Multiple ecological scales of host-parasite interactions using the three-spined stickleback and *Schistocephalus solidus* model system

Dissertation

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Summary

Parasites are powerful forces of selection shaping evolutionary and ecological processes such as maintenance of genetic polymorphism, species diversity, divergent selection, or even the evolution of sex. Studying host-parasite interactions offers a great way to catch evolution in action, yet much remains to be discovered about the underlying mechanisms. Indeed, host-parasite interactions are often the results of complex interactions at different ecological and evolutionary scales. Predicting in which direction reciprocal host-parasite interactions are driving resistance and virulence is challenging, but crucial for diverse fields of research such as epidemiology, conservation or speciation as it helps to foresee infectious diseases epidemics, population dynamics, or species diversification.

In this thesis I (and my co-authors) aimed at uncovering the underlying mechanisms of host-parasite interactions at different ecological scales for my model system. The three-spined stickleback (*Gasterosteus aculeatus*) and its specific tapeworm *Schistocephalus solidus* offer a unique opportunity to combine field observations and controlled experimentation in a vertebrate host. We used populations differing in ecology and coevolutionary history in field studies and experimental infections to investigate host-parasite interactions at the within-host, between-host, population and community scales.

In my first chapter I look at how the parasite community as a whole shapes host resistance by examining how relaxed parasite selection influence host immunocompetence and gene flow in a natural system. Over a 4-year field survey of the macroparasite community of two Norwegian three-spined stickleback populations, we found clear and stable patterns of drastically divergent parasite pressures potentially limiting the gene flow between locally adapted river and lake fish populations. We documented for the first time a macroparasite-free three-spined stickleback population and demonstrated experimentally its inferior resistance to two macroparasite species (*S. solidus* and *Diplostomum pseudospathaceum*) compare to the nearby parasite-rich population. These results confirmed theoretical predictions that while the population experiencing a relaxed parasite selection was found to be in better

general condition in its native habitat, it actually had a reduced resistance when exposed to parasites. This shows that divergent parasite communities can select for different immunocompetence and limit gene flow between divergent host populations.

In the second chapter, I disentangle the ecological and evolutionary components affecting *S. solidus* natural infection patterns in Canadian and European populations. By performing reciprocal infections of three-spined sticklebacks and *S. solidus* from the same or different continents, we were able to show that freshwater populations have recently evolved a global resistance to *S. solidus* infections when marine ancestral populations colonized new freshwater habitats. In those populations, *S. solidus* has counter adapted by evolving local infectivity to three-spined stickleback populations. The pattern of susceptibility/resistance observed in the different experimental combinations represents a departure from the main theoretical models of host-parasite interactions, "gene-for-gene" and "matching-allele". We proposed a hybrid conceptual model in which hosts first evolve global resistance by recognizing a conserved parasite motif (targeted-recognition), and in response, parasites counter adapt with different local infectivity strategies ("matching-allele").

In my third chapter, I investigate the genetic basis of three-spined sticklebacks resistance to *S. solidus* in two studied populations. Using experimental infections and gene expression measurements (RT-qPCR), we evaluated the differential expression of specific immune candidate genes between sympatric (coevolved) and allopatric (non-coevolved) host-parasite combinations at three time points. We identified different rates of host exploitation for the different infection combinations, reflecting the importance of coevolution for optimal parasite virulence and host resistance. In particular, the sympatric combinations reached a similar optimal relative level of host exploitation, while in contrast allopatric combinations resulted in either over- or under-host exploitation. Differential expression of immune genes between treatment groups revealed the manipulation of the host immune system by their coevolved parasites. These results indicate a complex interplay between parasite and host via the host immune system during infections. Coevolution

favoured local adaptation of both host and parasite genotypes through the selection for optimal host immune response and parasite evasion/manipulation.

In my fourth chapter I explored how parasite-parasite competition influences the expression of virulence in competing parasite genotypes. We used a highly virulent and a less virulent strain of *S. solidus* to measure individual parasite virulence in homologous and heterologous co-infections. We found that while virulence is strongly genetically determined, there is also a plastic dimension to this trait, as virulence depended on the co-infection competitor. This plasticity might reflect that *S. solidus* exploits its host through the production of a combination of common and strain-specific goods, which also mediates within-host competition. Plasticity through within-host interactions could affect the strength of host-parasite interactions as it reduces the phenotypic variation between different parasite genotypes. Hence, virulence plasticity could contribute to the maintenance of virulence polymorphism at a meta-population level.

This thesis highlights the complexity of factors shaping host-parasite interactions at different ecological and individual levels in the model system three-spined stickleback/*S. solidus*. Specifically, our results show a geographic structure of interactions as local environmental factors and coevolutionary histories create the conditions for local and reciprocal adaptation of host and parasite.

Zusammenfassung

Parasiten sind mächtige Kräfte der Selektion, die evolutionäre und ökologische Prozesse wie die Erhaltung genetischer Polymorphismen, Artenvielfalt, divergierende Selektion oder sogar die Entstehung sexueller Reproduktion prägen. Die Untersuchung von Wirt-Parasit-Interaktionen bietet eine großartige Möglichkeit Evolution in Aktion zu beobachten denn viele der zugrundeliegenden Mechanismen liegen noch immer im Verborgenen. Wirt-Parasit-Wechselwirkungen sind oft das Ergebnis komplexer Vorgängen, die auf verschiedenen ökologischen und evolutionären Ebenen stattfinden. Vorherzusagen, in welche Richtung diese Wechselwirkungen die Entwicklung von Resistenz und Virulenz treiben, ist schwierig aber wesentlich für viele verschiedene Forschungsgebiete und betrifft Bereiche wie zum Beispiel die Epidemiologie, Arterhaltung oder Speziation. Wissen um die Wirt-Parasit-Wechselwirkungen in diesen Bereichen ist wichtig um die Verbreitung von Infektionskrankheiten bei Epidemien, Populationsdynamik oder Artbildung vorauszusehen oder nachzuvollziehen.

Mit dieser Arbeit möchten ich (und meine Koautoren) die zugrundeliegenden Mechanismen von Wirt-Parasit-Interaktionen in verschiedenen ökologischen Maßstäben meines Modellsystems untersuchen. Der dreistachlige Stichling (*Gasterosteus aculeatus*) und sein spezifischer Bandwurm *Schistocephalus solidus* bieten die einmalige Gelegenheit, Feldforschung und kontrolliertes Experiment in einem Wirbeltierwirt zu kombinieren. Die hier untersuchten Fisch-Populationen unterscheiden sich in ihrer Ökologie und Evolutionsgeschichte und bilden daher ein ideales System um Wirt-Parasit-Wechselwirkungen in Feldstudien und experimentellen Infektionen auf Wirts-, Populations- oder Artengemeinschaftsniveau zu untersuchen.

In meinem ersten Kapitel gehe ich der Frage nach, wie die Parasitengemeinschaft die Wirtsresistenz beinflusst indem ich beschreibe wie schwache, durch Parasiten vermittelte Selektion die Immunokompetenz des Wirts und den damit verbundenen Genfluss in einem natürlichen System beeinflussen. Im Rahmen einer 4-jährigen Feldstudie wurde untersucht wie die Makroparasitengemeinschaft zweier norwegischer Stichlings-Populationen, die sich in dem durch sie ausgeübten Selektionsdruck massiv unterscheiden und den

Genfluss zwischen zwei lokal angepassten Fluss- und Seefischpopulationen potenziell einschränken können. Wir dokumentieren zum ersten Mal eine makroparasitenfreie dreistachlige Stichlingspopulation und zeigen experimentell, dass diese Population eine schwächer ausgeprägte Resistenz gegen zwei Makroparasitenarten (S*chistocephalus solidus* und *Diplostomum pseudospathaceum*) im Vergleich zu anderen parasitenreichen Population besitzen. Unsere Ergebnisse bestätigten theoretische Vorhersagen, dass eine Population die nur schwache Selektion durch Parasiten erlebt, in ihrem heimischen Lebensraum in einem besseren allgemeinen Zustand lebt als bei Selektion durch Parasiten gegen die sie nur schwach ausgeprägten Widerstand zu leisten vermögen. Diese Ergebnisse zeigen, dass divergierende Parasitengemeinschaften unterschiedliche Niveaus in Immunkompetenz selektieren und so den Genfluss zwischen sich so unterscheidenden Wirtspopulationen einschränken können.

Im zweiten Kapitel untersuche ich die ökologischen und evolutionären Komponenten natürlich vorkommender Infektionsmuster von *S. solidus* in kanadischen und europäischen Populationen. Durch wechselseitige Infektionen von dreistachligen Stichlingen mit *S. solidus* vom gleichen oder einem anderen Kontinent, konnten wir zeigen, dass Süßwasserpopulationen erst vor kurzem eine Weltweit verbreitete Resistenz gegen *S. solidus*-Infektionen entwickelt haben. Diese Resistenz entstand während der Kolonisation neuer Süßwasser-Lebensräume durch ursprünglich marine Populationen. An diese Populationen hat sich *S. solidus* wiederum durch die Fähigkeit lokale Stichlingspopulation zu infizieren angepasst. Das in den verschiedenen experimentellen Kombinationen beobachtete Muster von Anfälligkeit/Resistenz stellt eine Abweichung von den bekanntesten theoretischen Modellen für Wirt-Parasit-Wechselwirkungen, "gene-for-gene" und "matching-allele", dar. Wir haben dazu ein Hybrid-Modell vorgeschlagen, bei dem die Wirte zunächst eine globale Resistenz durch die Erkennung eines konservierten Parasitenmotivs (zielgerichtete Erkennung) entwickeln und Parasiten als Reaktion darauf mit unterschiedlichen lokalen Infektiositätsstrategien ("matching-allele") reagieren.

In meinem dritten Kapitel untersuche die genetische Basis der dreistachligen Stichlingsresistenz gegen *S. solidus* anhand von zwei Populationen. Durch experimentelle Infektionen und Genexpression (RT-qPCR)

wurde die differentielle Immun-Gen-Expression zwischen sympatrischen (koevolvierten) und allopatrischen (nicht-koevolvierten) Wirt-Parasit-Kombinationen an drei unterschiedlichen Zeitpunkten gemessen. Wir identifizierten unterschiedliche Raten der Wirtsausbeutung bei verschieden Infektionskombinationen, was die Bedeutung dieser Raten bei der Koevolution hin zu optimalen Parasitenvirulenzen und Wirtsresistenzen hervorhebt. Insbesondere sympatrische Kombinationen erreichten ein ähnliches optimales relatives Niveau hinsichtlich der Wirtsausbeutungrate. Im Gegensatz dazu führten allopatrische Kombinationen entweder zu einer übermässigen oder sehr geringen Beanspruchung des Wirts. Die sich zwischen den experimentellen Gruppen unterscheidenden Expressionsmuster von Immungenen wiesen eine Manipulation des Wirts-Immunsystems durch ihren Koevolvierten Parasiten nach. Diese Ergebnisse zeigen das komplexe Zusammenspiel von Parasit und Wirt durch das Wirtsimmunsystem während der Infektion. Koevolution begünstigte die lokale Anpassung sowohl der Wirts- als auch der Parasiten-Genotypen durch Selektion hin zu einer optimalen Wirtsimmunantwort.

In meinem vierten Kapitel habe ich untersucht, wie Parasiten-Parasiten-Konkurrenz die Ausprägung von Virulenz bei konkurrierenden Parasiten-Genotypen beeinflusst. Wir verwendeten einen sehr virulenten und einen weniger virulenten Stamm von *S. solidus*, um Parasitenvirulenz in homologen und heterologen Koinfektionen zu messen. Dabei haben wir festgestellt dass Virulenz stark genetisch determiniert ist, es jedoch auch eine plastische Dimension für dieses Merkmal gibt, da Virulenz auch von dem Koinfektions-Konkurrenten abhing. Diese Plastizität durch intra-Wirt-Interaktion könnte die Stärke der Wirts-Parasiten-Interaktion beeinflussen, da sie die phänotypische Plastizität zwischen verschiedenen Parasiten-Genotypen reduziert. Virulenz-Plastizität könnte also zur Beibehaltung von Virulenz Polymorphismen auf einer Meta-Populationsebene beitragen.

Diese Arbeit hebt die Komplexität der Faktoren hervor, die Wirt-Parasit-Interaktionen auf unterschiedlichen ökologischen und individuellen Ebenen im Modellsystem dreistachliger Stichling/*S. solidus* prägen. Im Einzelnen zeigen unsere Ergebnisse eine geographische Struktur von Interaktionen, da lokale Umweltfaktoren und koevolutionäre Geschichten die Voraussetzungen für die lokale und gegenseitige Anpassung von Wirt und Parasiten schaffen.

Introduction

1.1 On the ecological importance of parasites

Parasites can be broadly defined as micro- and macro-parasites (e.g. viruses, bacteria, protozoans and helminths) that inflict harm on their host (i.e. decrease their fitness), and need their host for one or all of the following- survival, reproduction and transmission. Parasitism is one of the most successful and ubiquitous lifestyle among living organisms. Not only the majority of species across all taxa are believed to be parasites, but virtually all free-living organisms are parasitized by several parasite species (Poulin 1996; Windsor 1998; Kuris *et al*. 2008). In the past thirty years, parasites have been increasingly recognized and studied as a key element in shaping ecosystem structure and functioning. The role of parasites in community dynamics, maintenance of biodiversity, and food web stability has led ecologists to consider a healthy ecosystem as one rich in parasites (Hudson, Dobson & Lafferty 2006; Wood *et al*. 2007; Lefèvre *et al*. 2009; Hatcher, Dick & Dunn 2012). Yet, some parasite strains can be highly virulent and threaten the ecological equilibrium (McCallum & Dobson 1995). With the spread of microbial resistance to antibiotics and the continuous emergence of new infectious diseases, understanding the determinants of host resistance and parasite virulence remains a major scientific challenge (Daszak, Cunningham & Hyatt 2000; Goldberg, Siliciano & Jacobs 2012).

1.2 Host-parasite coevolutionary dynamics

Parasites are a major ecological factor of selection. They live at the expense of their hosts, decreasing the host's fitness to various extents ranging from altered growth, fertility, to even death. Those adverse effects (virulence) act as an important force of selection, pushing hosts to evolve counter adaptive strategies (resistance), which urges the parasite's evolutionary response in return. The reciprocal fitness cost and adaptive pressure that hosts and parasites are exerting on each other can lead to fast antagonistic coevolutionary dynamics defined under "The Red Queen Hypothesis" (Van Valen 1973). Indeed, hosts and

parasites are engaged in an evolutionary arms race where they have to constantly adapt to stay competitive and survive, similarly to the Red Queen's race in Lewis Caroll's *Through the looking-Glass* (1871):
"Now, here, you see, it takes all the running you can do, to keep in the same place."

In this arms race, parasites are generally assumed to have an evolutionary advantage over their hosts as a result of shorter generation time and larger population size (Kaltz & Shykoff 1998; Gandon & Michalakis 2002). Because they adapt rapidly to their host, they are able to maintain their virulence, which can induce the escalation of the arm race. However, highly virulent parasites can induce the decline, or even the collapse of the susceptible host population. Since parasites interact and adapt more often with the most common host genotype, rare host genotypes can then be favoured and spread through the population. This frequency-dependent selection lead to fluctuating dynamics in which different genotypes of parasite and host alternate, maintaining genetic polymorphism (Anderson & May 1982). The outcomes of infections leading to coevolutionary feedbacks, depends primarily on the interaction between host and parasite genotypes (G_H x G_P) but several other factors influence the trajectory of this coevolutionary race (Lambrechts, Fellous & Koella 2006; Wolinska & King 2009; Rigaud, Perrot-Minnot & Brown 2010). In the following sections, I will introduce a few of these factors, specifically: i) intrinsic trade-offs in virulence and resistance; ii) environmental variability of ecological processes; and iii) within-host competition.

1.3 Virulence and evolutionary trade-off

The study of host-parasite interactions provides a theoretical framework to understand the evolution of parasite virulence (Sorci, Møller & Boulinier 1997). The paradox of why parasites harm their hosts if they depend on them for survival and transmission, first led scientists to view host-parasite interactions as the first step leading toward commensalism, or even mutualism (Ewald 1987; Leung & Poulin 2008). According to this early paradigm of the "avirulence hypothesis", only recent host-parasite associations were maladapted and

suffered from high virulence; to avoid driving host population to extinction and endure, parasite strains should progressively become avirulent (May & Anderson 1983). This "conventional wisdom", as coined by May & Anderson (1983), is supported by natural cases in which pathogens, benign to their coevolved hosts, are extremely virulent in recently introduced hosts but lessen their virulence over time to reach an endemic equilibrium (Bull 1994; Daszak *et al*. 2000; Berngruber *et al*. 2013). However, inter-host competition especially when parasite genotypes of different virulence are in direct competition, selects for a level of virulence that maximizes individual fitness and could maintain a certain level of virulence (Bremermann & Pickering 1983; Lenski & May 1994). Indeed, as virulence is often a necessity for parasite fitness via host infection and exploitation, virulence evolution started to be viewed as a compromise between parasite virulence and transmission (Alizon *et al*. 2009). Under the 'trade-off hypothesis', parasites are facing a trade-off between virulence (which allows for host exploitation) and transmission (which relies on sufficient host population density). In this theoretical framework, parasites should evolve an intermediate level of virulence ensuring an optimal level of host exploitation without compromising future transmission (Anderson & May 1982; Ewald 1983; Alizon *et al*. 2009).

The transmission modes and dependence of pathogens on hosts as disease reservoirs and vectors is variable. Non-vector-borne parasites can persist in the environment and rely less on host presence for their transmission (Ewald 1983, 2011). The "sit-and-wait" hypothesis predicts that such parasites could afford to be more virulent (Bonhoeffer, Lenski & Ebert 1996; Walther & Ewald 2004). On the contrary, for parasites with complex life cycles (which require the encounter of multiple hosts from different species) or vertical transmission (from mother to offspring), high virulence can limit the transmission success to the next host, in particular when it induces mortality of susceptible hosts (Yamamura 1993; Davies, Fairbrother & Webster 2002).

An emblematic case of attenuated virulence through host-parasite interactions is the case of the Myxoma virus introduced as a regulating agent in the European rabbit populations (Genus *Oryctolagus*) of Australia in 1950 (Best & Kerr 2000). Quite innocuous for its native populations of American rabbits (Genus

Sylvilagus), the Myxoma virus was highly lethal when first introduced in the naïve Australian population, initially killing >99% of infected hosts. It rapidly spread and dramatically reduced rabbit numbers, enforcing selection for less virulent strains inducing less morbidity and longer survival time (Fenner & Ratcliffe 1965; Best & Kerr 2000; Kerr *et al*. 2012). But shortly after introduction, the Myxoma strain with intermediate virulence (grade 3) became predominant. In part because it could outcompeted more virulent strains at the population level, as longer host survival favoured more efficient diseases transmission by its biting arthropod vectors (mosquitoes, fleas, *etc*.); but also because rabbit hosts evolved better resistance (Fenner 1983; Best & Kerr 2000).

