the copepod identity as a random factor and included DAY and PERIOD. Subsequently, we added the number of parasites a copepod was infected by on day 7 (NUMBER) and all its interactions with DAY and PERIOD. All copepods were infected by one parasite on day 0. Test statistics and MCMC-estimated p-values are for the comparison with the preceding model.

Factors	DF	Chisq	p
+ NUMBER	11,1	0.303	0.5817
+ DAY : NUMBER	12,3	0.675	0.4113
+ PERIOD : NUMBER	13,1	19.355	**<0.0001**
+ PERIOD : DAY : NUMBER	14,1	51.745	**<0.0001**
22500 observations on 105 copepods			

Table III. S7

Table III. S7: Outcome of multiple comparisons between treatments for each day and period in the recording (i.e. after a simulated predation attack (A) vs. after a recovery period (B)). Significant p-values are highlighted in bold. The comparison gives the numbers of parasites copepods were infected by on day 7. Every copepod was infected additionally by one parasite on day 0. No significant differences occurred while there should have been a conflict between parasites that infected their copepod host on day 0 and on day 7 (day 11 to 15)

A: After simulated predation attack								
Day	9		11		13		15	
Comparison	Z	p	Z	p	Z	p	Z	p
0-1	-2.62	**0.041**	0.83	0.834	0.87	0.815	0.79	0.856
0-2	-1.14	0.659	2.20	0.117	1.33	0.538	1.34	0.533
0-3	-0.98	0.754	1.75	0.289	2.23	0.111	0.80	0.849
1-2	1.52	0.419	1.60	0.371	0.54	0.947	0.65	0.914
1-3	0.79	0.856	1.29	0.562	1.71	0.309	0.30	0.991
2-3	-0.20	0.997	0.25	0.994	1.31	0.548	-0.15	0.999
Observations	3090		2580		2790		2670	
Copepods	103		86		93		89	
B: After recovery period								
Day	9		11		13		15	
Comparison	Z	p	Z	p	Z	p	Z	p
0-1	-2.12	0.142	-0.96	0.769	-1.14	0.656	-0.39	0.979
0-2	-0.67	0.908	0.46	0.967	-0.03	1.000	0.09	1.000
0-3	-0.41	0.975	0.61	0.927	-1.73	0.297	-0.48	0.962
1-2	1.50	0.432	1.48	0.440	1.15	0.648	0.48	0.962
1-3	1.04	0.718	1.26	0.578	-1.04	0.721	-0.24	0.995
2-3	0.05	1.000	0.30	0.990	-1.74	0.296	-0.54	0.946
Observations	3090		2580		2790		2670	
Copepods	103		86		93		89	

Appendix for chapter IV

Table IV. S1

Table IV. S1: Outcome of likelihood ratio tests for copepod activity (i.e., proportion of time spent moving within one minute). The initial model used whether or not a copepod moved within a two second interval as response and the day after the first infection (DAY), the Period in the recording (PERIOD), i.e. after a simulated predator attack vs. after a recovery period and the interaction between DAY and TIME as fixed effects. We used the copepod identity as a random factor and included DAY and PERIOD. Subsequently, we added the treatment (TREAT) and all its interactions with DAY and PERIOD. Test statistics and MCMC-estimated p-values are for the comparison with the preceding model.

Experiment I					Experiment II			
Factors	DF	Chisq	p		Factors	DF	Chisq	p
+ TREAT	15,5	88.522	<0.0001		+ TREAT	13,3	38.223	<0.0001
+ DAY : TREAT	20,5	80.475	<0.0001		+ DAY : TREAT	16,3	74.504	<0.0001
+ PERIOD : TREAT	25,5	27.401	<0.0001		+ PERIOD : TREAT	19,3	108.823	<0.0001
+ PERIOD : DAY : TREAT	30,5	53.011	<0.0001		+ PERIOD : DAY : TREAT	22,3	28.672	<0.0001
100800 observations on 240 copepods					62160 observations on 150 copepods			

Table IV. S2

Table IV. S2: Outcome of likelihood ratio tests for latency to resume moving after a simulated predator attack. The initial model used the time when a copepod first moved following a 10 second interval after the simulated predation attack and the day after the first infection (DAY) as fixed effect. We used the copepod identity as a random factor and included DAY. Subsequently, we added the treatment (TREAT) and its interactions with DAY. Test statistics and MCMC-estimated p-values are for the comparison with the preceding model.