1.4 The cost of host resistance

Host defences can take several forms including avoiding infection, reducing pathogenicity, elimination of pathogens and recovery from infections, to tolerance, or acquired immunity (Boots & Bowers 2004). Host immunocompetence, the ability of an individual to prevent and control an invasion by a parasite, is shaped by host-parasite interactions (Owens & Wilson 1999). However, immunocompetence is a costly trait imposing a trade-off in energy allocation on other important life history traits, namely growth, maintenance and reproduction ("immunocompetence handicap hypothesis"; Reznick 1992; Roberts *et al*. 2004). For example, in addition to the direct physiological cost of initiating and maintaining an immune response (energy, protein, nutrients), there are also indirect costs like immunopathology (oxidative stress, etc.) or autoimmune diseases (recognition of self as non-self) (Costantini & Møller 2009; Graham *et al*. 2010). The cost of immunity has been demonstrated for various life-history traits in many organisms (see the following reviews for examples: Lochmiller & Deerenberg 2000; Zuk & Stoehr 2002) and is most likely key in shaping the evolution of host immune defences.

Hence, host resistance should evolve, be maintained and expressed only if its benefits outweighed its cost, i.e. if the risk and the cost of parasitism are high (Lindström *et al*. 2004; Tschirren & Richner 2006). Like parasite virulence, host resistance is expected to evolve towards an optimum level shaped by the

frequency and intensity of host-parasite interactions, but also by intrinsic factors like life span and environmental factors like resources availability (Miller, White & Boots 2007; Houston *et al*. 2007).

1.5 Host-parasite local adaptation

Through the intrinsic cost of adaptation, host-parasite coevolution should lead to optimum levels of both resistance and virulence. However, natural populations do exhibit variability in host resistance and parasite virulence. This indicates that there might not be one universal optimal level of virulence and that external forces also shape the direction of the arms race. In fact, host-parasite coevolution is a potent force in creating and maintaining natural diversity (Clarke 1979; Eizaguirre & Lenz 2010). Variation in environmental factors creates spatial and temporal heterogeneity in species distribution and interaction (Blanford *et al*. 2002; Parratt, Numminen & Laine 2016). Through its cost, host resistance should be tailored to the parasitic pressure faced in a specific environment; different habitats presenting distinct parasite communities can act as a divergent ecological factor of selection for host defences (Auld *et al*. 2013). This can lead to local adaptation (i.e. specific genotypes perform better locally than away) in host resistance, parasite infectivity/virulence, or both (Kaltz & Shykoff 1998; Kawecki & Ebert 2004).

However, patterns of local adaptation are not always detected and local adaptation, no local adaptation, or maladaptation are observed in nature and laboratory populations (Kaltz & Shykoff 1998; Greischar & Koskella 2007; Hoeksema & Forde 2008). The "geographic mosaic theory of coevolution" suggests that the strength of natural selection varies in different geographical populations creating evolutionary hot spots with strong reciprocal antagonistic selection, and cold spots with no selection on one or both interacting species (Thompson 1999). Gene flow among such structured populations could influence local and overall host-parasite coevolutionary dynamics (Forde, Thompson & Bohannan 2004). Meta-analyses have identified three important factors in systems most likely to present local adaptation: i) gene flow (high gene flow can disrupt the fixation of adaptive genes in the host, but provides new adaptive

alleles for the parasite if gene flow is higher compared to its host); ii) specificity (i.e. specialist vs. generalist parasite); and iii) intensity of the interaction (high prevalence and virulence) (Gandon & Michalakis 2000; Lajeunesse & Forbes 2002; Greischar & Koskella 2007; Hoeksema & Forde 2008). Consequently epidemiology (pattern of disease prevalence and intensity) reflects the complex interaction of ecological processes, host-parasite interactions and coevolutionary history (Penczykowski *et al.* 2016, and see fig. 1 where I summaryzed these concepts).

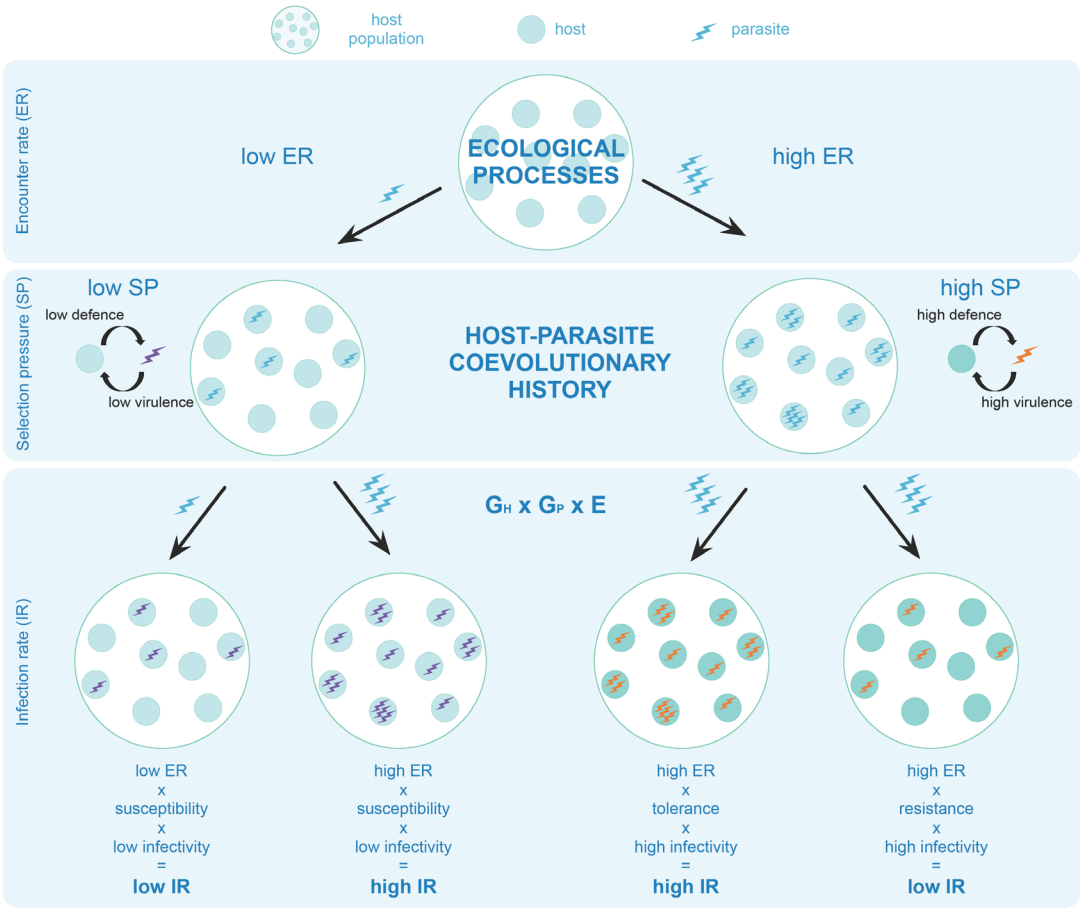

Figure 1

Ecological processes (environmental conditions, resource availability, diversity of parasite community, parasite transmission strategy, *etc*.) determine host and parasite **encounter rate**, which can vary in different host populations. Divergent strengths of selection pressure in different host populations leads to different **coevolutionary histories** selecting for different resistance-virulence optima. Ultimately, host genotype x parasite genotype x environment interactions (G_H **x** G_P **x E**), which determine encounter rate, parasite infectivity and host defences, are responsible for the patterns of infection observed in natural populations.

1.6 Within-host parasite interactions

While individual interactions between host and parasite are often studied to determine the ecological and evolutionary determinants of virulence, in nature host are rarely infected with a single parasite (Petney & Andrews 1998; Rigaud *et al*. 2010). At the individual host level, several parasite species and genotypes can interact and compete within the same host for space, resources or manipulation. Within the host, higher virulence can be associated to higher replication or growth rates through higher exploitation of host resources by one or several parasites. The expression of virulence might differ in the context of single or multiple infections, and this can impact the evolution of virulence (Bremermann & Pickering 1983; Bull 1994; van Baalen & Sabelis 1995). Surprisingly both in theory and in practice, multiple infections have the potential to direct virulence evolution toward both increased and decreased virulence (Cressler *et al*. 2016).

Following kin selection theory, relatedness can influence the outcome of within-host interactions: mixed infections with low relatedness promote competition, while high relatedness promotes cooperation (Lewontin 1970; Bremermann & Pickering 1983; Ewald 1983; Chao *et al*. 2000; Buckling & Brockhurst 2008). Davies *et al*. (2002) tested this theoretical assumption and found that mixed-strain schistosome infections in snails did show higher virulence than single-strain infections. Indeed, direct competition between parasites can give a competitive advantage to more virulent genotypes, and thus select for increased virulence (Nowak & May 1994; Levin & Bull 1994; Bull 1994; Frank 1996; de Roode *et al*. 2005). But competition could also favour virulence plasticity, when parasites can adjust their host exploitation upon detection co-infecting parasite (Gower & Webster 2005; Choisy & de Roode 2010; Leggett, Brown & Reece 2014). On the other hand, cooperation through collective action can take the form of prudent host exploitation and select for decreased virulence; or on the contrary, cooperative exploitation of more host resources which lead to increased virulence (Chao *et al*. 2000; Leggett *et al*. 2014, and see fig.2 and Box 1 where I summaryzed these concepts). A well-studied example of pathogen cooperation is the production of siderophores by bacteria. Those iron-scavenging molecules give a collective benefit by enhancing bacterial growth in

iron-limited environment but represent a metabolic cost for the producers (West & Buckling 2003; Buckling & Brockhurst 2008).

Just like among-host interactions, within-host interactions can be subjected to fluctuating dynamics as cooperation through public goods production (such as siderophores) is vulnerable to cheater genotypes (Griffin, West & Buckling 2004; Harrison *et al*. 2006; Sandoz, Mitzimberg & Schuster 2007), and competitive interference (such as bacteriocins) will selects for resistant genotypes (Gardner, West & Buckling 2004; Mideo 2009). Accordingly, parasite within-host interactions are probably contributing to the maintenance of parasite diversity and deserve to be more closely investigated (Bashey 2015). Within-host interactions and dynamics are often studied experimentally in the context of parasites that multiply within their host such as microbial pathogens as it allows comparing the relative abundance of different genotypes in mixed infections (Gower & Webster 2005; de Roode *et al*. 2005). But parasites are extremely diverse in their modes of transmission, life cycle and reproduction. And life history-traits, such as whether parasites replicate asexually, sexually or only grow in their host, surely should affect the type of pressures, interactions and strategies developed by co-infecting parasites (Barrett *et al*. 2008). Replicating parasites can readily mutate or adapt to host immunity, and increased virulence might arise from within-host selection without long term adaptive value (short-sighted evolution, Levin & Bull 1994). For example, parasites with complex life cycles that use hosts only to optimize growth and transmission might respond differently to within-host competition.

Multiple infections can also affect the evolution of host resistance as defences might act differently against single and multiple infections. For instance, host immunity can be overwhelmed by mixed infection as it pays a higher cost of mounting a response against genetically diverse parasite infections, which can facilitate other infections (Bull 1994; Alizon 2008; Choisy & de Roode 2010; Telfer *et al*. 2010). Host immunity could also discriminate and attack parasite genotypes differentially, creating apparent competition or concomitant immunity (Cox 2001; Choisy & de Roode 2010; Benesh & Kalbe 2016). Thus, within-host interactions influence host resistance evolution and have important implications on host-parasite interactions. This highlights the complexity of the ecological and evolutionary determinants of virulence and

resistance, and how both between- and within-host scales play a role and need to be studied to understand host-parasite interactions and disease dynamics in any systems (Telfer *et al*. 2010; Susi *et al*. 2015; Cressler *et al*. 2016).

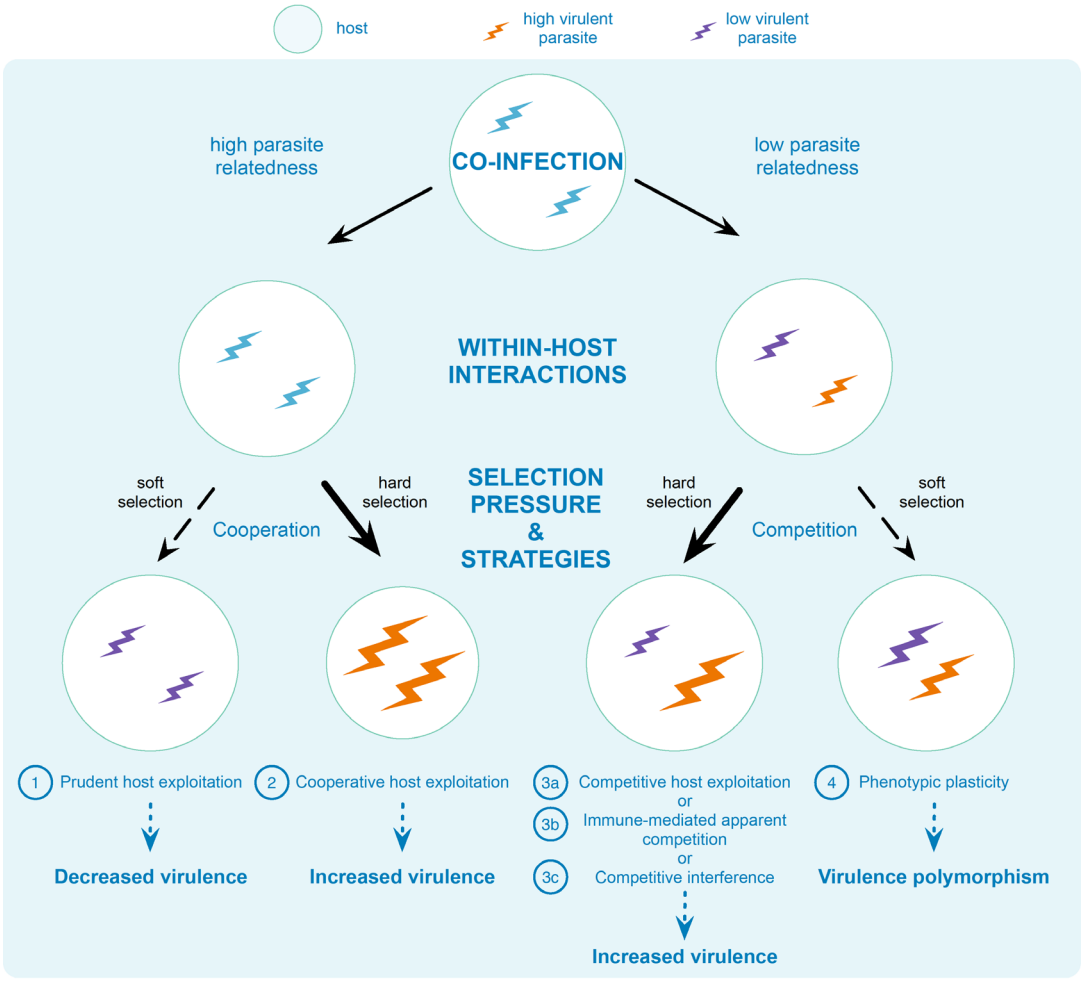

Figure 2

Box 1 & Figure 2

Depending on the **relatedness** of co-infecting parasite, different interactions strategies can emerge. High relatedness will select for cooperative strategies through kin selection, while low relatedness will promote competition between co-infecting parasites. The following strategies are possible under either **hard** or **soft selection**:

Cooperative strategies

1) Prudent host exploitation: co-infecting parasites cooperate to decrease their individual host exploitation for the benefit of each other (self-restraint), which increase inclusive fitness in compensation.

2) Cooperative host exploitation: co-infecting parasites cooperate to increase their host exploitation, for instance via public goods production*****.

Competitive strategies

Resources, host immune response, or direct interference could mediate within-host competition (Read & Taylor 2001; Mideo 2009):

3a) Competitive host exploitation: co-infecting parasites compete to access host resources without self-restraint, with the more virulent parasite out-competing the low virulent one.

3b) Immune-mediated apparent competition: the host immune response is more efficient at containing the low virulent parasite and/or the more virulent parasite is more efficient at evading the host immune response (indirect interaction via the host immune system).

3c) Competitive interference: direct inhibition of low virulent parasite by more virulent parasite (direct interaction between co-infecting parasites).

4) Phenotypic plasticity: increase of virulence and competitive ability upon the detection of co-infecting parasites.

In theory, these different outcomes can lead to the evolution of decreased virulence (**1**), increased virulence (**2**, **3a**, **3b**, **3c**) or maintenance of virulence polymorphism (**4**). In extreme cases, cooperative exploitation and competition can lead to over-exploitation with depletion of host resources or even premature death of the host, creating an ecological dead-end (short-sighted evolution of increased virulence, Levin & Bull 1994).

Public goods: compounds with an individual cost of production but which procure collective benefits in host exploitation.

1.7 The model host: three-spined stickleback

1.7.1 Rapid adaptation to new environment

The three-spined stickleback (*Gasterosteus aculeatus*) is a small teleost fish widely distributed through the temperate Northern hemisphere in a range of coastal marine, anadromous and freshwater habitats. It has been extensively used as a model organism in behavioural ecology (Milinski & Bakker 1992; Barber, Hoare & Krause 2000; Jennions & Petrie 2007), ecological genetics (Colosimo *et al*. 2005; Jones *et al*. 2012; Feulner *et al*. 2015), and evolution (Rundle & Nosil 2005; Gibson 2005), in particular because of its astonishing array of morphological and genetic diversity. Indeed, after the last Pleistocene glaciation ca. 12000 years ago, sticklebacks have undergone rapid ecological radiation by repeatedly colonising newly accessible freshwater habitats from estuarine and marine refuges (McKinnon & Rundle 2002). In response to ecological variation (hydrodynamic conditions, predation risk, feeding regime, etc.), freshwater three-spined stickleback diverged morphologically (body armour, pigmentation and general form) and ecologically (behaviour, benthic/limnetic morph, *etc*.) into different ecotypes constituting a species complex.

1.7.2 Parasite mediated selection

The rapid habitat-specific adaptation of sticklebacks highlights their extreme adaptability to fast changing environment, which makes them particularly suitable to study fast adaptation processes like host-parasite coevolution (Bell, Aguirre & Buck 2004; Gelmond, Von Hippel & Christy 2009; Barber 2013). Three-spined sticklebacks have a well-characterized and diverse macro-parasite fauna with divergent parasite communities found in different ecotypes (Kalbe 2002; Wegner, Reusch & Kalbe 2003; Scharsack *et al*. 2007a; Poulin *et al*. 2011; De Roij & MacColl 2012; Barber 2013). For instance, in Northern Germany, habitat-specific parasite mediated selection plays an important role in the ecological divergence and speciation of three-spined stickleback populations from lake and stream habitats (Reusch, Wegner & Kalbe 2001b). The immunocompetence adaptation to specific parasite pressure

constitutes a mating barrier that limits the gene flow between neighbouring populations of distinct ecotypes (Eizaguirre *et al*. 2011, 2012).

This offers the perfect ground to study host local adaptation in natural populations exposed to divergent selection pressures from different parasite communities. Moreover, three-spined sticklebacks are easy to breed and maintain in the laboratory, which allow the exposition of naïve hosts in controlled experimental infections (Barber & Scharsack 2010). Due to its involvement as a model species in other fields, stickleback's ecology and biology are well documented. This means the availability of a sequenced genome and many molecular tools that allow to use molecular approaches such as population genetics and gene expression measurements, in particular for immune assays, in the field and the laboratory on a vertebrate model (Peichel *et al*. 2001; McKinnon *et al*. 2004; Robertson, Bradley & MacColl 2016).

1.8 The parasite model, part 1: *Schistocephalus solidus*

1.8.1 Host specificity implies close coevolution

The tapeworm *Schistocephalus solidus* (Müller, 1776) is a trophically transmitted pseudophyllidea cestode with a three-host complex life cycle (fig 3) and an ideal system to study host-parasite reciprocal adaptation. *S. solidus* virulence is expressed through resource exploitation and manipulation of its intermediate hosts. While *S. solidus* can infect several cyclopoid copepod species (as first intermediate host) and fish-eating bird species (as final host), it is highly specific to its second intermediate host, the three-spined stickleback (Bråten 1966; Orr, Hopkins & Charles 1969; Henrich & Kalbe 2016). In its obligatory fish host, *S. solidus* completes its entire growth and survives for several weeks until the fish is eaten by the final host or dies. To become infective to its final host, *S. solidus* needs to reach a weight threshold of 50 mg and can represent between 20 and 50% of the host weight (Arme & Owen 1967). Indeed, *S. solidus* reproductive success is directly correlated to the size reached in the fish host, as potential sexual partners tend to prefer larger worms and

reproductive output increases with worm size (Wedekind, Strahm & Schärer 1998; Lüscher 2002).

The spectacular size of the tapeworm is not without consequences for the fish host. Not only successful transmission to the final host is inevitably lethal, but parasite growth can severely limit competitive abilities and reproductive success of infected fish (Barber & Ruxton 1998; Barber 2005; Heins & Baker 2008). Large parasites can limit fish food consumption, and restrict the egg production in female, even leading to castration in some populations (Milinski 1984; Barber & Huntingford 1995; Heins & Baker 2003; Heins, Birden & Baker 2010b).

Infected sticklebacks also display signs of parasite phenotypic and behavioural manipulation that increase transmission to the final host (Hammerschmidt & Kurtz 2005b; Barber & Scharsack 2010; Barber 2013). This strong antagonistic interaction between *S. solidus* and sticklebacks is likely to lead to fast reciprocal adaptation through coevolution. In particular, *S. solidus* is likely to interact closely with the fish immune system during its establishment and long survival in the fish body cavity. While an independent activation of the host innate immunity can clear the infection in its early stages, in general *S. solidus* seem to evade this immune response, potentially through mimicry or immune manipulation (Wedekind & Little 2004; Hammerschmidt & Kurtz 2005a; Scharsack, Koch & Hammerschmidt 2007b). Multiple infections with worm of different sizes (i.e. from different infection events) are common in nature and highlights that previous infections do not immunize the host from re-infection (Heins, Baker & Martin 2002; Jäger & Schjørring 2006). As parasite burden is so tightly linked to host exploitation and virulence, relative parasite size can be used as a proximal measure of both parasite virulence and host resistance.

1.8.2 The pace of the evolutionary race

In nature, sticklebacks are generally infected in one single wave of *S. solidus* (McPhail & Peacock 1983; Tierney, Huntingford & Crompton 1996; Heins, Baker & Green 2011). Indeed, copepods are small prey items preferred by juvenile sticklebacks of the year which are therefore preferential exposed to the parasite (Christen & Milinski 2005). Sticklebacks can live up to 3-4 years in the

wild but reproduce mostly annually as several studies report that only a small portion of individual survive to their second autumn (Baker 1994; Clavero, Pou-Rovira & Zamora 2009). As a consequence, *S. solidus* and its specific host have similar generation time, and thus similar potential for adaptation. This allow for selection of optimal host resistance and parasite virulence through concomitant reciprocal adaptation (Kalbe *et al*. 2016).

1.8.3 Heterogeneous distribution of S. solidus *infections*

S. solidus is commonly found in natural three-spined stickleback populations, but infection incidence, intensity and virulence vary spatially and temporally (Wootton 1976; MacColl 2009; Morozińska-Gogol 2011; De Roij & MacColl 2012). The factors shaping the distribution of *S. solidus* infections are likely to be environmental. For instance, *S. solidus* eggs and the first coracidium larval stage are viable in salinities of up to 12.5‰ which restrains the distribution of infections to freshwater and brackish water stickleback populations; particularly lake habitats where the abundance of cyclopoid copepods and bird predation insure the completion of the life cycle (Confer *et al*. 2012; Simmonds & Barber 2016). And finally, infected sticklebacks exhibiting a conspicuous belly or demelanization, motility issues, and reduced anti-predator behaviour due to parasite manipulation have an increase predation risk not only by fish-eating birds but likely by predatory fish as well (Giles 1987; Ness & Foster 1999; Blake, Kwok & Chan 2006). High prevalence of predatory fish can hinder successful transmission to the final host and is an ecological dead-end for the completion of *S. solidus* life cycle. As a matter of fact, in populations where predatory fish have been recently introduced, *S. solidus* infections often decline in the following years (Jakobsen, Johnsen & Larsson 1988).

Those different ecological contexts have the potential to create different coevolutionary histories and send the host-parasite interactions on different trajectories, sticklebacks populations differing in *S. solidus* epidemiology offers an excellent opportunity to study host-parasite interactions. While *S. solidus* populations can be genetically structured, the dispersal of genotypes across long distances by the final host should ensure high gene flow between distant populations, which is a factor of local adaptation (Sprehn *et al*. 2015; Kalbe *et*

al. 2016). Additionally, within-host interactions might play a role in disease epidemiology and evolution, as it is not uncommon to find fish infected with multiple worms. And as a manipulative parasite, it is likely that at least some interactions between co-infecting tapeworms could be mediated by the host immune system, which makes *S. solidus* a promising model to study apparent competition.

1.8.4Complete life cycle in the laboratory

Finally, *S. solidus* can easily be cultured in the laboratory and used in controlled experimental infections. The complete life cycle of *S. solidus* can be replicated by infecting the intermediates hosts and using an *in vitro* breeding system that replaces the final bird host (Smyth 1946; modified by Wedekind 1997). Procercoids freshly extracted from the fish body cavity are placed in a mesh bag in suspension in a medium and continuously shake at +40°C. This mimics the condition of the digestive tract of the final host and after 48h worms start to produce eggs that can be collected, rinsed and stored at +4°C for long periods. These proceedings allow to breed and to maintain specific *S. solidus* strains in the laboratory. Another advantage of the system is the convenience of measuring the relative weight of the tapeworm (parasite index) to assess its individual host exploitation and virulence (Arme & Owen 1967; Kalbe *et al*. 2016).

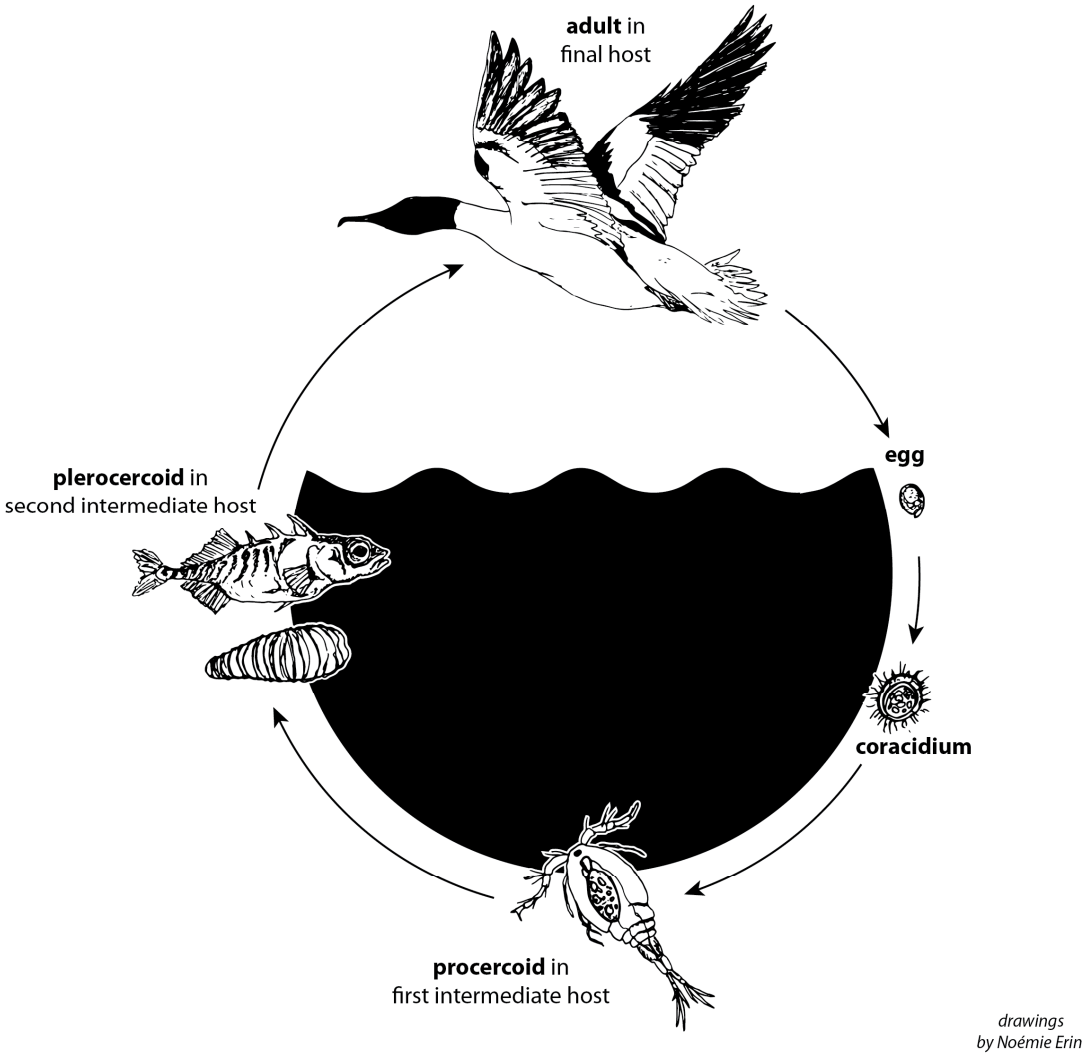

Figure 3

The life cycle of *Schistocephalus solidus* starts when birds infected with mature tapeworms disperse *S. solidus* eggs in their faeces. After two to three weeks in freshwater, the eggs hatch and release the free-swimming larval stage, **coracidium**, which has to be ingested by **cyclopoid copepods** within a few hours to continue the cycle. Once established in the copepod's haemocoel, *S. solidus* will develop into the **procercoid** larval stage and become infective to the next host within a few days. When a **three-spined stickleback** (*Gasterosteus aculeatus*) feed on an infected copepod, the procercoid penetrates the gut wall to establish in the fish body cavity. Through this process, the procercoid shades it outer tegument and develop into a **plerocercoid** larva which will grow for several weeks in its intermediate host before becoming infective to its final host. Fish-eating birds preying on infected sticklebacks will complete the cycle, allowing the simultaneous hermaphrodite tapeworms to reproduce sexually (by selfing or cross-fertilization) in the bird digestive tract.

1.9 The parasite model, part 2: *Diplostomum pseudospathaceum*

To a lesser extent than *S. solidus*, I also used the eye fluke *Diplostomum pseudospathaceum* (Niewiadomska, 1984) as a model to investigate fish innate immune defences in chapter 1. It is a digenean trematode able to infect a large range of host species at each step of its three-host complex life cycle (fig. 4). Multiple infections, i.e. when several cercariae (the fish-infecting larval stage) reached and established in the same fish eye lens, are common in nature. By inducing cataract formation, eye flukes increase the host predation risk and the chance of transmission to the final host (Crowden & Broom 1980; Seppälä, Karvonen & Valtonen 2004). Multiple infections can be a selected trait in this parasite species, while the parasite mediated increased risk of predation should be strongly selected against in the fish host population (Kalbe & Kurtz 2006). In the second intermediate fish host, the cercariae have to reach the immune privileged region of the eye lens within 24h to establish successfully in the host (Whyte, Secombes & Chappell 1991). Thus *D. pseudospathaceum* cercariae infecting a naïve fish host should only be subjected to the innate immune system and the efficiency of this immune response should be reflected through prevalence and parasite load (Kalbe & Kurtz 2006; Wegner, Kalbe & Reusch 2007; Rieger *et al.* 2013; Haase *et al.* 2014). Naturally infected snails can easily be used to retrieve cercariae clones for controlled experimental infections in the laboratory. After extraction of the eye lens of exposed fish, metacerariae are visible and can be counted to assess the precise infection rate.

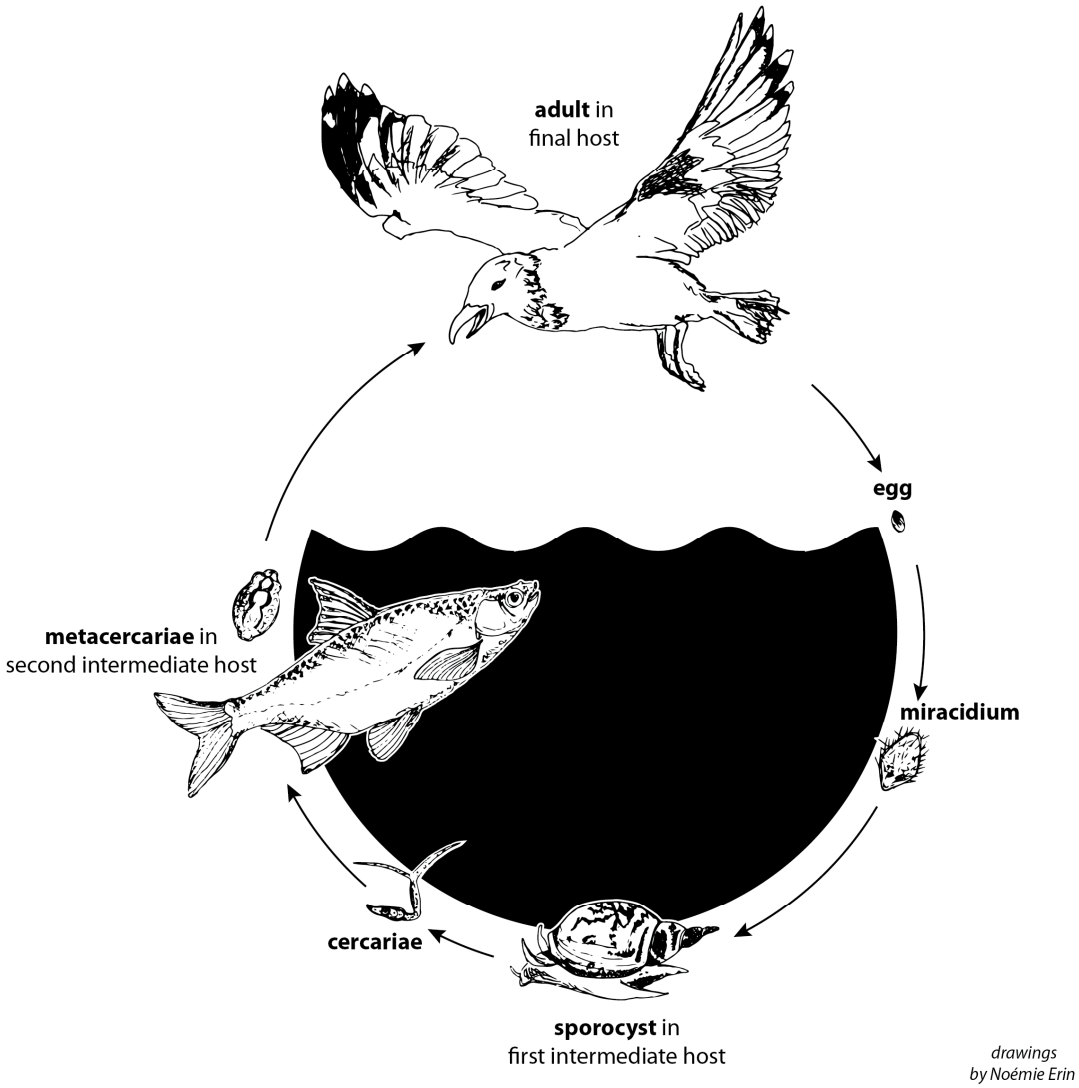

Figure 4

The life cycle of *Diplostomum pseudospathaceum* starts when infected birds release eggs into water through their faeces. When they hatch, the free-swimming **miracidium** larvae infect the first intermediate host, the pond snail *lymnaea stagnalis*, to grow and multiply asexually (**sporocyst**); *D. pseudospathaceum* is highly specific to its first intermediate host. Infected snails release **cercariae** clones that can infect a large range of fish species as second intermediate host by penetrating the fish skin and migrating through the tissues to the eye lens where they establish, feed on crystallized proteins and develop into **metacercariae**. *D. pseudospathaceum* is able to mature and reproduce sexually in the intestine of fish-eating birds.

Thesis outline & authors contributions

The aim of my thesis was to explore the determinants of host resistance and parasite virulence in the model system three-spined stickleback-*Schistocephalus solidus*. I assessed underlying mechanisms of host-parasite coevolution by comparing different locally adapted populations that took divergent evolutionary routes. This thesis is organized in four independent chapters. From field studies to controlled experiments in the laboratory, I (and my co-authors) investigated the following aspects of the host-parasite coevolution of my model system:

1) Chapter 1: Relaxed parasite-mediated selection reduces resistance and limits gene flow between lake and river stickleback ecotypes. How divergent parasite-mediated selections can select for different level of host immunocompetence in natural three-spined stickleback populations? In Chapter 1, we investigate the logical corollary of the cost of immunocompetence, i.e. that in the absence of parasite-mediated selection, host should reduce their energy investment in the costly arms race against parasite and consequently decrease their immunocompetence (Auld *et al*. 2013). Using a combination of empirical and experimental studies on a fish population naturally devoid of macroparasites, we look at the effect of adaptation to a macroparasite-free environment on the ability of fish migrants to invade a high parasite-pressure environment.

Chapter 1 - Author contributions*: NIE and MK designed research. **NIE**, TH, IES, PJJ and MK collected field data; **NIE** and MK performed experiments; **NIE** analysed data; **NIE** drafted the manuscript; **NIE**, TH, IES, PJJ and MK revised and contributed to the final manuscript.

2) Chapter 2: Resist globally, infect locally: A transcontinental test of adaptation by stickleback and their tapeworm parasite. What is the relative importance of environmental factors (constraining species distribution and

encounter rates), and host-parasite coevolution (susceptibility/infectivity of populations) on epidemiology? In Chapter 2, we explore determinants of *S. solidus* infection patterns by using trans-continental cross-infections of host population of western Canada and northern Europe with differing infection abundance.