Experiment I					Experiment II			
Factors	DF	Chisq	p		Factors	DF	Chisq	p
+ TREAT	11,5	84.623	<0.0001		+ TREAT	9,3	33.871	<0.0001
+ DAY : TREAT	16,5	60.699	<0.0001		+ DAY : TREAT	12,3	58.168	<0.0001
1680 observations on 240 copepods					1036 observations on 150 copepods			

Table IV. S3

Table IV. S3: Outcome of multiple comparisons between days for each treatment. Results from experiment I. Significant p-values are highlighted in bold. Control: Uninfected control copepods, CAM: Copepods infected with *C. lacustris* on day 0, cam: Copepods infected with *C. lacustris* on day 7, sch: Copepods infected with *S. solidus* on day 7, CAM-cam: Copepods infected with one *L. lacustris* on day 0 plus one on day 7, CAM-sch: Copepods infected with one *L. lacustris* on day 0 plus one *S. solidus* on day 7.

Activity after simulated predator attack												
Treatment	Control		CAM		cam		sch		CAM-cam		CAM-sch	
Comparison	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
day9 - day11	0.34	1	24.50	**<0.001**	-11.34	**<0.001**	-9.63	**<0.001**	20.05	**<0.001**	21.03	**<0.001**
day11 - day13	-2.98	**0.045**	3.94	**0.002**	-5.43	**<0.001**	-9.35	**<0.001**	2.61	0.122	2.91	0.055
day13 - day15	3.29	**0.018**	-2.76	0.080	-2.85	0.064	1.88	0.497	-3.48	**0.009**	-1.77	0.569
day15 - day17	-1.97	0.434	-2.61	0.118	17.80	**<0.001**	7.89	**<0.001**	-2.50	0.155	-6.70	**<0.001**
day17 - day19	2.14	0.327	-0.82	0.982	10.35	**<0.001**	-3.38	**0.013**	2.92	0.053	-3.81	**0.003**
day19 - day21	-3.16	**0.026**	-0.77	0.987	-0.96	0.961	5.55	**<0.001**	2.05	0.378	-1.54	0.721
Observations	8400		8400		8400		8400		8400		8400	
Copepods	40		40		40		40		40		40	
Activity after a recovery period												
Treatment	Control		CAM		cam		sch		CAM-cam		CAM-sch	
Comparison	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
day9 - day11	-0.64	0.996	21.26	**<0.001**	-4.67	**<0.001**	-7.06	**<0.001**	18.22	**<0.001**	21.34	**<0.001**
day11 - day13	-2.61	0.122	3.24	**0.019**	-6.04	**<0.001**	-4.95	**<0.001**	-1.12	0.920	0.04	1
day13 - day15	4.39	**<0.001**	-1.75	0.568	-2.08	0.350	0.76	0.989	-0.75	0.989	0.38	1
day15 - day17	0.68	0.994	1.58	0.683	15.69	**<0.001**	5.02	**<0.001**	-2.05	0.378	-3.81	**0.003**
day17 - day19	0.34	1	-3.83	**0.002**	7.20	**<0.001**	2.16	0.317	2.89	0.059	-3.51	**0.008**
day19 - day21	-2.30	0.246	-1.11	0.922	2.99	**0.042**	3.98	**0.001**	-0.96	0.962	1.11	0.924
Observations	8400		8400		8400		8400		8400		8400	
Copepods	40		40		40		40		40		40	

Latency to resume moving after a simulated predator attack												
Treatment	Control		CAM		cam		sch		CAM-cam		CAM-sch	
Comparison	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
day9 - day11	-0.28	1	-12.68	**<0.001**	2.08	0.366	1.72	0.604	-8.07	**<0.001**	-10.32	**<0.001**
day11 - day13	0.18	1	-1.83	0.526	1.21	0.891	2.26	0.261	0.30	1	-0.48	0.999
day13 - day15	-0.81	0.984	0.71	0.992	0.74	0.990	-2.20	0.293	0.78	0.987	-0.42	1
day15 - day17	0.01	1	-0.92	0.969	-6.41	**<0.001**	-0.18	1	-0.10	1	1.52	0.731
day17 - day19	-0.02	1	0.73	0.991	-2.04	0.388	-1.85	0.516	-1.02	0.949	-0.24	1
day19 - day21	0.26	1	0.67	0.994	-0.02	1	0.20	1	0.62	0.996	0.39	1
Observations	280		280		280		280		280		280	
Copepods	40		40		40		40		40		40	

Table IV. S4

Table IV. S4: Outcome of multiple comparisons between days for each treatment. Results from experiment II. Significant p-values are highlighted in bold. Control: Uninfected control copepods, SCH: Copepods infected with S. solidus on day 0, cam: Copepods infected with C. lacustris on day 7, SCH-cam: Copepods infected with one S. solidus on day 0 plus one C. lacustris on day 7.