Chapter 2 - Author contributions*: JNW, MK, **NIE**, DIB and designed research; JNW, **NIE,** MK and NS, LM, KCS performed experiments; JNW analysed data; JNW and DIB interpreted the data and wrote the manuscript.

3) Chapter 3: Differences in host immune response in reciprocal sympatric and allopatric parasite infections. What is the genetic basis of host resistance in two differentially adapted populations with similar optimal level of *S. solidus* virulence and three-spined stickleback resistance? In chapter 3, we investigate the genetic determinants of host resistance in two populations that have reached the same resistance/virulence optimum through different coevolutionary routes. In experimental combinations of sticklebacks infected with sympatric (coevolved) and allopatric (non-coevolved) *S. solidus*, we compare the differential expressions of eight immune genes (RT-qPCR) involved in mounting the innate and adaptive response against parasites in two host immunologically relevant organs (head-kidney and spleen).

Chapter 3 - Author contributions*: MK, IES, and PJ designed research; MK and IES performed experiments. **NIE** performed RT-qPCR assays; **NIE** analysed data; **NIE** drafted and wrote the manuscript.

4) Chapter 4: Intraspecific within-host parasite competition alters virulence in a fish-cestode system. What is the effect of within-host competition on the expression of *S. solidus* virulence? In Chapter 4, we compare the competitive ability in the stickleback host of two parasite strains of differing virulence in controlled experimental co-infections.

Chapter 4 - Author contributions*: NIE, TH, and MK designed research; TH, **NIE**, LP and MK performed experiments; TH, **NIE** and LP analysed data; **NIE**

interpreted the phenotypic results; TH drafted the manuscript; **NIE**, TH, LP and MK wrote the manuscript.

Table O1: Summary of authors' contributions to the different chapters

Chapter	Conception & design	Conducted the research	Data analysis	Interpretation & writing
1	**NIE**, MK	**NIE**, TH, IES, PJJ, MK	**NIE**	**NIE**, MK
2	JNW, MK, **NIE**, DIB	JSW, **NIE**, MK, NS, LM, KCS	JNW	JNW, DIB
3	MK, IES, PJJ	MK, IES, **NIE**	**NIE**	**NIE**
4	TH, **NIE**, MK	TH, **NIE**, LP, MK	TH, **NIE**, LP	TH, **NIE**, LP, MK

*Authors are given in alphabetical order:

Daniel I. Bolnick (DIB); **Noémie I. Erin (NIE)**; Tina Henrich (TH); Per J. Jakobsen (PJJ); Martin Kalbe (MK); Lei Ma (LM); Luke Phelps (LP); Irene E. Samonte (IES); Kum Chuan Shim (KCS); Natalie Steinel (NS); Jesse N. Weber (JNW).

1 Chapter 1

Relaxed parasite-mediated selection reduces resistance and limits gene flow between lake and river stickleback ecotypes

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Submitted manuscript

1.1 Abstract

Parasites are important selective agents that can shape species' distributions and gene flow amongst host populations by selecting for specific and local immunocompetence. To test for their role as a divergent selective pressure, we used a combination of field data and controlled experimental infections of three-spined sticklebacks (*Gasterosteus aculeatus*) from a Norwegian lake-river system with contrasting parasite prevalence. In this system, the river population is remarkably devoid of macroparasites. As a result, river fish have lower parasite resistance yet are in better general condition compared to the lake population. This contrast is revealed when laboratory-bred river and lake fish are experimentally exposed to a generalist trematode parasite, *Diplostomum pseudospathaceum* and a host-specific cestode, *Schistocephalus solidus*. A waterfall between the two habitats creates unidirectional migration between the two populations. Although we detect ~10% of first generation immigrants in the river below the waterfall, the two populations remained genetically distinct over four years of survey. These results suggest strong parasite-mediated selection against river immigrants in the habitat below the waterfall. Our results support the possibility of reproductive isolation induced by local adaptation to different parasite pressure between lake and river stickleback ecotypes and further demonstrate the rapid action of parasite-mediated selection.

1.2 Introduction

Parasites exert strong selective pressures and play important roles in the maintenance of genetic and species diversity of their hosts (Clarke 1979; Hudson *et al*. 2006; Eizaguirre & Lenz 2010). As a result, hosts have to constantly evolve immune defences. In this evolutionary arms race referred to as the Red Queen dynamics (Van Valen 1973), host immunocompetence is shaped by host-parasite interactions (Owens & Wilson 1999). Immunocompetence, however, is costly and imposes trade-offs in energy allocation on other life history traits, such as growth or reproduction (Schmid-Hempel 2003). Further, there are indirect costs linked to immunopathology (e.g. oxidative stress) and autoimmune diseases (Graham, Allen & Read 2005). Hence, the selection on resistance is expected to relax in a host population experiencing a long-term absence of parasite infection, and resistance might decrease or be lost (Zuk & Stoehr 2002).

Trade-offs involved in parasite resistance can also influence the success of migrants, shaping dispersal patterns and gene flow (Schmid-Hempel & Ebert 2003). For example, studies of invasive species highlight the importance of host-parasite local adaptation and high immunocompetence in successful migration and colonization (Prenter *et al*. 2004). The 'enemy release hypothesis' connects the success of invasive species to the absence of their native predators, pathogens, and parasites (Colautti *et al*. 2004). And indeed, successful invasive species often have superior immune systems enabling them to deal with the novel parasites of the colonized habitat (Lee & Klasing 2004; Vilcinskas, Mukherjee & Vogel 2012). Conversely, hosts originating from parasite-free habitats are expected to suffer more than natives when migrating to a high parasite-pressure habitat, and consequently should fail to invade. Host-parasite coevolution can thus create a geographic mosaic of adaptation, restricting gene flow between locally adapted populations, leading to divergent evolution and potentially speciation (Thompson 1999; Eizaguirre *et al*. 2009a; Eizaguirre & Lenz 2010).

The three-spined stickleback (*Gasterosteus aculeatus*) is ideal to study the role of parasite communities in shaping population divergence as demonstrated by their ecological radiation into various ecotypes (Lavin &

Mcphail 1993; Bell & Foster 1994; Reusch *et al*. 2001b; Hendry & Taylor 2004; Eizaguirre *et al*. 2009a; Raeymaekers *et al*. 2010; Deagle *et al*. 2012). In particular, stickleback populations from lake and river habitats experience contrasting parasite communities with higher prevalence and diversity in lakes (Kalbe 2002; Eizaguirre *et al*. 2011; Feulner *et al*. 2015). Divergent parasite-mediated selection might reinforce reproductive barriers by decreasing migration success and gene flow between neighbouring populations (Reusch *et al*. 2001b; Scharsack *et al*. 2007a; Eizaguirre *et al*. 2011).

Here we focus on two three-spined stickleback populations of a Norwegian lake-river system. Part of the river presents the particularity to naturally be devoid of macroparasites. A waterfall creates a natural barrier and unidirectional migration from the low parasitized river towards a highly parasitized lake population. This system enables us to test whether relaxed parasite-mediated selection induces a loss of genetic diversity and superior condition - at the cost however of higher susceptibility in the parasite-rich lake habitat. Over four years of field survey, we assessed parasite pressure and gene flow between those populations. To validate field observations, we performed experimental infections using laboratory-bred fish and measured differences in resistance to a generalist parasite, the trematode *Diplostomum pseudospathaceum*, as well as to the highly host-specific cestode *Schistocephalus solidus*. We expected the macroparasite-free river fish to exhibit lower resistance, which prevents them from successfully invading the lake population upon migrating to this environment with higher parasite pressure.

1.3 Material and Methods

1.3.1 Field sampling and parasite screening

In the fall 2009 to 2013, we sampled three-spined sticklebacks from the Skogseidvatnet Lake and its headwater Orraelva River (Hordaland, Fusa, Norway, 60° 14' 38" N, 05° 54' 51" E; see fig. S1). A 6 m waterfall at approximately 1.5 km upstream of the lake generates unidirectional gene flow from the river to the lake. We therefore characterized three distinct sampling sites: the 'River Above' the waterfall (RA), the 'River Below' the waterfall (RB) and the lake (L) (see table S1). Each year, three-spined sticklebacks (1+ years old) were caught with minnow traps and dipnets, killed with an overdose of MS222 (tricaine methanesulfonate, 1 mg/ml) and sampled for DNA. We dissected a subset of fish and recorded standard body length (±0.01 mm), body weight, and weight of all internal organs (±0.1 mg). We screened eyes and all inner organs for macroparasites, according to a standard protocol (Kalbe 2002). In total 663 fish were collected and genetically analysed; 420 fish had their inner organs weighed and 389 out of 420 fish were screened for parasites (see table S1).

1.3.2 Fish genotyping

We extracted genomic DNA from the caudal fin using the DNeasy® 96 Blood & Tissue Kit (Qiagen, Germany). To assess population genetic structure and gene flow among the three sampling sites and across years, we estimated genetic variation at nine neutral microsatellite loci (*Gac1097, Gac1125, Gac4170, Gac5196, Gac7033, STN18, STN32, STN75, STN84*) (Largiadèr *et al*. 1999; Peichel *et al*. 2001). We amplified microsatellites (Kalbe *et al*. 2009) and performed the fragment analysis with GeneMarker 1.95 (SoftGenetics).

1.3.3 Population genetic diversity, differentiation and Bayesian structure

We assessed genetic diversity and differentiation among sampling sites for each year by calculating the number of alleles per locus (*A*), observed heterozygosity (*Ho*), expected heterozygosity (*Hs*) and pairwise-F_{st} values using

Arlequin 3.5 (Excoffier & Lischer 2010) and identified private alleles using CONVERT 1.31 (Glaubitz 2004).

We tested all microsatellite loci for neutrality within each sampling site using the default parameters on Lositan (Antao *et al*. 2008), and for deviations from Hardy-Weinberg equilibrium and for linkage disequilibrium at each sampling site for each year using Genepop 4.2 on the Web (Rousset 2008). We performed genetic clustering for each survey year independently and combined using STRUCTURE 2.3.4 (Pritchard, Stephens & Donnelly 2000). We ran admixture model, with 100,000 burn-in followed by 1,000,000 Markov-Chain Monte-Carlo simulations and 20 iterations for each K (K = 1-13 for the complete dataset or K = 1-4 for a given sampling year). Results were retrieved using STRUCTURE Harvester (Earl & VonHoldt 2012), CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007) and Distruct (Rosenberg 2003).

Lastly, we used GeneClass2 (Piry *et al*. 2004) to detect first generation immigrants and NewHybrids 1.0 (Anderson & Thompson 2002) to detect potential hybrids.

1.3.4 Fish lab-breeding

To generate laboratory-bred sticklebacks, we crossed Norwegian fish wild caught from the RA and L populations in fall 2012, kept in the lab under standardized conditions and bred in spring and summer 2013. To test for differences in parasite resistance and tolerance between Skogseidvatnet fish populations, independent pure river (RAxRA) and pure lake (LxL) first generation laboratory-bred fish families were generated. The same parents were also used to generate reciprocal hybrid families LxRA (river mothers) and RAxL (lake mothers). Hereafter, we refer to the classes of laboratory-bred fish (RAxRA, LxL, LxRA, RAxL) as 'genetic types' and to the hybrid families as 'RA-' or 'L-maternal hybrid' (LxRA and RAxL, respectively).

1.3.5Experimental infection with **Diplostomum pseudospathaceum**

To study innate resistance, we experimentally exposed laboratory-bred fish to the generalist parasite *Diplostomum pseudospathaceum* (Kalbe & Kurtz 2006). Cercariae reach within 24h the eye lens where they successfully establish, escape the immune system and develop into metacercariae (Whyte *et al*. 1991). The number of metacerariae in the eye lens serves as a proxy for the efficacy of the innate immunity (Scharsack & Kalbe 2014).

We created a mix of *D. pseudospathaceum* clones by pooling cercariae from five naturally infected *Lymnaea stagnalis* snails from northern Germany, collected in October 2013 (Plön, Germany, 54° 9' 42.17" N, 10° 22' 45.09" E). Around 10 fish each from five RA fish families, five L fish families, five LxRA fish families and four RAxL fish families were individually exposed to 100 cercariae (see table S2).

1.3.6 Experimental infection with Schistocephalus solidus

To assess specific immunity, we experimentally exposed laboratory-bred fish to the cestode *Schistocephalus solidus*. *S. solidus* is highly specific to the three-spined stickleback; it completes almost its entire growth in the body cavity of the fish until it is eaten by the final bird host. While the innate immune system can clear early infections within the first two weeks after exposure (Scharsack *et al*. 2007b), parasite growth in the fish host depends on the host adaptive immune genetic diversity (Kurtz *et al*. 2004).

We used six *S. solidus* tapeworms collected from naturally infected three-spined sticklebacks from the Skogseidvatnet lake population to produce three independent *S. solidus* laboratory-bred families in an *in vitro* system (Smyth 1946; Wedekind 1997). We bred parental worms in size-matched pairs (Lüscher & Milinski 2003). Laboratory cultured copepods (*Macrocyclops albidus*) were singly exposed to one coracidium (for details see Scharsack *et al*. 2007b) and checked for infection under the microscope. Each fish was individually exposed to a single procercoid. To control for family effects, we used a balanced design and exposed around 30 fish each from three RA fish families and three L fish families to each of the three *S. solidus* families. As a control group, around 10 fish from each family were handled similarly to exposed fish but were not given an

infected copepod (see table S3). Due to insufficient sample sizes, no RAxL and LxRA hybrids could be used in the *S. solidus* experimental infection.

1.3.7 Fish dissections

At 4 and 8 weeks post-exposure, for *D. pseudospathaceum* and *S. solidus* exposures respectively, fish were sacrificed with an overdose of MS222 and dissected following the same protocols as the dissection in the field. Metacercariae of *D. pseudospathaceum* were counted in each eye lens, and plerocercoid weight was recorded for *S. solidus*.

1.3.8 Fish condition parameters

We calculated the fish condition factor (CF) to assess individual condition (Frischknecht 1993). As an estimator of energy reserves, we used the hepatosomatic index (HSI) which is the ratio of fish liver weight to body weight (Wootton, Evans & Mills 1978). The splenosomatic index (SSI) is the ratio of fish spleen to body weight. An enlarged spleen can indicate immune activation in response to parasite infections (MacNab, Katsiadaki & Barber 2009).

1.3.9 Parasite index

As a measure of *S. solidus* virulence, we calculated the parasite index (PI) as the relative weight of the worm over the somatic weight of the fish (Arme & Owen 1967), which can be used as an estimate of the efficiency of host adaptive immunity (Kurtz *et al.* 2004).

1.3.10 Statistical analyses

All data visualization (ggplot2 library) and statistics were carried out using R 3.2.3 (The R Foundation for Statistical Computing, 2015). When necessary, we used Box-Cox transformations and excluded extreme values to help meet normality and homoscedasticity assumptions.

To compare the parasite communities in the different habitats from field surveys, we performed a principal component analysis (PCA) (dudi.pca function,

ade4 library) followed by a permutation test (randtest function, ade4 library). We used the individual parasitation index (I_{PI}) (Kalbe 2002) to compare fish from the three sampling sites (RA, RB and L) over time and from the different genetic clusters found in the different habitats (RA, RA in RB, L in RB, L; hereafter refer to as 'genotype-by-habitat'). As the I_{PI} (continuous variable) showed a zero-inflated Poisson distribution, we transformed the data by adding a small value (0.1) to all I_{PI} to shift the distribution before using a generalized mixed model for Gamma family (glmer function, lme4 library). We used linear mixed models (lmer function, lme4 library) to compare fish condition (log transformed CF) and health condition (4^{th} square root of SSI) within the three sampling sites (RA, RB and L) over time and among different genetic populations within each habitat (RA, RA in RB, L in RB, L). In all models, sex, sampling date, dissection date and handling condition (dissected shortly after capture, transferred alive or frozen) were included as random factors.

To compare the effect of the fish genetic type (RA, RAxL, LxRA, L) on susceptibility to *D. pseudospathaceum* (number of parasites that managed to infect a fish), we used a generalized mixed effect model for Poisson family (glmer function). We used linear mixed effect models to assess the effect of fish 'genetic type' on fish condition (log transformed CF, log transformed HSI) and health (4^{th} square root of SSI) parameters. In all models, we used sex and parental identity as random factors (identification number used of female and male for a given breeding pair).

For *S. solidus* exposure, we used linear mixed effect models to test for the effect of the fish genetic type (RAxRA or LxL) and infection status (control, uninfected or infected) on epidemiological (PI), condition (CF, HSI) and health (4^{th} square root of SSI) parameters. We included sex, fish family and *S. solidus* family as random factors. We used Chi-squared tests to assess the effect of the fish 'genetic type' on the proportion of dead (mortality) and infected fish.

Tukey *post-hoc* tests were used for all post hoc comparisons (lsmeans function in R, lsmeans library).

1.4 Results

1.4.1 Population genetic diversity

Microsatellite loci showed an average of 3, 19 and 18 alleles per loci in RA, RB and L respectively. RA had extremely reduced diversity and was also characterized by half the heterozygosity (0.47 on average) observed in RB or L (see table S4). The RA population shared all its alleles with the populations below the waterfall (RB and L). By contrast, rare alleles were found in several loci for both RB and L (17 private alleles in 7 loci in RB, 10 private alleles in 6 loci in L; see table S4).

1.4.2 Genetic linkage

Within each sampling site and for each year, no locus deviated significantly from neutrality or from Hardy-Weinberg equilibrium after Bonferroni correction except for *STN32* in RB2012 and RB2013, and for *STN75* in RA2009 and RB2013 (see table S5). Tests revealed genotypic linkage disequilibrium only for a few locus pairs in RB2010, RB2012, RB2013 and L2012 (see table S5).

1.4.3 Population genetic differentiation and Bayesian population structure

While the RA population is consistently and significantly differentiated from both RB and L, the latter two do not show signs of divergence in pairwise F_{ST} estimates (see fig. S2). Bayesian population structure analysis indicates there are two source populations (K = 2; see fig. S3). For each year, the results showed a similar pattern where the RA fish and the fish below the waterfall (RB and L) formed two distinct clusters with a few immigrants from the RA cluster found in RB but not in L (see fig. S3). We found the same result when combining the four sampling years (K = 2; fig. 1).

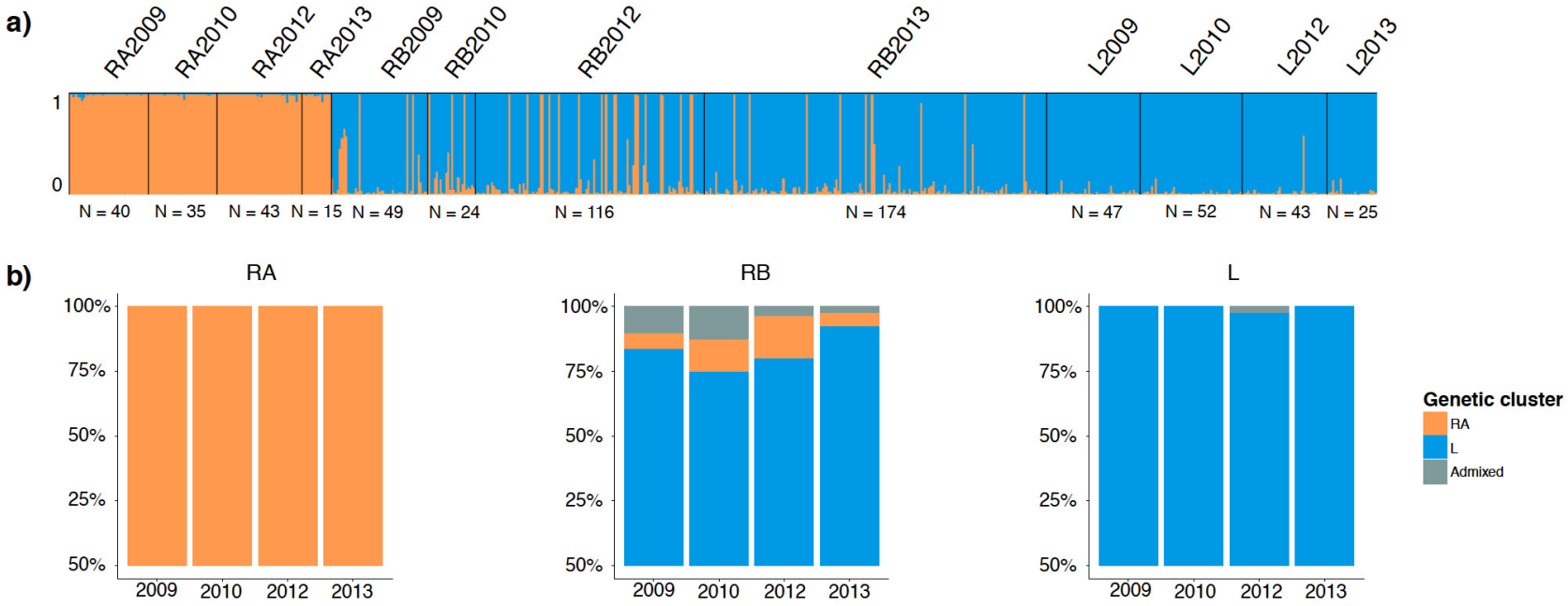

Figure 1

a) Results of Bayesian population structure analysis (using STRUCTURE 2.3.4) of microsatellite data from three sampling sites (RA, RB, L) of three-spined sticklebacks over four survey years (2009, 2010, 2012, 2013). There are two genetic clusters (K = 2), corresponding to populations above (RA, in orange) and below the waterfall (RB and L, in blue). Individual probabilities of assignment are shown on the y-axis and are grouped by sampling populations. **b)** Proportion of individuals from each genetic cluster (RA, L and admixed) in each sampling sites (RA, RB, L) over four survey years (2009, 2010, 2012, 2013).

1.4.4 Detection of immigrants and hybrids

Each year, between 5.2 and 16.4% (with an average of 10.0% ±5.3 of fish over the four sampling years) of the fish sampled in RB belonged to the RA genetic cluster. In total, 34 of 363 fish sampled in RB were putative first generation immigrants from RA. No fish from the RA cluster were identified in samples from L. 17 fish had genotypes (16 in RB and 1 in L) that NewHybrid could not unambiguously assigned to only one of the four possible genotypes (pure RA, pure L, F1 hybrid, F2 hybrid, RA backcrossed, L backcrossed). These 17 admixed individuals suggest a low level of admixture between immigrants from RA and RB/L fish. Since we wanted to focus on the differences between genetic clusters in different habitats, the genetically admixed individuals (N = 17) were excluded from further statistical analyses.

1.4.5 Field parasite community

The RA fish population was consistently devoid of macroparasites, whereas RB and L harboured diverse and different macroparasite communities (Monte-Carlo test, $P = 0.001$) with four species of cestodes, four trematodes and two nematodes (fig. 2, see fig. S4, table S6). The mean parasite prevalence and intensity as well as individual parasitisation index I_{PI} in L fish were significantly higher than of RB fish in all survey years except 2009 (ANOVA, $F_{2,442} = 11.344$, $P = 0.003$; Tukey's HSD, $P < 0.001$; fig. 2, fig. 3). The immigrants from RA sampled in RB had a significantly lower I_{PI} than RB residents, which had themselves a significantly lower I_{PI} than L residents (ANOVA, $F_{3,441} = 178.053$, $P < 0.001$; fig. 3).

1.4.6 Field population condition parameters

While every year RA fish had a higher CF than RB or L fish, this difference was never significant (ANOVA, $F_{2,430} = 5.008$, $P = 0.082$). There was also no effect of the year (ANOVA, $F_{3,438} = 5.300$, $P = 0.151$) but an effect of the interaction 'habitat*year' (ANOVA, $F_{11,430} = 18.010$, $P = 0.006$). Overall, there was a significant difference in CF between the different 'genotype-by-habitat' (ANOVA, $F_{3,438} = 48.775$, $P < 0.001$). Both RA residents and putative immigrants

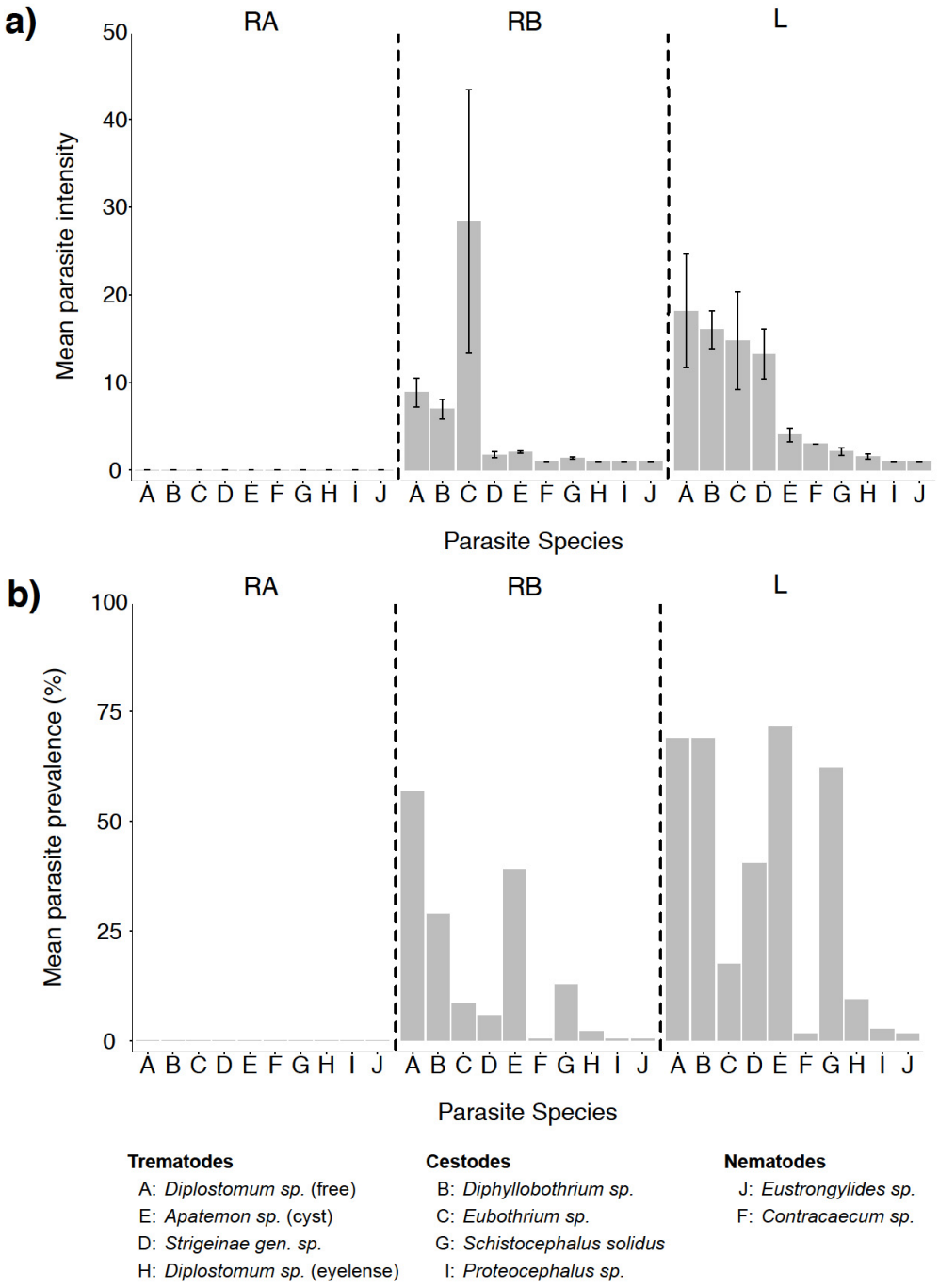

Figure 2

a) Mean parasite intensity (± SD) and **b)** mean parasite prevalence in each sampling sites (RA, RB, L) for four survey years (2009, 2010, 2012, 2013) (RA-L admixed individuals are excluded; see table S1 for sample sizes).

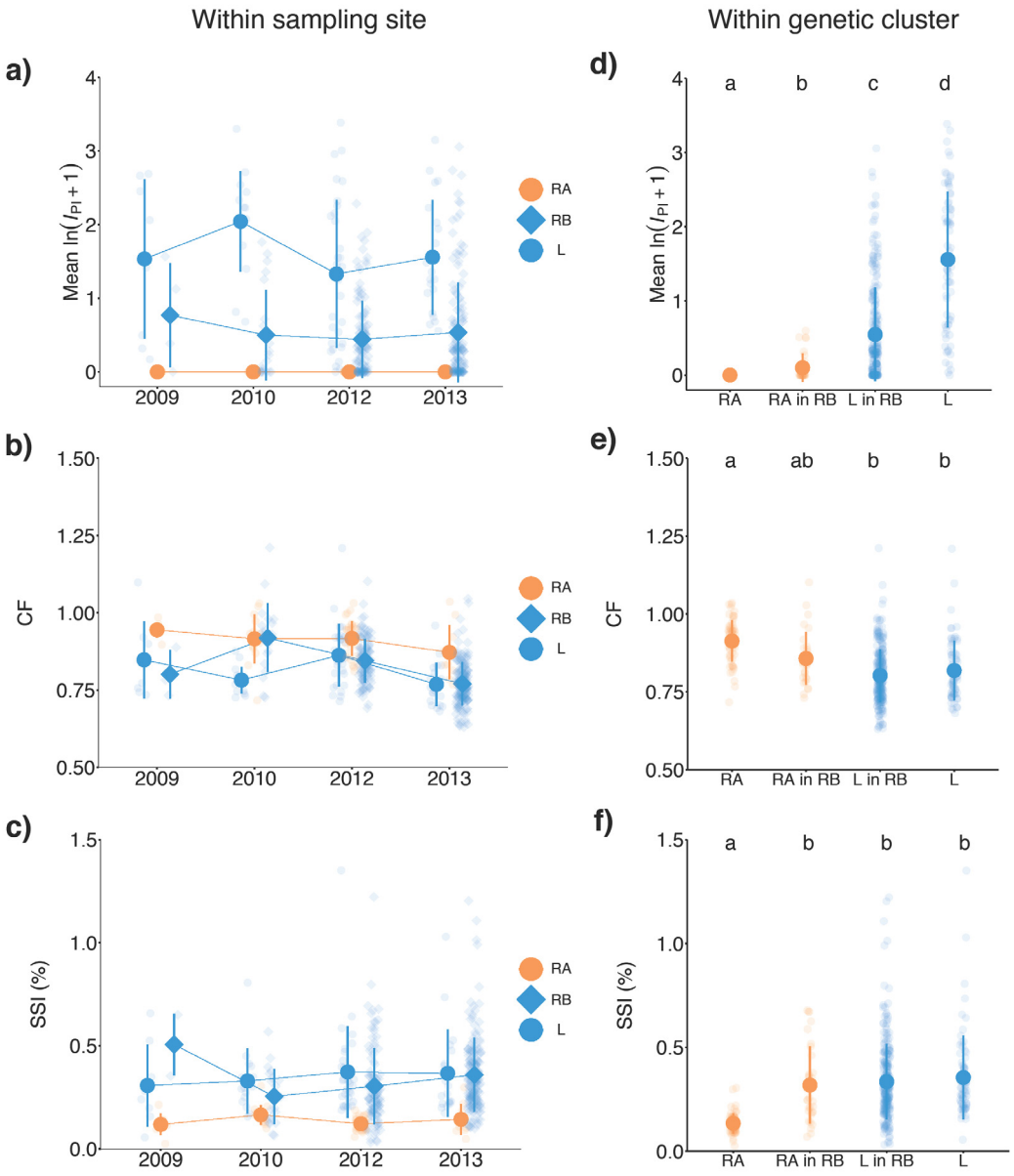

Figure 3

Fish parasitation and condition **a-c)** within each sampling site (RA, RB, L) over four survey years (2009, 2010, 2012, 2013) and **d-f)** within each genetic cluster (RA, L) in each sampling sites (RA, RB, L) overall years: **a)/d)** Log-transformed mean individual parasitation index ($\ln(I_{PI} + 1)$) (± SD); **b)/e)** Mean fish condition factor CF (± SD) (three extreme values were excluded); **c)/f)** Mean splenosomatic index SSI (± SD). Means annotated with different letters are significantly different (Tukey's HSD, $P < 0.050$) (RA-L admixed individuals are excluded; see table S1 for sample sizes).

had higher condition than lake fish in RB or L habitat, this difference being significant for RA resident (Tukey's HSD, $P < 0.001$; fig. 3).

The population of origin had a significant effect on SSI (ANOVA, $F_{2,442} = 26.023$, $P < 0.001$) as RA showed reduced SSI compared to RB and L (Tukey's HSD, $P < 0.001$). This pattern was stable as there was no effect of the survey year (ANOVA, $F_{2,441} = 0.410$, $P = 0.938$) nor 'habitat*year' interaction (ANOVA, $F_{2,433} = 8.939$, $P = 0.177$) on SSI. There was a significant of 'genotype-by-habitat' in SSI (ANOVA, $F_{3,441} = 144.25$, $P < 0.001$). There was no difference in SSI between fish found in L or in RB, including both L river resident and putative RA immigrants (Tukey's HSD, $P > 0.050$). However, fish in RA had a consistently lower mean SSI than fish found bellow the waterfall (Tukey's HSD, $P < 0.001$). This indicates a higher immune activation of fish below the waterfall (fig. 3).

1.4.7Experimental infection with Diplostomum pseudospathaceum

We found the laboratory-bred RA fish to be more susceptible than L fish to the generalist parasite *D. pseudospathaceum*. Even though all fish were infected with at least one metacercaria, the mean number of metacercariae per fish was significantly different among genetic types (ANOVA, $F_{3,205} = 15.338$, $P = 0.001$): RA fish had more parasites than L fish (Tukey's HSD, $P = 0.003$), or L-maternal hybrids (Tukey's HSD, $P = 0.003$). The hybrid families (RAxL and LxRA) had an intermediate number of parasites compared to the pure families (RA and L), but did not depart significantly from their respective pure maternal genetic types (Tukey's HSD, $P > 0.050$, fig. 4).

There was no significant effect of the genetic types on CF (ANOVA, $F_{3,203} = 1.208$, $P = 0.751$). There was a significant effect of genetic type on HSI (ANOVA, $F_{3,205} = 21.353$, $P < 0.001$) but we could not detect significant differences among the different types (Tukey's HSD, $P > 0.050$; see fig. S5). We found a significant effect of genetic type on SSI (ANOVA, $F_{3,205} = 19.282$, $P < 0.001$), with the RA-maternal hybrids having a significantly higher SSI than L fish (Tukey's HSD, $P = 0.019$; see fig. S5).

1.4.8Experimental infection with Schistocephalus solidus

The proportion of fish infected with *S. solidus* was significantly higher in laboratory-bred RA fish compared to L fish ($x^2_{1,180} = 9.817$, $P = 0.001$; fig. 4). The parasite index of worms infecting pure RA fish was significantly higher than for pure L fish (ANOVA, $F_{1,46} = 26.842$, $P < 0.001$) with worms infecting RA fish being almost twice as big as those infecting L fish (24.0% vs. 14.6% of the fish somatic weight; fig. 4).

There was no effect on CF of the infectious status (ANOVA, $F_{2,206} = 4.955$, $P = 0.084$), but an effect of the genetic type (ANOVA, $F_{1,207} = 8.327$, $P = 0.004$); while infected fish did not differ from the controls (Tukey's HSD, $P > 0.050$), infected RA fish had lower CF than infected L fish (Tukey's HSD, $P = 0.018$).

There was a significant effect on HSI of infectious status (ANOVA, $F_{2,206} = 9.982$, $P = 0.007$) and genetic type (ANOVA, $F_{1,207} = 5.183$, $P = 0.023$). Infected fish had a significantly lower HSI than both control (Tukey's HSD, $P = 0.049$) and uninfected fish (Tukey's HSD, $P < 0.001$).

There was no significant difference in SSI between infectious status (ANOVA, $F_{1,199} = 4.832$, $P = 0.089$) or genetic types (ANOVA, $F_{1,200} = 3.523$, $P = 0.060$; see fig. S5).

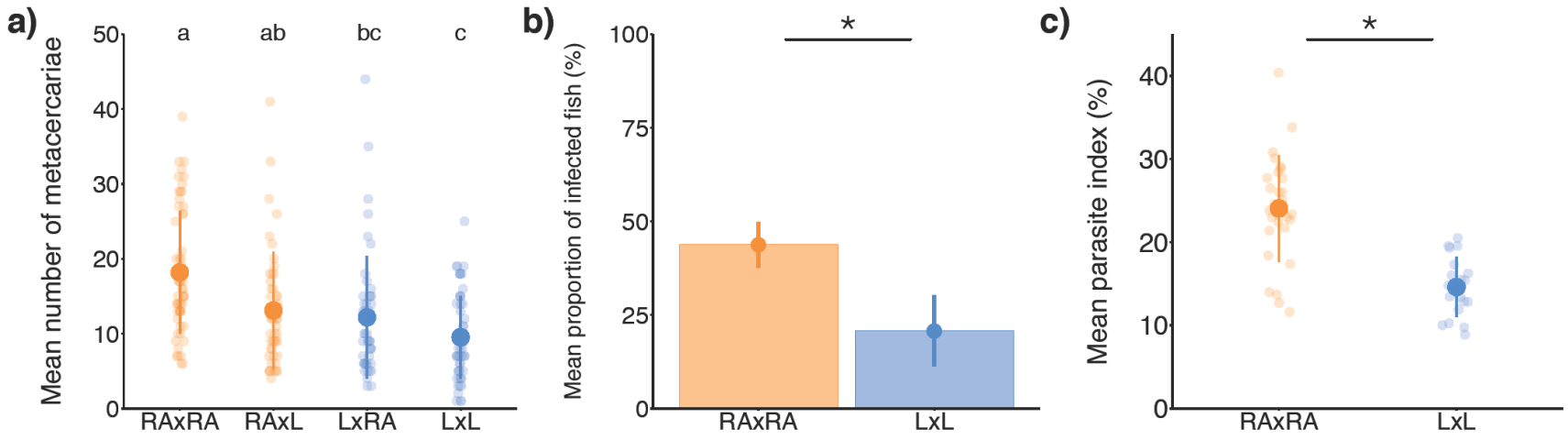

Figure 4

Results of the experimental infections for the different Skogseidvatnet laboratory-bred fish genetic types ('River Above' RAxRA, 'River Above' maternal hybrid RAxL, lake maternal hybrid LxRA, lake LxL). In **a)** mean number of *D. pseudospathaceum* metacercariae in the eye lenses (± SD) (RAxRA N = 58, RAxL N = 47, LxRA N = 48, LxL N = 56) **b)** mean proportion of fish infected with *S. solidus* per fish family (± SD) (RAxRA N = 89, LxL N = 91) and **c)** mean *S. solidus* parasite index (± SD) (RAxRA N = 29, LxL N = 19). Means annotated with different letters or asterisks are significantly different (Tukey's HSD, $P < 0.050$).