Activity after simulated predator attack								
Treatment	Control		SCH		cam		SCH-cam	
Comparison	Z	p	Z	p	Z	p	Z	p
day9 - day11	3.78	**0.003**	2.24	0.274	-4.17	**0.001**	0.98	0.957
day11 - day13	-1.78	0.560	5.47	**<0.001**	-4.79	**<0.001**	1.58	0.687
day13 - day15	1.68	0.629	0.89	0.974	0.80	0.984	4.55	**<0.001**
day15 - day17	1.79	0.554	-1.49	0.751	18.48	**<0.001**	8.62	**<0.001**
day17 - day19	-2.09	0.358	1.98	0.429	6.42	**<0.001**	5.33	**<0.001**
day19 - day21	-0.13	1	-2.72	0.094	-3.33	**0.014**	-1.17	0.903
Observations	8340		6240		8250		8250	
Copepods	40		30		40		40	
Activity after a recovery period								
Treatment	Control		SCH		cam		SCH-cam	
Comparison	Z	p	Z	p	Z	p	Z	p
day9 - day11	1.97	0.434	1.54	0.721	-7.86	**<0.001**	-0.29	1
day11 - day13	2.20	0.294	0.68	0.994	-1.67	0.617	0.22	1
day13 - day15	-0.48	0.999	0.16	1	-2.81	0.068	-3.20	**0.022**
day15 - day17	0.65	0.995	3.39	**0.012**	18.73	**<0.001**	14.67	**<0.001**
day17 - day19	-2.58	0.131	1.53	0.728	8.13	**<0.001**	8.17	**<0.001**
day19 - day21	-1.38	0.811	1.94	0.455	-3.17	**0.023**	-0.61	0.996
Observations	8340		6240		8250		8250	
Copepods	40		30		40		40	
Latency to resume moving after a simulated predator attack								
Treatment	Control		SCH		cam		SCH-cam	
Comparison	Z	p	Z	p	Z	p	Z	p
day9 - day11	-2.54	0.145	-1.24	0.880	1.36	0.823	-0.33	1
day11 - day13	2.18	0.304	-0.46	0.999	3.17	**0.025**	-1.68	0.631
day13 - day15	-0.47	0.999	-0.86	0.978	0.27	1	-0.68	0.994
day15 - day17	-0.62	0.996	0.54	0.998	-9.15	**<0.001**	-4.16	**0.001**
day17 - day19	0.07	1	-1.11	0.926	-2.02	0.404	-0.27	1
day19 - day21	-0.82	0.983	0.65	0.995	1.16	0.909	0.04	1
Observations	278		275		208		275	
Copepods	40		40		30		40	

Table IV. S5

Table IV. S5: Outcome of multiple comparisons between treatments for each day. Results from experiment I. Significant p-values are highlighted in bold. Control: Uninfected control copepods. CAM: Copepods infected with *C. lacustris* on day 0. cam: Copepods infected with *C. lacustris* on day 7. sch: Copepods infected with *S. solidus* on day 7. CAM-cam: Copepods infected with one *L. lacustris* on day 0 plus one on day 7. CAM-sch: Copepods infected with one *L. lacustris* on day 0 plus one *S. solidus* on day 7.

Activity after simulated predator attack														
Day	9		11		13		15		17		19		21	
	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
Control _CAM	-9.41	<**0.001**	0.79	0.969	3.03	**0.030**	0.73	0.978	1.22	0.828	-0.35	0.999	0.10	1
Control _cam	-4.30	<**0.001**	-6.71	<**0.001**	-8.64	<**0.001**	-11.40	<**0.001**	-3.64	**0.004**	-0.65	0.987	1.38	0.738
Control _sch	0.34	0.999	-1.84	0.442	-3.16	**0.019**	-4.50	<**0.001**	-2.66	0.084	-2.06	0.307	0.28	1
Control _CAM-cam	-6.52	<**0.001**	0.69	0.983	1.19	0.844	-0.69	0.983	-1.56	0.623	-0.64	0.988	-0.18	1
Control _CAM-sch	-7.95	<**0.001**	1.23	0.820	2.21	0.234	0.91	0.944	-0.73	0.978	-2.35	0.174	-0.89	0.949
CAM_cam	-5.60	<**0.001**	7.44	<**0.001**	11.37	<**0.001**	12.02	<**0.001**	4.85	<**0.001**	0.30	1	-1.28	0.795
CAM_sch	9.70	<**0.001**	-2.62	0.093	-6.14	<**0.001**	-5.22	<**0.001**	-3.87	**0.002**	-1.71	0.525	0.18	1
CAM_CAM-cam	3.43	**0.008**	-0.10	1	-1.84	0.437	-1.42	0.716	-2.79	0.060	-0.29	1	-0.28	1
CAM_CAM-sch	1.79	0.469	0.44	0.998	-0.83	0.962	0.18	1	-1.95	0.370	-2.00	0.344	-0.99	0.921
cam_sch	4.64	<**0.001**	4.95	<**0.001**	5.67	<**0.001**	7.43	<**0.001**	1.00	0.918	-1.42	0.717	-1.10	0.883
cam_CAM_cam	-2.32	0.186	7.35	<**0.001**	9.72	<**0.001**	10.80	<**0.001**	2.11	0.283	0.01	1	-1.56	0.627
cam_CAM-sch	-3.93	**0.001**	7.87	<**0.001**	10.65	<**0.001**	12.19	<**0.001**	2.94	**0.039**	-1.70	0.530	-2.27	0.207
sch_CAM-cam	6.85	<**0.001**	-2.52	0.118	-4.33	<**0.001**	-3.81	**0.002**	-1.11	0.878	-1.42	0.713	0.45	0.998
sch_CAM_sch	8.27	<**0.001**	-3.06	0.027	-5.34	<**0.001**	-5.40	<**0.001**	-1.94	0.376	0.29	1	1.16	0.855
CAM-cam_CAM_sch	-1.67	0.551	0.54	0.995	1.02	0.911	1.60	0.598	0.84	0.961	-1.71	0.526	-0.71	0.981
Observations	7200		7200		7200		7200		7200		7200		7200	
Copepods	240		240		240		240		240		240		240	

Activity after a recovery period														
Day	9		11		13		15		17		19		21	
	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
Control _CAM	-10.57	**<0.001**	0.22	1	2.98	**0.034**	1.03	0.875	0.49	0.997	-0.60	0.991	0.31	1
Control _cam	-3.17	**0.019**	-8.26	**<0.001**	-9.88	**<0.001**	-6544.67	**<0.001**	-3.04	**0.029**	0.09	1	0.93	0.939
Control _sch	0.79	0.969	-2.69	0.076	-5.31	**<0.001**	-7.72	**<0.001**	-1.98	0.354	-4.41	**<0.001**	-0.94	0.936
Control _CAM-cam	-8.63	**<0.001**	-0.78	0.971	1.00	0.918	-1.82	0.380	-1.36	0.751	-1.21	0.830	0.57	0.993
Control _CAM-sch	-8.80	**<0.001**	-0.47	0.997	1.73	0.511	-0.01	1	-1.68	0.544	-4.23	**<0.001**	-3.13	**0.022**
CAM_cam	-7.79	**<0.001**	8.44	**<0.001**	12.46	**<0.001**	18.86	**<0.001**	3.51	**0.006**	-0.69	0.983	-0.61	0.990
CAM_sch	11.24	**<0.001**	-2.90	**0.043**	-8.18	**<0.001**	-6.26	**<0.001**	-2.46	0.135	-3.82	**0.002**	-1.25	0.813
CAM_CAM-cam	2.40	0.156	-1.00	0.920	-1.98	0.352	-2.02	0.269	-1.85	0.435	-0.62	0.990	0.26	1
CAM_CAM-sch	2.07	0.302	-0.69	0.983	-1.26	0.808	-0.74	0.967	-2.17	0.254	-3.64	**0.004**	-3.43	**0.008**
cam_sch	3.95	**0.001**	5.70	**<0.001**	4.93	**<0.001**	9.52	**<0.001**	1.07	0.894	-4.50	**<0.001**	-1.86	0.427
cam_CAM_cam	-5.65	**<0.001**	7.50	**<0.001**	10.76	**<0.001**	15.95	**<0.001**	1.69	0.538	-1.30	0.783	-0.35	0.999
cam_CAM-sch	-5.89	**<0.001**	7.82	**<0.001**	11.39	**<0.001**	17.86	**<0.001**	1.37	0.745	-4.31	**<0.001**	-4.04	**0.001**
sch_CAM-cam	9.35	**<0.001**	-1.91	0.396	-6.28	**<0.001**	-4.27	**<0.001**	-0.62	0.989	-3.21	**0.017**	-1.51	0.659
sch_CAM_sch	9.50	**<0.001**	-2.23	0.225	-6.98	**<0.001**	-5.54	**<0.001**	-0.30	1	-0.19	1	2.18	0.248
CAM-cam_CAM_sch	-0.32	1	0.31	1	0.73	0.978	1.28	0.739	-0.32	1	-3.02	**0.030**	-3.69	**0.003**
Observations	7200		7200		7200		7200		7200		7200		7200	
Copepods	240		240		240		240		240		240		240	