1.5 Discussion

Through four years of field survey, we found a difference in parasite pressure above and below the waterfall: the RA stickleback population has been consistently devoid of macroparasites whereas both the RB and L populations experience high parasite pressure. While river sticklebacks usually have few macroparasites, this is the first well documented case of a population with no evidence of macroparasites (Feulner *et al*. 2015). All parasite species detected have complex life cycles and depend on the presence of first intermediate hosts to be transmitted to sticklebacks. The absence of parasites in RA fish is likely the result of a combination of factors, including specific species community, absence of key intermediate hosts, and fast currents preventing free-swimming infective parasite stages or invertebrate hosts to remain in contact with fish (Thieltges, Jensen & Poulin 2008).

The RA population is separated by a waterfall from the RB and L populations, which creates unidirectional gene flow likely at the source of the reduced and distinct genetic diversity (Crispo *et al*. 2006; Castric, Bonney & Bernatchez 2007). The populations above the waterfall has probably only recently been separated from the one below by the post-glacial isostatic rebound that shaped the Norwegian landscape (Svendsen & Mangerud 1987) and has experienced a bottleneck - explaining why the RA genetic cluster shares all its microsatellite alleles with the L cluster. While neutral variation is expected to decline after a bottleneck, theory predicts that the diversity of adaptive markers can be maintained or restored through selection (Oosterhout *et al*. 2006). With the remarkable absence of selection by macroparasites in the RA habitat, it is likely that the loss of immune adaptive diversity would be permanent.

To evaluate survival and reproduction of RA fish below the waterfall, we first tested for genetic differentiation and identified two genetic clusters (RA vs. RB and L). Furthermore, we detected unidirectional fish migration from RA into RB through the presence of up to 16% immigrants but limited introgression. Indeed, only 17 fish had admixed genotypes, suggesting that cross-population mating occurs rarely in RB. Whereas it is difficult to estimate a hybridization

rate, with an average of 4.4% admixed individuals and the fact that the RB population stayed genetically distinct from RA over four years, it appears that the RA fish do not effectively reproduce and invade the RB environment.

In sticklebacks mate selection relies on the assessment of immunocompetence (Milinski & Bakker 1990) or immunogenetic background (Reusch *et al*. 2001a; Aeschlimann *et al*. 2003; Jäger *et al*. 2007) limiting gene flow between ecotypes with distinct parasite communities (Eizaguirre *et al*. 2011). For instance, the Major Histocompatibility Complex (MHC) genes have pleiotropic role in immune function and mate choice, and present distinct allele pools in stickleback ecotypes experiencing divergent parasite pressure (Milinski *et al*. 2005; Eizaguirre *et al*. 2009a; b, 2012). Therefore parasite-mediated local adaptation might not only hinder RA fish survival in RB environment but also favour mating barriers between immigrants and residents (Nosil, Vines & Funk 2005; Tobler *et al*. 2009).

As immunity is a costly trait, we expected the RA population to be locally adapted to its extremely low parasite pressure by reducing its investment into immune functions while investing more into growth and reproduction (Lochmiller & Deerenberg 2000; Zuk & Stoehr 2002). The higher splenosomatic index of RA migrants could indicate that even a low level of infection elicits a high immune response and is more detrimental to the condition of immigrants. By contrast, fish in the RA habitat have a consistently higher body condition than RA immigrants, RB or L fish found below the waterfall. This pattern is in line with our predictions, suggesting low parasite exposure in the RA habitat allows for higher growth and resource storage compare to the less advantageous habitat below the waterfall.

As both RA and RB are typical river habitats with similar ecological conditions, the remarkable difference in parasite pressure is most likely to be the strongest ecological factor responsible for RA fish failing to establish below the waterfall. However we did not measure other environmental factors, nor could we know of the life history of field-sampled individuals. As such, it is difficult to disentangle pre-adaptation to habitat-specific parasite pressure from other parameters potentially influencing migration success from field data

alone. Therefore, we compared the resistance of fish from the RA and L populations to *D. pseudospathaceum and S. solidus* in controlled experimental conditions.

We showed that the first generation of laboratory-bred RA fish is indeed less resistant to both parasite species than the L population. Experiencing a relaxed parasite pressure, the RA population did not develop an adequate innate resistance to *D. pseudospathaceum*, nor an adaptive resistance to *S. solidus*. Our results are consistent with previous studies of Northern German stickleback populations, which showed that laboratory-bred river fish have lower resistance compared to lake fish (Kalbe & Kurtz 2006; Lenz *et al*. 2013) and a higher susceptibility to lake parasites when translocated into lake habitat (Scharsack *et al*. 2007a). The significantly larger parasite burden of laboratory-bred RA fish infected with *S. solidus* had a detrimental effect on fish condition (lower CF than L fish). Such effects were not observed with *D. pseudospathaceum* infection because the relative physiological toll of a single exposure to *D. pseudospathaceum* infection is expected to be lower than for a chronic infection with *S. solidus*. We also found that hybrids had an intermediate level of susceptibility and showed signs of potential maternal effects with a splenosomatic index similar to the one of their pure maternal line. First generation hybrids in the natural ecosystem are thus likely to be less competitive than pure L fish in the presence of parasites. Consequently, lower immunocompetence and ecological hybrid inferiority is likely to limit genetic flow from RA to L genetic clusters (Nosil *et al*. 2005).

While we demonstrated lower resistance of RA fish and expected them to be heavily parasitized when migrating to the 'River below' environment, immigrants were found to exhibit relatively low levels of parasitism compared to the RB residents. There are several possible explanations for this observation. First, RB residents were exposed for a longer time than immigrants to the high parasite pressure environment and accumulated parasites including during the early months of their development, when sticklebacks predominantly prey on the small invertebrate hosts of macroparasite species (Christen & Milinski 2005). Second, recent RA immigrants not only spent part of their life in a macroparasite-free habitat, but are also probably better adapted to swimming in

the river current (Bolnick *et al*. 2009; Jiang *et al*. 2015), where infection risk is lower than in the lake. Finally, RA fish residing for a longer period in the RB habitat might reach a fatal level of infection and die before we could sample them. In any case, the high immunological migration costs to RA fish in RB could explain why they fail to establish.

Our study demonstrates the potential of divergent parasite pressures to contribute to reproductive barriers through selection against immigrants. Field surveys and experimental evidence both indicate that RA fish are adapted to extremely low parasite pressure and cannot cope with the high parasite pressure they encounter when migrating below the waterfall. Therefore parasite pressure is a plausible agent preventing admixture in the lake population. Our results provide evidence for an important role of host-parasite coevolution in the ecological isolation of three-spined stickleback populations in this system.

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2 Chapter 2

Resist globally, infect locally:

A transcontinental test of adaptation by stickleback and their tapeworm parasite

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Resist Globally, Infect Locally: A Transcontinental Test of Adaptation by Stickleback and Their Tapeworm Parasite

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abstract: Parasite infections are a product of both ecological processes affecting host-parasite encounter rates and evolutionary dynamics affecting host susceptibility. However, few studies examine natural infection variation from both ecological and evolutionary perspectives. Here, we describe the ecological and evolutionary factors generating variation in infection rates by a tapeworm (*Schistocephalus solidus*) in a vertebrate host, the threespine stickleback (*Gasterosteus aculeatus*). To explore ecological aspects of infection, we measured tapeworm prevalence in Canadian stickleback inhabiting two distinct environments: marine and freshwater. Consistent with ecological control of infection, the tapeworm is very rare in marine environments, even though marine fish are highly susceptible. Conversely, commonly infected freshwater stickleback exhibit substantial resistance in controlled laboratory trials, suggesting that high exposure risk overwhelms their recently evolved resistance. We also tested for parasite adaptation to its host by performing transcontinental reciprocal infections, using stickleback and tapeworm populations from Europe and western Canada. More infections occurred in same-continent host-parasite combinations, indicating parasite "local" adaptation, at least on the scale of continents. However, the recently evolved immunity of freshwater hosts applies to both local and foreign parasites. The pattern of adaptation described here is not wholly compatible with either of the common models of host-parasite coevolution (i.e., matching infection or targeted recognition). Instead, we propose a hybrid, eco-evolutionary model to explain the remarkable pattern of global host resistance and local parasite infectivity.

Keywords: susceptibility, resistance, threespine stickleback, helminth, infection.

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Introduction

Parasite prevalence often varies substantially among host populations, both globally (Marzal et al. 2011) and at local spatial scales (Laine et al. 2011). For example, the prevalence of a fungal leaf rust (*Microbotryum* sp.) varies dramatically between patches of its host plant (*Viscaria* sp.; Carlsson-Granér and Thrall 2015). Similarly, the prevalence of many helminth parasites varies greatly among fish populations inhabiting adjoining lakes. Understanding the epidemiology of such patchily distributed parasite metapopulations requires data on rates of host-parasite encounters and on the probability of infection following such outcomes. Host-parasite encounters are a largely ecological process and depend on factors such as the abundance of suitable intermediate hosts (for parasites with complex life cycles; Parker et al. 2015), host foraging behavior (Hall et al. 2007), and abiotic control of parasite viability (Decker et al. 2001). Conversely, the infection rate following an encounter depends strongly on host immunity and parasite infectivity, which are subject to strong genetic control and are therefore involved in evolutionary and perhaps coevolutionary interactions (although environmental conditions may additionally affect immune function; Stutz et al. 2015). So pressing questions in host-parasite biology include the following: To what extent is spatial variation in parasite prevalence due to ecologically regulated encounter rates or to localized evolution of hosts and parasites (Gandon and Nuismer 2009)? If infection prevalence mostly depends on encounter rates, what ecological processes dictate those encounters? If prevalence depends on (co)evolutionary dynamics, does spatial variation in susceptibility primarily reflect host or parasite adaptation?

There is a substantial literature on the ecological determinants of infection rates (Johnson et al. 2015), clearly showing that abiotic conditions and biotic community structure regulate the risk of parasite encounters. For example, in the case of louping-ill virus (Gilbert et al. 2001), infection

rates and virus persistence depend strongly on host diversity and abundance, both of which are affected by abiotic conditions. There is an equivalently large literature concerning the relative strength of local adaptation by hosts to their parasites or adaptation of parasites to their local hosts (Greischar and Koskella 2007; Hoeksema and Forde 2008). Although the results on host adaptation are equivocal, there is a common observation about parasite evolution: parasites evolve local adaptation when they have higher rates of gene flow than their hosts.

Evolutionary and ecological perspectives on infection are by no means independent. Instead, selection for host resistance presumably depends on the frequency of parasite encounters. More specifically, resistance evolves when parasitism is frequent and substantially decreases host fitness (Boots and Haraguchi 1999). Where infection is rare, selection for resistance will be correspondingly weak and may be counteracted by costs of resistance (Sheldon and Verhulst 1996; Duffy et al. 2008, 2012; Duncan et al. 2011; Auld et al. 2013) or drift. This selection for resistance may feed back to alter host-parasite ecology, by reducing parasite reproductive rates or infection success rates. At the most extreme, highly exposed populations might evolve strong resistance that leads them to have the lowest observed infection rates (Stutz et al. 2014), whereas rarely exposed populations might remain susceptible. Thus, evolution of host resistance can negate or even reverse the relationship between exposure risk and observed infection prevalence. Eco-evolutionary feedbacks may therefore generate positive or negative covariation between parasite exposure, host resistance, and infection prevalence. To provide an empirical estimate of this covariation, we describe the relationship between exposure risk, resistance, and infection prevalence in a natural host-parasite system.

Study system. We examine patterns of infection by a tapeworm (*Schistocephalus solidus*) in its threespine stickleback (*Gasterosteus aculeatus*) host. The closely related genera *Schistocephalus* and *Ligula* are historically important, as they were among the first parasites shown to have a complex life history, initially through comparative analyses of morphology in 1829 and 1839 and subsequently through experiments in 1876 (full history and citations in Dubinina 1980). *Schistocephalus solidus* eggs hatch in freshwater (but rarely hatch in brackish water; Simmonds and Barber 2016), where they infect copepods. When a threespine stickleback eats an infected copepod, the tapeworm penetrates the intestinal wall and enters the body cavity, where it grows to its final size. If a bird eats the stickleback, the parasite rapidly becomes reproductively mature in the bird's intestine, where it reproduces via selfing or outcrossing (Orr and Hopkins 1969; Dubinina 1980), and eggs are defecated to initiate a next generation. *Schistocephalus solidus* can be experimentally bred in vitro (bypassing the bird host by using artificial culture media), allowing laboratory studies of the parasite's genetics and infection rates (Smyth 1946; Orr and Hopkins 1969). The tapeworm can infect a variety of copepod and bird species but is specialized on threespine stickleback. *Schistocephalus solidus* is genetically distinct from both *Schistocephalus cotti* (Chubb et al. 2006) and *Schistocephalus pungitii* (Nishimura et al. 2011), which respectively infect bullheads (*Cottus gobio*) and ninespine stickleback (*Gasterosteus pungitius*). *Schistocephalus solidus* also failed to establish lasting infections in two experiments with congeneric ninespine stickleback, one using freshwater fish (Henrich et al. 2013) and the other using hosts of unknown origin (Orr et al. 1969).

When *S. solidus* infections occur, they can pose substantial burdens on stickleback fitness, decreasing body condition and energy reserves (Arme and Owen 1967; Tierney et al. 1996) and reducing gonad development (Heins et al. 1999). In addition, *S. solidus* infection reduces sticklebacks' antipredatory responses (Giles 1983). Although *S. solidus* virulence does vary among populations (Heins and Baker 2008), there is limited evidence that stickleback mount strong immune responses to clear the parasite (Barber and Scharsack 2010). Indeed, some studies report that *S. solidus* is capable of strong suppression of host immunity (Scharsack et al. 2004).

Stickleback are a leading model organism for studies of the ecology and genetics of adaptation and are well known for exhibiting local adaptation to a wide variety of selective pressures (Hendry et al. 2002; Bolnick et al. 2008; Jones et al. 2012; Spence et al. 2013; DeFaveri and Merilä 2014), including parasites (Konijnendijk et al. 2003; Kalbe and Kurtz 2006; Scharsack et al. 2007; de Roij et al. 2011; Lenz et al. 2013). Many studies capitalize on the fact that stickleback evolution has a clear directionality: the species is predominantly marine, but after Pleistocene deglaciation stickleback frequently colonized and adapted to freshwater habitats. Extant marine stickleback are believed to resemble the ancestral form that colonized freshwater, because of the marine populations' large effective population size and minimal phylogeographic structure (Bell and Foster 1994; Taylor and McPhail 1999; Hohenlohe et al. 2010; Jones et al. 2012; Catchen et al. 2013; but see DeFaveri et al. 2013).

Here, we evaluate the causes of extreme differences in *S. solidus* prevalence across stickleback populations. The putatively "ancestral" marine stickleback are rarely infected by *S. solidus*, which might reflect (1) a low encounter rate with the tapeworm, which hatches poorly in salt water, (2) effective resistance by this large and well-mixed host population, (3) parasite specialization on lake stickleback, or (4) combinations of these factors. Similarly, differences in infection prevalence among freshwater populations could result from differences in host exposure, resistance, and/or parasite specificity. Using field observations and lab infection trials of marine and freshwater stickleback exposed to both native and foreign parasites, we aim to disentangle

ecological and evolutionary explanations for natural infection prevalence.

To partition any effects of host and parasite evolution, our infection assays included a reciprocal-infection experiment using stickleback and *S. solidus* from both western Canada and northern Europe (7,000 km apart). Because birds are the final host for *S. solidus*, this tapeworm likely has a large dispersal potential, which may confound interpretations of local adaptation when examining parasites from nearby lakes. However, transcontinental gene flow is unlikely, because the tapeworm typically breeds within days of entering the bird gut and survives only a short time thereafter (Dubinina 1980). Gene flow is therefore restricted to birds' regular migration routes, at distances that birds can cover within *S. solidus*'s short window of sexual maturity. Accordingly, there is appreciable genetic divergence between *S. solidus* from different continents (Nishimura et al. 2011). Local as well as continent-scale genetic divergence is well documented in the stickleback host (Colosimo et al. 2005; Jones et al. 2012). We collected allopatric *S. solidus* from Canada and Norway and exposed these tapeworms to both same- and different-continent stickleback hosts. We use these experimental infections to test two key predictions. First, we predict that host populations with higher parasite exposure risk (e.g., in freshwater) will tend to be more resistant than the low-risk marine populations. This resistance might be specific to local parasites or may apply to all *S. solidus* generally. Second, we predict that this host evolution may be undermined by parasite evolution, which might result in *S. solidus* that more readily infect local than foreign stickleback.

Material and Methods

Survey of Tapeworm Prevalence in Canadian Marine and Lake Stickleback

In June 2009 and June 2013, we sampled a total of 38 lake and 5 marine populations of threespine stickleback from northern Vancouver Island, British Columbia. Between 60 and 100 fish were caught per site with unbaited minnow traps set in the water for at most 3 hours. Specimens were preserved in 10% neutral buffered formalin and subsequently dissected to determine the number of *Schistocephalus solidus* per fish. We estimated infection prevalence (percent of individuals infected) per site and used a binomial generalized linear model (GLM) to test whether prevalence differed between marine and lake populations.

Stickleback Collection for Experimental Infections

We used unbaited minnow traps to collect threespine stickleback from three populations on Vancouver Island, Canada (Scientific Fish Collection permit NA12-77018). Gosling and Roberts Lakes (hereafter GOS and ROB, respectively; 50° 03′47″N, 125°30′07″W and 50°13′38″N, 125°33′01″W) are in separate watersheds and contain isolated freshwater populations of stickleback with no current access to the ocean and limited gene flow with other populations in the watershed. Like all freshwater populations on Vancouver Island, GOS and ROB fish are derived from marine ancestors that colonized new habitats after deglaciation less than 12,000 years ago (Caldera and Bolnick 2008). *Schistocephalus solidus* prevalence in GOS is consistently high (estimated at 50%–80%, depending on the year), while infection has been absent (or undetected) in ROB for the past decade (2009 data in fig. 1). Second, we sampled anadromous marine stickleback that migrate, in early June, to breed in brackish water in Sayward Estuary (SAY; 50°22′46″N, 125°56′43″W). SAY fish spend most of their life at sea and carry correspondingly few *S. solidus* (<2% prevalence for each of 5 years, 2009–2014). The SAY marine fish represent a proxy for the phenotype of ancestral stickleback that likely colonized freshwater lakes such as GOS and ROB. By comparing *S. solidus* susceptibility of the ancestral-like SAY with that of derived GOS and ROB stickleback, we test whether the transition from marine to freshwater entailed evolution of immunity to this predominantly freshwater-associated parasite.