Latency to resume moving after a simulated predator attack														
Day	9		11		13		15		17		19		21	
	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
Control _CAM	8.05	**<0.001**	-0.25	1	-2.13	0.270	-0.82	0.964	-1.76	0.491	-1.38	0.740	-0.72	0.980
Control _cam	2.91	**0.042**	6.59	**<0.001**	8.53	**<0.001**	9.85	**<0.001**	1.78	0.477	-1.23	0.823	-1.38	0.739
Control _sch	-0.09	1	1.74	0.502	4.10	**0.001**	2.36	0.170	2.28	0.203	0.43	0.998	0.40	0.999
Control _CAM-cam	4.41	**<0.001**	-1.04	0.906	-0.97	0.928	0.38	0.999	0.30	1	-0.90	0.948	-0.34	0.999
Control _CAM-sch	6.13	**<0.001**	-0.80	0.967	-1.46	0.690	-1.20	0.835	0.21	1	-0.02	1	0.19	1
CAM_cam	5.14	**<0.001**	-6.84	**<0.001**	-10.66	**<0.001**	-10.67	**<0.001**	-3.54	**0.005**	-0.15	1	0.66	0.986
CAM_sch	-8.14	**<0.001**	1.99	0.348	6.23	**<0.001**	3.18	**0.018**	4.04	**0.001**	1.81	0.462	1.12	0.874
CAM_CAM-cam	-3.64	**0.004**	-0.79	0.969	1.16	0.854	1.20	0.836	2.06	0.309	0.48	0.997	0.38	0.999
CAM_CAM-sch	-1.92	0.391	-0.56	0.994	0.67	0.985	-0.38	0.999	1.97	0.358	1.36	0.750	0.91	0.945
cam_sch	-3.00	**0.032**	-4.85	**<0.001**	-4.43	**<0.001**	-7.49	**<0.001**	0.50	0.996	1.66	0.561	1.78	0.477
cam_CAM_cam	1.50	0.666	-7.63	**<0.001**	-9.50	**<0.001**	-9.47	**<0.001**	-1.48	0.674	0.33	0.999	1.04	0.903
cam_CAM-sch	3.22	**0.016**	-7.40	**<0.001**	-9.99	**<0.001**	-11.06	**<0.001**	-1.57	0.618	1.21	0.832	1.57	0.616
sch_CAM-cam	-4.50	**<0.001**	2.78	0.061	5.06	**<0.001**	1.98	0.355	1.98	0.354	1.32	0.772	0.74	0.977
sch_CAM_sch	-6.22	**<0.001**	2.55	0.111	5.56	**<0.001**	3.57	**0.005**	2.07	0.305	0.44	0.998	0.21	1
CAM-cam_CAM_sch	1.72	0.518	0.23	1	-0.49	0.996	-1.59	0.607	-0.09	1	0.88	0.952	0.53	0.995
Copepods	240		240		240		240		240		240		240	

Table IV. S6

Table IV. S6: Outcome of multiple comparisons between treatments for each day. Results from experiment II. Significant p-values are highlighted in bold. Control: Uninfected control copepods, SCH: Copepods infected with S. solidus on day 0, cam: Copepods infected with C. lacustris on day 7, SCH-cam: Copepods infected with one S. solidus on day 0 plus one C. lacustris on day 7.

Activity after simulated predator attack														
Day	9		11		13		15		17		19		21	
	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
Control _SCH	-1.17	0.646	-1.79	0.280	0.69	0.900	0.67	0.908	-0.53	0.952	1.10	0.692	0.19	0.998
Control _cam	-4.12	**<0.001**	-7.07	**<0.001**	-8.44	**<0.001**	-8.21	**<0.001**	-0.65	0.914	2.66	**0.039**	1.42	0.489
Control _SCH-cam	-5.68	**<0.001**	-6.93	**<0.001**	-5.09	**<0.001**	-4.00	**<0.001**	-1.70	0.323	1.13	0.672	0.84	0.833
SCH_cam	2.70	**0.035**	4.85	**<0.001**	8.56	**<0.001**	8.29	**<0.001**	0.07	1	-1.37	0.515	-1.13	0.670
SCH_SCH-cam	-4.21	**<0.001**	-4.85	**<0.001**	-5.39	**<0.001**	-4.35	**<0.001**	-1.04	0.726	-0.05	1	0.59	0.934
cam_SCH-cam	-1.67	0.340	-0.16	0.999	3.78	**0.001**	4.57	**<0.001**	-1.05	0.718	-1.53	0.417	-0.58	0.937
Observations	4470		4320		4500		4500		4410		4470		4410	
Copepods	149		144		150		150		147		149		147	
Activity after a recovery period														
Day	9		11		13		15		17		19		21	
	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
Control _SCH	-3.96	**<0.001**	-4.01	**<0.001**	-4.74	**<0.001**	-4.61	**<0.001**	-3.41	**0.004**	-2.48	0.064	-1.30	0.564
Control _cam	-5.3	**<0.001**	-8.16	**<0.001**	-10.3	**<0.001**	-11.56	**<0.001**	-3.23	**0.007**	0.29	0.992	-0.39	0.980
Control _SCH-cam	-7.66	**<0.001**	-8.35	**<0.001**	-9.97	**<0.001**	-11.04	**<0.001**	-5.19	**<0.001**	-1.77	0.288	-1.46	0.459
SCH_cam	1.00	0.749	3.79	**0.001**	5.24	**<0.001**	7.02	**<0.001**	-0.44	0.971	-2.73	**0.032**	-0.92	0.792
SCH_SCH-cam	-3.34	**0.004**	-4.16	**<0.001**	-5.01	**<0.001**	-6.44	**<0.001**	-1.42	0.489	0.85	0.833	-0.06	1
cam_SCH-cam	-2.51	*0.058*	-0.5	0.960	0.18	0.998	0.69	0.899	-2.01	0.184	-2.05	0.170	-1.06	0.715
Observations	4470		4320		4500		4500		4410		4470		4410	
Copepods	149		144		150		150		147		149		147	

Latency to resume moving after a simulated predator attack														
Day	9		11		13		15		17		19		21	
	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p	Z	p
Control _SCH	0.66	0.910	1.15	0.656	-0.53	0.952	-1.04	0.726	-0.17	0.998	-2.16	0.136	-0.11	0.999
Control _cam	2.41	0.075	5.32	**<0.001**	6.48	**<0.001**	7.69	**<0.001**	-0.51	0.957	-4.22	**<0.001**	-0.88	0.815
Control _SCH-cam	5.31	**<0.001**	6.62	**<0.001**	3.41	**0.004**	3.34	**0.005**	-0.79	0.860	-1.89	0.231	-0.57	0.942
SCH_cam	-1.57	0.395	-3.76	**0.001**	-6.53	**<0.001**	-8.15	**<0.001**	0.29	0.991	1.77	0.287	0.71	0.893
SCH_SCH-cam	4.26	**<0.001**	4.98	**<0.001**	3.69	**0.001**	4.13	**<0.001**	-0.55	0.946	0.40	0.978	-0.41	0.976
cam_SCH-cam	2.87	**0.021**	1.37	0.516	-3.07	**0.012**	-4.34	**<0.001**	-0.29	0.992	2.35	0.088	0.32	0.988
Copepods	149		144		150		150		147		149		147	

Appendix for chapter VI

Supplementary information VI: Confirmation of the effect of feeding treatment on parasite performance

Statistical analysis

To verify that our feeding treatments had an effect on parasite performance in our study, we used general linear models within the stats package in R (R Development Core Team 2010). We modelled the presence or absence of a cercomer on 9 dpi, the parasite size 10 dpi (day-11 copepods) or 16 dpi (day 17 copepods) and the infection success in fish on day 11 (day-11 copepods) or day 17 (day-17 copepods) as response variables and the feeding treatment as fixed factor. For the presence or absence of a cercomer and the infection success we used a binomial error structure, for parasite size we used a Gaussian error structure. For an overview of treatment groups and variables measured, see Figure 1 in the main text.