We also collected stickleback from two freshwater lake populations in northern Europe: Großer Plöner See, Plön, Ger-

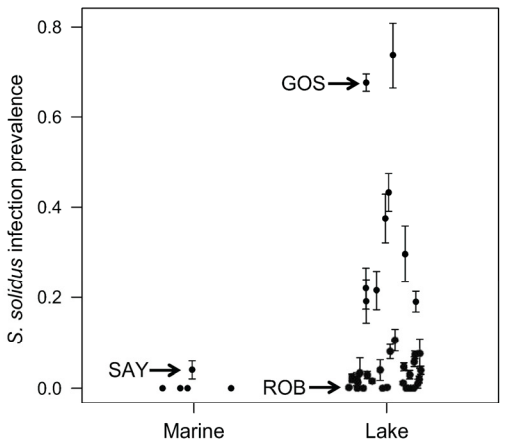

Figure 1: *Schistocephalus solidus* prevalence (% fish infected) is higher in recently evolved freshwater lake populations of threespine stickleback on Vancouver Island than in putatively ancestral marine populations. Each marine population ($N = 5$) and lake population ($N = 38$) is represented by a prevalence estimate and standard error confidence interval, with horizontal jitter added. The focal populations in this article are indicated with text (SAY = Sayward Estuary; GOS = Gosling Lake; ROB = Roberts Lake).

many (GPS; 54°08′48″N, 10°24′31″E), and Lake Skogseidvatnet, Norway (SKO; 60°14′44″N, 5°55′03″E). The GPS population has low prevalence of *S. solidus* (estimated prevalence of <1% over 14 years), similar to the rarely infected Canadian marine fish (SAY). The SKO population has a high prevalence of *S. solidus* (between 20% and 60% in the past 8 years), much like Canadian GOS fish. Unlike the Canadian population comparison, GPS is not a proxy ancestor for SKO. Nevertheless, we are able to compare experimental infections of rarely infected stickleback (SAY, ROB, GPS) and commonly infected stickleback (GOS, SKO) to test whether host resistance is stronger in the former or the latter category.

We bred between 3 and 12 families of fish from each population (table 1). We generated Canadian fish families in the field by using in-vitro fertilization and shipped fertilized eggs to the University of Texas at Austin for rearing (Transfer License NA12-76852). All Canadian fish were between 22 and 24 months of age at the time of exposure to *S. solidus*. Norwegian and German sticklebacks were bred by mating wild-caught sticklebacks in the lab. These European fish families were reared according to previously described methods (Kalbe and Kurtz 2006) at the Max Plank Institute for Evolutionary Biology in Plön and were used for experiments at an age between 8 and 14 months.

Table 1: Summary of experimental exposures

Tapeworm origin and family, stickleback families exposed	Fish per population
Echo Lake, British Columbia, Canada:	
Can1:	
Gosling 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 (2 fish)	29
Sayward	3
Skogseidvatnet 1, 2, 3	30
Großer Plöner See 1, 2, 3 (9 fish)	29
Can2:	
Gosling 1, 3, 4, 5, 6, 8, 9, 10, 11, 12	30
Sayward	3
Skogseidvatnet 1, 2, 3	30
Großer Plöner See 1, 2, 3	30
Can3:	
Gosling 1, 2, 3, 4, 5 (1 fish), 6, 8, 9, 10, 11	28
Sayward (6 fish)	6
Skogseidvatnet 1, 2, 3	30
Großer Plöner See 1 (8 fish), 2 (7 fish), 3 (7 fish)	22
Roberts (5 fish per family unless noted) 1, 2 (3 fish), 3, 4, 5 (4 fish), 6, 7	32
Lake Skogseidvatnet, Norway:	
Nor1:	
Gosling 1, 2 (2 fish), 3, 4, 5, 6, 8, 9 (2 fish), 10, 11	28
Sayward	3
Skogseidvatnet 1 (12 fish), 2, 3	32
Großer Plöner See 1, 2, 3	30
Roberts 6, 7, 8, 9, 10	15
Nor2:	
Gosling 1, 3, 4, 5, 6, 8, 9, 10, 11, 12	30
Sayward	3
Skogseidvatnet 1, 2 (9 fish), 3	29
Großer Plöner See 1, 2 (11 fish), 3	31
Roberts 6, 7, 8, 9, 10	15
Nor3:	
Gosling 3, 4, 5, 6 (2 fish), 7, 8, 9, 10, 11, 12	29
Sayward	3
Skogseidvatnet 1, 2, 3	30
Großer Plöner See 1, 2, 3	30
Roberts 6, 7, 8, 9, 10	15

Note: Three fish from each Canadian family and 10 fish from each European family were exposed. Deviations from these sample sizes are noted after stickleback family name.

Tapeworm Collection and Experimental Infections

We exposed the five fish populations to *S. solidus* from both Canada and Europe, allowing us to test whether infection rates depend on parasite genotype (controlling for host). We obtained mature *S. solidus* from live fish captured in Echo Lake in British Columbia, Canada (49°59′11″N, 125° 24′36″W) or in Lake Skogseidvatnet (SKO) in Norway. We generated full-sibling families of *S. solidus* from each population, using previously described protocols (Smyth 1946; Wedekind 1997). We paired each adult tapeworm with another sympatric individual of similar mass to encourage outcrossing (Lüscher and Milinski 2003), placed the pairs in nylon biopsy bags (Fisherbrand, catalog no. 15-182-501), and then flame-sealed the bags. We then submersed the bags in media that stimulated release of gametes and mating (Smyth 1946, 1990; Jakobsen et al. 2012) and harvested eggs for the next 6 days. To minimize fungal growth, we rinsed the eggs several times with sterile water to remove residual media. Before use, we repeatedly sterilized and conditioned the nylon bags by submersing them in boiling water for several minutes, followed by a round of autoclaving.

The infection experiments were performed concurrently in two different labs for the Canadian and European fish (GOS, ROB, and SAY at the University of Texas at Austin, GPS and SKO at the Max Planck Institute in Plön). We incubated rinsed eggs in darkness at 4°C for 9–12 months. To stimulate hatching, we increased the incubation temperature to 20°C for 14–22 days and then exposed eggs to an alternating light/dark schedule (additional details in "Methods" in the appendix). We fed one or two hatched tapeworm coracidia to each of a large number of juvenile copepods (*Macrocyclops albidus*). We screened copepods 4–14 days later to identify infected individuals, retaining copepods with fully developed tapeworm procercoids (determined by presence of a cercomere), following Benesh and Hafer (2012). These infected copepods were fed to individually housed stickleback (after 12–24 hours acclimation), with one singly infected copepod per fish (SKO and GPS), three per fish (ROB Norway worm exposure, GOS, SAY), or five per fish (ROB Echo worm exposure). After all the copepods were consumed, we cohoused full-sibling fish in standard aquaria for each lab. At least 47 days after exposure, we dissected each fish and recorded the presence or absence of tapeworm infection. In addition, for Canadian fish we recorded the number of parasites, and we measured parasite mass for European fish.

As a result of inevitable differences in equipment, there were slight differences in the rearing and experimental procedures (see "Methods" in the appendix for additional details). For the majority of our statistical comparisons, we analyzed data from the Canadian and European fish separately.

In total, we exposed 12 GOS stickleback families, two families from SAY, and three families from both SKO and GPS. We exposed these fish to six different families of worms—three from Norway and three from Canada. Because ROB fish produce small clutches, we exposed five ROB families to a single Canadian tapeworm family, and five different families were exposed to the three Norwegian tapeworm families. All combinations of exposures between particular fish families and parasite populations are described in table 1, along with sample sizes. All components of this experiment were approved by the University of Texas Institutional Animal Care and Use Committee as part of AUP-2010-00024 and by the Ministry of Energy, Agriculture, the Environment and Rural Areas of the state of Schleswig-Holstein, Germany (reference no. V 313-72241. 123-34). Data from natural surveys and laboratory infections are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.mv5c0 (Weber et al. 2016).

Analysis of Laboratory Infection Success

We first focused on Canadian stickleback, whose recent evolutionary history allows us to designate an ancestor-descendent relationship. We used a binomial generalized linear model (GLM) to test whether the rarely infected ancestral population (SAY) is more or less susceptible to *S. solidus* than stickleback from a commonly infected derived freshwater population (GOS) or a never-infected freshwater population (ROB). We also used a Poisson GLM to compare tapeworm abundance per fish in SAY with that in GOS (or ROB) genotypes. In each GLM we used likelihood ratio tests (LRTs) to evaluate whether the addition of a population effect explains infection data better than a null model. Because siblings do not represent statistically independent samples, we also tested for family-level difference within each population. We found limited evidence for among-family differences within populations and thus focused on tests that used individuals as units of replication. Family-based analyses are included in the appendix. We similarly tested for and found no effects of sex or sex × population interactions on any infection trait ("Results" in the appendix). All analyses were performed in R (ver. 3.3.1; R Development Core Team 2014), and the package multcomp, version 1.4-5 (Hothorn et al. 2008), was used to calculate *P* values from Tukey HSD–corrected post-hoc comparisons.

We repeated these comparisons for European fish, contrasting the infection prevalence in naturally low-infection GPS with that in commonly infected SKO fish. We also used linear models to test whether the parasite index (PI; 100 × parasite mass/fish mass) differed between GPS and SKO. As noted above, because of methodological differences between labs we avoided comparing data from Canadian and European fish in a single statistical model.

We next used binomial GLMs to test whether, within each host population, infection incidence depends on par-

asite genotype (source population). In Canadian fish, we also tested whether parasite genotype affects parasite load (tapeworms per fish; Poisson GLM). Within each continent, we tested for an interaction between host and parasite genotype. Similar to host-specific comparisons, we also tested whether there were family-level infection differences with Canadian and Norwegian tapeworms.

Finally, we tested for parasite local adaptation, by categorizing each parasite-host combination as either sympatric (Canadian host and parasite or European host and parasite) or allopatric (host and parasite from different continents). Parasite local adaptation would lead to a pattern in which infection rate is higher for sympatric (coevolved) than for allopatric pairs. Host local adaptation would entail lower infection rates for sympatric combinations. Note that, although we use the term "local adaptation," this is tested at the scale of continents, not local populations within each continent.

Results

Natural Tapeworm Prevalence in Canadian Stickleback Populations

Schistocephalus solidus infection prevalence is rare in estuary samples of marine stickleback and appreciably higher in many (but not all) freshwater lakes (fig. 1). Overall, infection prevalence differs significantly between marine and freshwater fish ($LRT_{1,41}$: $\chi^2 = 9.41$, $P = 3.8e^{-3}$). There is no significant year effect, consistent with our observations that *S. solidus* prevalence is relatively stable in the few populations that we have sampled repeatedly over a decade (D. I. Bolnick, unpublished data). This scarcity of *S. solidus* in marine populations is consistent with prior observations that marine stickleback are rarely exposed to this tapeworm, which cannot hatch in water with ocean-level salinity (Simmonds and Barber 2016).

Evolution of Tapeworm Resistance in Canadian Freshwater Stickleback

We found evidence that marine stickleback's colonization of freshwater lakes coincided with the evolution of increased resistance to *S. solidus*. The rarely infected SAY population is highly susceptible to experimental *S. solidus* infection (91.7% infection incidence in the laboratory). In contrast, fish from a freshwater Canadian lake with high tapeworm prevalence (GOS) show a high degree of resistance to tapeworms from nearby Echo Lake (11 out of 87 exposures [12.6%] resulted in infection). Fish from a very-low-infection lake (ROB) also show a high degree of resistance (only 2 of 32 animals were infected). Our GLM analysis showed that fish population explained a significant proportion of infection variance (deviance $[D] = 36.9$, df $= 2$, $P = 9.81e^{-9}$; fig. 2) but that *S. solidus* family had no effect ($D = 0.95$, df $= 2$, $P = .62$). In post-hoc tests, both freshwater populations had significantly lower susceptibility (higher resistance) than the putatively ancestor-like marine fish (GOS-SAY: $z = 3.96$, $P < .001$; ROB-SAY: $z = 4.01$, $P < .001$; fig. 2). The same trend was observed when evaluating parasite load across populations ($D = 48.9$, df $= 2$, $P = 2.41e^{-11}$), with both lake populations having significantly fewer parasites per fish (GOS-SAY: $z = 6.97$, $P < 1e^{-5}$; ROB-SAY: $z = 4.83$, $P = 1e^{-5}$). Most infected SAY fish (7/11) had two or three tapeworms, while this was true of only two GOS and a single ROB fish.

Infection Susceptibility in High- and Low-Prevalence Host Populations

In European comparisons, stickleback from a frequently infected population were more resistant to local *S. solidus* than

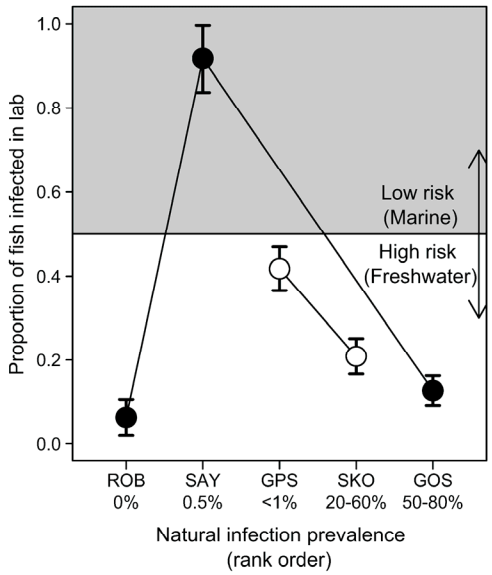

Figure 2: Within both Canada (black points) and Europe (white points), laboratory exposures to same-continent parasites reveal significant infection variation among stickleback populations ($P = 9.81e^{-9}$ in Canada; $P = 2.22e^{-3}$ in Europe). Freshwater fish have a higher risk of *Schistocephalus solidus* exposure and are generally less susceptible (more resistant) than marine fish. However, infection prevalence does not cleanly explain differences in resistance. Approximate prevalence of natural infection in wild fish is indicated below each population's abbreviation (ROB = Roberts Lake; SAY = Sayward Estuary; GPS = Großer Plöner See; SKO = Lake Skogseidvatnet; GOS = Gosling Lake). Points represent the mean infection prevalence per population (±SE).

were stickleback from a rarely infected population (fig. 2). Norwegian (SKO) stickleback (high infection prevalence in the wild) showed substantial resistance to sympatric *Schistocephalus* (only 19 out of 91 exposures [20.9%] succeeded), while German (GPS) stickleback (low infection in the wild) were significantly more susceptible (41.8% infection susceptibility; LRT: $\chi^2 = 9.36$, $P = 2.22e^{-3}$). There was also a significant difference in relative parasite mass (parasite index, PI) between GPS and SKO fish infected with Norwegian tapeworms ($F_{1,54} = 45.4$, $P = 1.10e^{-8}$). The mean PI in SKO fish was 11.3%, compared to 15.5% in GPS fish.

When the European and Canadian data are combined, naturally high-infection populations (GOS and SKO) tend to be more resistant in the laboratory, while low-infection populations are susceptible (SAY and GPS, but with ROB as an exception). GOS and ROB fish were equally resistant to infection (post-hoc test: $z = 0.971$, $P = .586$), although the former is heavily infected whereas we have never found an infected ROB fish in the wild. We elaborate on this exception in "Discussion," but here we briefly note that ROB fish are likely to be exposed to *S. solidus* in nature (all intermediate and final hosts are present). When we reanalyzed previously published diet data from these lakes (Snowberg et al. 2015), GOS and ROB stickleback had nearly identical frequencies of cyclopoid copepods in their stomach contents (2.56% vs. 2.43% by mass; $t_{49} = 0.08$, $P = .932$). Also, the tapeworm is common in nearby lakes that resident piscivorous birds regularly visit, so parasite eggs should be frequently imported into Roberts Lake. We propose that both ROB and GOS have high rates of *S. solidus* exposure and that both are resistant, but the former is essentially completely resistant.

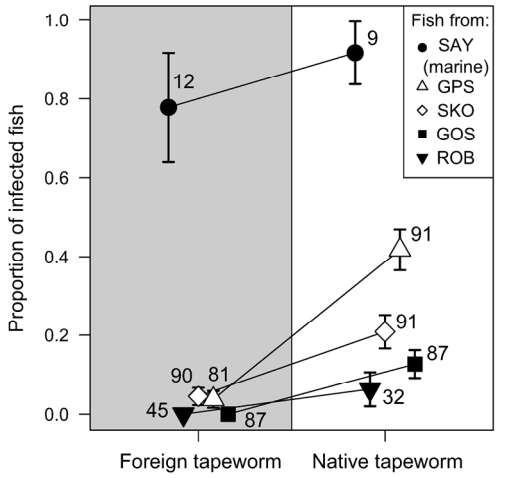

Figure 3: Infection results from exposing Canadian (black symbols) and European (white symbols) threespine stickleback to either local (same continent) or foreign (different continent) *Schistocephalus solidus* tapeworms. Symbols represent population means (±SE), and adjacent numbers are sample sizes. SAY = Sayward Estuary; GPS = Großer Plöner See; SKO = Lake Skogseidvatnet; GOS = Gosling Lake; ROB = Roberts Lake.

Infection Success of Local versus Foreign Tapeworms

Controlling for host population, we found that infection incidence was higher when fish were exposed to same-continent parasites (fig. 3). In GPS fish, Canadian tapeworms infected 4% of fish, whereas Norwegian tapeworm success was 10-fold higher. The same trend was observed in SKO fish, albeit less strongly (fig. 3). Applying a GLM with both host and tapeworm genotype shows that infection incidence in European fish depended on additive effects of both fish genotype and parasite genotype. Including tapeworm origin (continent) as a second, fixed effect was significantly better than a model considering fish population alone (LRT: $D = 49.5$, df = 1, $P = 2.01e^{-12}$). Even more dramatically, Canadian tapeworms infected 13% of the local GOS fish and 6% of ROB fish, but Norwegian tapeworms had 0% infection success in both Canadian lake populations (0 of 87 trials in GOS and 0 of 45 in ROB).

Although SAY marine fish were highly susceptible to all tapeworms, infection success was slightly higher (nonsignificantly) for native tapeworms. As a result, in Canadian fish we observed significant effects of both host and parasite genotypes (LRT, "host type" vs. "host + parasite types": $D = 17.3$, df = 1, $P = 3.20e^{-5}$). We found no significant evidence for host × parasite interactions in either Canadian or European fish.