Effect of feeding treatment on day 11 copepods

Parasites in day-11 copepods in the high food treatments developed significantly faster than those in the low food treatment. On day 9 60 % of copepods in the high vs. 18 % in the low food treatment possessed a cercomer ($Z_{1,119}$=-4.520, p<0.0001). Parasites in a high food treatment were also larger 10 dpi (mean +/- 95 % CI: 20425 +/- 862 um^2 vs. 17199 +/- 671 um^2, $t_{1,120}$=-5.79, p<0.0001) and more likely to infect fish on day 11 (15 % vs. 3 %, $Z_{1,119}$=-2.005, p=0.045).

Effect of feeding treatment on day 17 copepods

In the day 17 copepods differences between feeding treatments were less pronounced. There were no significant differences in whether parasites possessed a cercomer 9 dpi (High vs. low food treatment: 47 vs. 52 %, $Z_{1,120}$=0.496, p=0.620) and how likely they were to infect fish (High vs. low food treatment: 88 vs. 76 %, $Z_{1,120}$=-1.583, p=0.114). However, parasites in a high food treatment again grew larger than those in a low food treatment (mean +/- 95 % CI: 25201+/- 851 um vs. 23514 +/- 890, $t_{1,100}$=-2.685, p=0.0085). These results are consistent with previous claims that S. solidus growth is more responsive than ontogeny to resource availability (Benesh 2010).

In summary, the feeding treatment was sufficiently aggressive to create variation in parasite traits considered fitness relevant.

Table VI. S1

Table S1: Post hoc tests for the effect of the interaction between feeding treatment and infection for the distance copepods moved. Test statistics and p values were obtained using general linear hypotheses within the multcomp package in R (Hothorn et al. 2008). Feeding treatment and infection were combined into a single factor with four different levels comprising all possible combinations between these two factors (FEED_INF): Uninfected control, high food (c_H); uninfected control, low food (c_L); infected, high food (inf_H); infected, low food (inf_L). The comparisons were based on the following models (see Table 1 for more details): Day-11 copepods: FEED_INF + INTERVAL + (INTERVAL|RE) + (1 | ID), Day 17: FEED_INF + INTERVAL + INFECTIVITY + INTERVAL:INFECTIVITY + (INTERVAL|RE) + (INFECTIVITY | ID). Significant p-values have been marked in bold.

	Day-11 copepods		Day-17 copepods	
Comparison	z	p	z	p
c_H – c_L	-4.771	**<0.001**	2.896	**0.0194**
inf_H – inf_L	-2.140	0.140	-0.556	0.9444
c_H – inf_H	-8.518	**<0.001**	-6.445	**< 0.001**
c_L – inf_L	-5.383	**<0.001**	-3.413	**0.0038**
c_H – inf_L	-10.571	**<0.001**	-6.918	**< 0.001**
c_L – inf_H	3. 383	**0.004**	2.953	**0.0164**

Table VI. S2

Table S2: Associations between parasite performance and host activity and distance. Mixed models used whether or not a copepod moved within a two second interval (Activity) or, if it moved, how far it moved (distance, log transformed) as response variables. Copepod identity (ID), the recording event (RE, i.e. a combination of copepod identity and the day of the recording), and the time interval in the recording (i.e. before vs. after the simulated predator attack, INTERVAL) were incorporated into the models' random effect structure. INTERVAL, feeding treatment (FEED), and their interaction were additionally included as fixed effects. For day 17-copepods we included whether or not parasites were infective for fish as both fixed and random effect (INF, together with ID) and its interaction with FEED and INTERVAL. Subsequently, we separately added measures of parasite performance (PERFORM (i.e. presence or absence on a cercomer on day 9 as an indicator of development, parasite size on day 10 (day-11 copepods) or day 16 (day-17 copepods) and infection success in fish) and all their pairwise interactions with INTERVAL, FEED and INF (day-17 copepods). Test statistics and MCMC-estimated p-values are for the comparison with the preceding model. Null models: Day-11 copepods: INTERVAL + FEED + INTERVAL: FEED + (INTERVAL|RE) + (1 | ID), day-17 copepods: INTERVAL + FEED + INF+ INTERVAL: FEED + INTERVAL:INF+FEED:INF (INTERVAL|RE) + (INFECTIVITY | ID). Since we used multiple tests, according to bonferroni adjustment only p-values below 0.0042 should be considered significant at α=0.05. They have been marked in bold. P-values significant only prior to adjustment have been put in italics.

		Parasite size						Development (Cercomer present or absence 9 dpi)						Infection success in fish					
		Activity			Distance			Activity			Distance			Activity			Distance		
Day-11 copepods	Factors	DF	Chisq	p	DF	Chisq	p	DF	Chisq	p	DF	Chisq	p	DF	Chisq	p	DF	Chisq	p
	+PERFORM	1,9	0.447	0.5040	1,10	9.883	**0.0017**	1,9	6.591	*0.0103*	1,10	0.417	0.5186	1,9	3.217	0.0729	1,10	7.934	*0.0049*
	+PERFORM: INTERVAL	1,10	3.490	0.0617	1,11	0.898	0.3433	1,10	0.950	0.3298	1,11	3.884	*0.0488*	1,10	0.332	0.5644	1,11	3.056	0.0805
	+PERFORM: FEED	1,11	0.048	0.8272	1,12	1.564	0.2111	1,11	0.216	0.6418	1,12	0.089	0.7651	1,11	0.280	0.5964	1,12	0.902	0.3424
		31240 observations, 355 RE, 122 copepods			10949 observations, 352 RE, 122 copepods			30712 observations, 349 RE, 121 copepods			10609 observations, 346, and 121 copepods			30976 observations, 352 RE, 121 copepods			10801 observations, 348 RE, 121 copepods	

		Parasite size						Development (Cercomer present or absence 9 dpi)						Infection success in fish					
		Activity			Distance			Activity			Distance			Activity			Distance		
Day-17 copepods	Factors	DF	Chisq	p	DF	Chisq	p	DF	Chisq	p	DF	Chisq	p	DF	Chisq	p	DF	Chisq	p
	+PERFORM	1,14	12.323	**0.0004**	1,15	9.099	**0.0026**	1,14	0.382	0.5368	1,15	0.012	0.9113	1,14	0.030	0.8637	1,15	1.242	0.2651
	+ERFORM: INTERVAL	1,15	0.046	0.8306	1,16	3.617	0.0572	1,15	0.077	0.7813	1,16	5.435	*0.0197*	1,15	0.067	0.7951	1,16	1.954	0.1621
	+PERFORM: INF	1,16	0.001	0.9752	1,17	0.022	0.8830	1,16	1.574	0.2096	1,17	0.518	0.4717	1,16	5.598	*0.0180*	1,17	0.519	0.4713
	+PERFORM: FEED	1,17	5.722	*0.0168*	1,18	0.696	0.4041	1,17	0.980	0.3221	1,18	1.458	0.2273	1,17	0.734	0.3917	1,18	1.421	0.2332
		50512 observations, 574 RE, 102 copepods			18433 observations, 557 RE, 102 copepods			50072 observations, 569 RE, 101 copepods			18316 observations, 552 RE, 101 copepods			48928 observations, 556 RE, 99 copepods			17603 observations, 539 RE, 99 copepods	

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Declaration

Hereby I declare that

1) Apart from my supervisor's guidance, the content and design of this dissertation is the product of my own work. The co-authors' contributions to specific paragraphs are listed in the thesis outline section.

2) This thesis has not been submitted either partially or wholly as part of a doctoral degree to another examination body. Parts of the introduction and of the conclusion have been submitted as a book chapter with M. Milinski as coauthor, chapter I has been published in the Journal of Fish Biology with T. Henrich as first and K. Mobley as last author, chapter II and IV have been submitted to scientific journals with M. Milinski as coauthor, chapter III has been published in Evolution with M. Milinski as coauthor, chapter V has been published in Parasites & Vectors with D. Benesh as first author and chapter VI has been submitted to a scientific journal with D. Benesh as coauthor. Details on each publication have been indicated at the beginning of the appropriate chapter. No other materials are published or submitted for publication.

3) The preparation of the thesis has been subjected to the Rules of Good Scientific Practice of the German Research Foundation.

Plön, 13th July 2015