The PI measurements showed the same pattern of increased infection success in local fish-tapeworm pairings. European tapeworms had a ~38% higher PI in their local European fish than did Canadian tapeworms ($F_{1,60} = 15.07$, $P = 2.6e^{-4}$). Because *Schistocephalus* from Norway and Canada show infection differences when exposed to the same stickleback populations, with highest infection rates and higher PI in local host-parasite pairings, we conclude that the tapeworms have evolved adaptations to improve their infection success on their local hosts. We could not test the effect of *Schistocephalus* origin on tapeworm load because there were no successful infections of GOS or ROB fish by Norwegian tapeworms. But, consistent with our previous finding that marine fish generally lack immunity to tapeworms, there was no difference in parasite load between SAY fish infected with foreign and those infected with local tapeworms (LRT: $\chi^2 = 0.12$, $P = .73$).

Discussion

Our results demonstrate that *Schistocephalus solidus* infection prevalence depends on both ecological and evolutionary factors, including parasite exposure risk as well as host and parasite genotype. When parasite genotype is held constant, hosts from a rarely infected marine population are more susceptible than hosts from freshwater populations. This result implies that infection outcome is subject to host genetic control and that stickleback in high-infection-risk freshwater habitats evolve greater resistance (albeit based on only a few host populations). When host genotype is held constant, native parasites have a higher infection success than foreign parasites. This indicates that infection outcome is also subject to parasite genetic control. We found no instances of host × parasite interaction when evaluating hosts from a single continent, but there were indications of a cross-continent interaction: freshwater fish were almost exclusively infected by same-continent parasites. This interaction suggests that each species is subject to selection to improve infection rates (for the hosts, reducing infection; for the parasite, increasing infection). Note, however, that this may not require coevolution in the strict sense of iterative reciprocal selection by each species on the other. In addition to providing evidence for evolution by both species, our results provide several insights into the species' evolutionary history and host-parasite evolutionary ecology more generally.

Host Resistance to S. solidus *Is Recently Derived*

We propose that threespine stickleback were, historically, susceptible to *S. solidus* infection. Marine stickleback are thought to have changed little in the past 12,000 years (or even longer) and so likely reflect the genotypes that may have originally colonized the postglacial landscape. The data presented here clearly indicate that marine stickleback from a single site in western Canada (SAY) are highly susceptible to *S. solidus* infection (91% of 12 fish exposed to Canadian tapeworms, with a comparable infection rate for Norwegian tapeworms). Lending further credence to our proposal, Kalbe et al. (2016) also found that Baltic Sea stickleback from a brackish lagoon (<1% infection prevalence) are highly susceptible. When each of these marine fish was exposed to a single tapeworm (the same Norwegian genotype we use here), 47% were infected. Although samples of additional marine populations would be valuable, these results clearly rule out the hypothesis that low natural tapeworm prevalence is due to heritable resistance in marine stickleback (fig. 1). Instead, low infection prevalence must reflect a low rate of *S. solidus* exposure in marine environments. This low exposure rate is consistent with another recent study showing that *S. solidus* eggs are unable to hatch in salinities above 20‰ (Simmonds and Barber 2016), and marine water has a salinity of 30‰–35‰. The few infections that we do observe most likely occur when anadromous marine fish enter freshwater to breed (Confer et al. 2012). Note that we cannot rule out the possibility that some marine populations are, or were previously, resistant. It is also possible that resistance alleles occur at low frequency in marine populations and were not sampled for our study. To the extent that resistance alleles do exist in marine populations, they may be purged if resistance carries some additional energetic or immunological costs (Auld et al. 2013).

In stark contrast to this ancestral susceptibility, we find that most recently evolved freshwater lake stickleback resist infection. This inference is supported by our comparisons of (1) two Canadian freshwater lake populations versus a nearby marine population (SAY) and (2) two European freshwater lake populations (SKO and GPS) with a Baltic Sea population (Kalbe et al. 2016; SKO had significantly lower susceptibility; GPS's was lower but not significantly different). This supports our contention that the transition into freshwater entailed an increased risk of exposure to the freshwater-hatching *S. solidus*, followed by stickleback evolution toward greater resistance. Thus, the higher infection prevalence in freshwater fish occurs despite higher resistance, not because of greater susceptibility. This strongly implies that ecological control of infection risk may frequently override the freshwater hosts' evolved resistance (or lack thereof).

If selection for resistance increases with exposure risk, this would also explain why, in both Canada and Europe, susceptibility to *S. solidus* in the lab negatively covaries with natural rates of infection (fig. 2), with the exception of ROB fish, which we discuss below. Host populations that are most frequently infected with tapeworms show the lowest susceptibility (highest resistance) in our laboratory assay. In addition, stickleback resistance also appears to involve postinfection responses. Local tapeworms grew less well (lower PI) in the European stickleback with higher natural infection prevalence, which may result from increased leukocyte responses in high-infection populations (Franke et al. 2014). This PI difference was also replicated by Kalbe et al. (2016), using independent sets of parasite and fish families (e.g., worms grew less in SKO and GPS fish than in Baltic Sea fish).

However, many freshwater lakes still exhibit high infection prevalence, despite our observation that multiple populations convergently evolved increased resistance. For most populations (except perhaps ROB), this resistance is clearly incomplete. Complete elimination of the parasite may be prevented by as-yet-unknown costs to resistance, lack of suitable genetic variation, or counterevolution by the parasite (see below).

What could explain the high resistance of ROB fish despite an apparent lack of natural infections? Although the observed prevalence and intensity of *S. solidus* infection in threespine stickleback populations can vary both within and across years (e.g., Heins et al. 2010, 2016), this is unlikely to explain the absence of infections across our 17 years of sampling in Roberts Lake. In addition, we must remember that infection prevalence is not a direct measure of exposure risk. In fact, we suspect that ROB fish are exposed to the tapeworm. ROB is ecologically similar to nearby high-infection lakes, such as GOS. Like most lakes in the Pacific Northwest (Shih and Chengalath 1994), both ROB and GOS contain abundant freshwater cyclopoid copepods (2.16 and 5.1 individuals per liter of lake water, respectively, in a June 2009 plankton tow). ROB and GOS fish also consume these copepods at similar rates. Both lakes also harbor resident piscivorous birds (loons and mergansers), which should regularly import *S. solidus* from nearby lakes. Indeed, *S. solidus* is moderately common in three of four different lakes within 0.5 km of Roberts Lake. These circumstantial arguments will in the future be tested with environmental DNA surveys of *S. solidus.*

If, as we suspect, *S. solidus* occurs in Roberts Lake, then the resident fish must be either rarely exposed (e.g., behavioral avoidance) or immune to infection. Given the prevalence of cyclopoid copepods in ROB fish stomachs, behavioral avoidance must entail an aversion to infected copepods, which appears to be unlikely (Hammerschmidt et al. 2009). Although ROB and GOS fish have similar infection success rates (parasite present/absent) when exposed to Echo Lake tapeworms, we have evidence that ROB fish are indeed more effective at eliminating the parasite population after infection (J. N. Weber, N. C. Steinel, K. C. Shim, and D. I. Bolnick, unpublished manuscript). In laboratory trials, ROB fish suppress tapeworm growth dramatically, encase the worms in cysts, and frequently kill the encysted tapeworm. This resistance applied to *S. solidus* from three geographically disparate sites in British Columbia, indicating that the evolved resistance applies to this parasite across the region as a whole. We may have simply overlooked such atypically tiny tapeworms in surveys of wild fish. Indeed, subsequent collections did reveal a low frequency of microscopic tapeworms in ROB.

Alternatively, resistance in low-infection ROB fish might be a vestigial trait, a "ghost of infections past" inherited from ancestors (in Roberts Lake or downstream McCreight Lake) that were more frequently infected. Finally, ROB resistance to *S. solidus* might be a result of correlated selection in response to other parasites that do occur in Roberts Lake.

Infect Locally, Resist Globally

Importantly, the immunity of freshwater lake stickleback applies to both local and foreign tapeworms. In fact, Norwegian tapeworms are, apparently, unable to infect GOS or ROB stickleback (0% infection success). However, those same foreign tapeworms are capable of infecting the closely related SAY stickleback at a very high rate. This latter point strongly suggests that complete resistance to Norwegian tapeworms is a genetic feature of the recently evolved GOS and ROB fish and is not an artifact of either transporting parasite eggs over a large geographic distance or laboratory procedures. Similarly, SKO and GPS fish are much less susceptible to infection by Canadian tapeworms than by the European parasites. Together, these results demonstrate that stickleback on both continents evolved generalized resistance (relative to their marine ancestors) that is effective against both local parasite genotypes and parasite genotypes from 7,000 km away. The mechanisms underlying this global resistance have yet to be determined.

An important caveat is that our observations are based on a limited number of tapeworm families, drawn from only one high-infection lake per continent. We were unable to expand this to include parasites from both low- and high-infection lakes because, by definition, the former do not yield sufficient parasites for us to culture and breed.

Despite the near-complete resistance of freshwater stickleback (from either continent) to foreign parasites, these fish can be infected by their local parasites. When we separately examine each of four lakes (GOS and ROB in Canada, GPS and SKO in Europe) and thereby hold host genotypes constant, the European and Canadian parasite genotypes differ significantly in their infection success. Specifically, in every case the local (same-continent) parasites have a higher infection incidence than the foreign (different-continent) parasites.

We cannot definitively prove, at present, that this parasite local adaptation is due to coevolution in the strict sense of a series of tit-for-tat evolutionary changes by each species in response to changes in the other. The evolution of increased host resistance appears to be a response to an ecological transition during colonization, leading to increased exposure rates, rather than a response to tapeworm evolution. It is plausible that this increased host resistance then selected for more infectious parasites, resulting in local adaptation. Or it is possible that increased infectiousness on local hosts might have evolved anyway, with or without the host evolution.

Previous studies have shown that *S. solidus* may inhibit host immune responses during infection (Scharsack et al. 2004). We propose that this inhibition may be most effective for native parasite-host combinations, because of local adaptation by parasites to their sympatric host genotypes.

We reiterate that several caveats may affect our conclusions. First, our categorizations of high-/low-prevalence populations are based on a relatively brief window of data (~10 years); low infection prevalence (e.g., in Roberts Lake)

may not imply low exposure rates. Second, we tested very few stickleback and tapeworm populations from each continent, and it has previously been shown that even geographically proximate populations can vary in parasite resistance (Kalbe and Kurtz 2006; Eizaguirre et al. 2012; Scharsack and Kalbe 2014). In particular, we provide no data on parasite evolution within continents, and it will be interesting to compare geographically adjacent parasites to test whether infectivity covaries with infection prevalence (Thrall and Burdon 2003) or geographic distance between host or parasite populations. Indeed, we expect to observe variation in resistance among neighboring lake populations of stickleback, given that infection prevalence does vary substantially among lakes on Vancouver Island (fig. 1). Finally, laboratory infection trials are unlikely to fully duplicate population differences in host immunity arising from nutritional intake, (a)biotic stressors, and other ecological causes of immunosuppression or activation (Stutz et al. 2015). We cannot speak to whether maternal effects play a role in host resistance, as the animals in this experiment had wild parents. For example, it is certainly possible that the stark absence of tapeworms in marine environments could have contributed to the high susceptibility of SAY fish. Regardless of other features of this system, all freshwater stickleback that we tested clearly showed effective resistance to tapeworms from two very distinct populations (from more than 7,000 km apart) and local parasite counteradaptation.

Theoretical Implications

Theoretical models of host-parasite coevolution typically invoke one of four "infection matrices," which convey whether infections can occur for various combinations of host and parasite genotypes. In the haploid "matching-alleles" (MA) model, the *i*th parasite genotype can infect only the *i*th host genotype, so every parasite has one and only one suitable host. In the haploid "inverse matching-alleles" (IMA) model the *i*th parasite genotype can infect all hosts except the *i*th genotype. The "gene-for-gene" model (GFG) resembles the IMA, except that there can be a universally susceptible host genotype and a universally infectious parasite. The "inverse gene-for-gene" model resembles the MA but allows for a universally resistant host.

Recently, Dybdahl et al. (2014) argued that these four models can be elegantly condensed into two alternatives. Matching-infection models focus on the parasite's capacity to recognize and infect particular hosts, and each parasite genotype is therefore specialized on a subset of hosts (the MA model is a special case; fig. 4*A*). Targeted-recognition models focus on the hosts' capacity to recognize and fend off particular pathogens, so that a match between the host and parasite genotypes enables a host immune response (e.g., host toll-like receptors bind to highly specific pathogen-associated molecular patterns). The IMA model is a special case of targeted recognition, as each host genotype recognizes and resists particular pathogens (fig. 4*B*).

In actuality, empirical studies of infection genetics do not always yield infection matrices that cleanly adhere to these classic models (Barrett 1985; Luijckx et al. 2011). This holds for our results as well. Namely, we observe a universally susceptible host genotype (SAY marine fish), which is typical of targeted-recognition models (particularly the GFG model; fig. 4*A*). And yet the infection matrix from our data (fig. 4*C*) otherwise resembles a matching-infection model (fig. 4*B*), as we observe higher infection incidence in same-continent host-parasite combinations. In addition, our infection matrix is not perfectly binary, which may indicate that host populations harbor genetic variation for infection-related traits or that this system may be similar to the "partial-infection" model examined by Agrawal and Lively (2002).

To explain this resemblance to multiple models, we argue that the evolution of host resistance (in freshwater) and parasite local counteradaptation likely entailed multiple distinct molecular changes. Hosts' ability to recognize pathogens and mount an immune response and pathogen immune evasion entail a complex network of cell types, signaling molecules, and many genes that readily generate epistatic effects. Consequently, different components of the host-parasite interaction could fit different infection matrices, and their joint effect would resemble no single model.

To illustrate a possible hybrid model (fig. 4*D*), consider the possibility that marine fish were susceptible to all extant parasite genotypes (e.g., the top row in fig. 4*A*). In transitioning to freshwater, the host evolved a generic defense that recognized an invariant, conserved feature of *S. solidus*, conferring global resistance (e.g., the infection matrix would immediately switch to the top row of fig. 4*B*). Although all hosts recognize conserved features of *S. solidus*, distinct populations may use different molecular tools to detect or eliminate the parasite (Kurtz et al. 2004). Tapeworms could respond by either evading (Hammerschmidt and Kurtz 2005) or suppressing (Franke et al. 2014) host immune responses (e.g., fig. 4*B*). To the extent that host populations differ in their immune pathways used to detect a generic parasite signal, parasites would have to evolve specialized strategies to counter the immunity of their local native host. If parasite immunosuppression targets host loci different from the genes for parasite recognition, coevolution will entail multiple distinct epistatic interactions between host and parasite loci. Some of these genetic interactions may resemble a targeted-recognition model while others resemble a matching-infection model, leading to a complex intermediate infection matrix. This hypothesis is similar to suggestions of Dybdahl et al. (2014), except that it is independent of infection stage (e.g., pre- vs. postinfection).

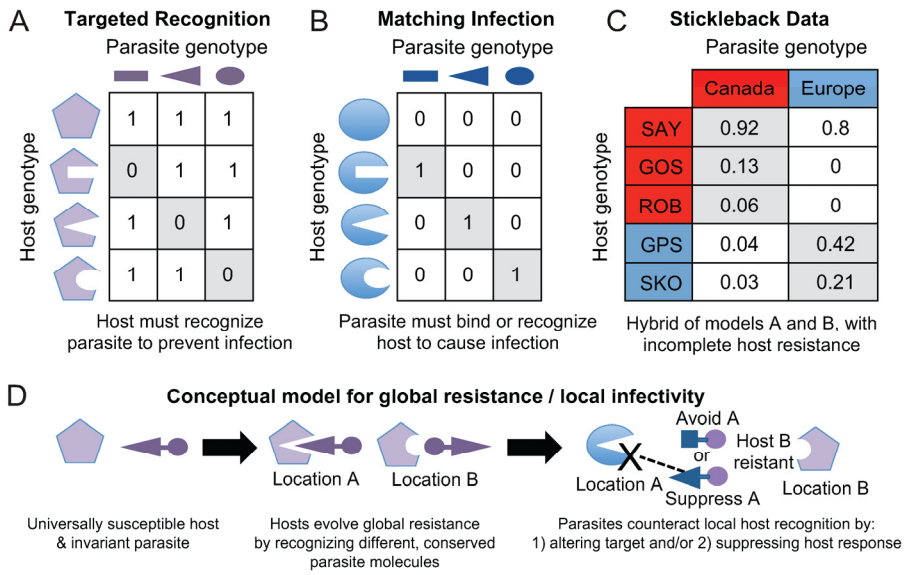

Figure 4: Illustrations of the infection matrices for targeted-recognition (*A*) and matching-allele (*B*) models, following Dybdahl et al. (2014). Cartoon molecular structures (on left) represent host protein receptors that must be bound by a parasite molecule (top) to either initiate (*A*) or prevent (*B*) infection. Combinations that can generate this binding are lightly shaded, and successful infections are indicated by 1's and unsuccessful infections by 0's. In *C*, we present the analogous infection matrix for the host and parasite genotypes sampled in this study, color-coding the fish and parasites by their continent of origin (red for Canada, blue for Europe). Matched host-parasite combinations (same-continent pairs) are shaded in the matrix, as in *A* and *B*. Numbers in the matrix represent the infection rates measured in our experimental infection trials. SAY = Sayward Estuary; GOS = Gosling Lake; ROB = Roberts Lake; GPS = Großer Plöner See; SKO = Lake Skogseidvatnet. *D*, Given the mismatch between our results and either of the standard infection-matrix models, we propose a hybrid version in which host-parasite coevolution involves evolution of two separate molecular interactions between host and parasite. First, a targeted-recognition locus (*A*) evolves to allow a universally susceptible host to recognize and resist a globally fixed parasite motif. Next, parasite evolution (at a separate matching-infection locus) allows host-specific avoidance or immunosuppression. If spatially separate host populations differ in their target motif, parasites would evolve local adaptations resembling a matching-infection model.

Orr et al. (1969) also found, similar to our data, that stickleback-tapeworm interactions may involve a diversity of mechanisms. In that study, *S. solidus* failed to infect a distant cyprinid relative of threespine stickleback, indicating there was a barrier to infection. In contrast, the tapeworm navigated into the peritoneal cavity of ninespine stickleback and differentiated into its plerocercoid stage but was unable to sustain infection past 14 days, presumably as a result of recognition and degradation by the host immune system. The ecological origin of the ninespine stickleback was not reported, so we cannot evaluate whether marine-freshwater immune transitions are a convergent feature of multiple stickleback species. But this study suggests that examining early time points of infection may be important for understanding the mechanics of stickleback-tapeworm interactions. We also anticipate that further dissection of the molecular mechanisms of host-parasite coevolution (e.g., Ebert et al. 2016) may help guide the development of more realistic infection matrix models that better represent the multistep process of invasion and resistance (e.g., Nuismer and Dybdahl 2016). Specifically, we expect quantitative trait locus mapping of host resistance (or parasite infectivity) to reveal multiple independent loci controlling various facets of infection success.

Local Adaptation

The structure of the infection matrix can influence whether theoretical models predict local adaptation by the host or by the parasite. Local adaptation also depends on the rate of gene flow, but in a counterintuitive manner: in spatially structured host-parasite coevolution, the more dispersive species is often expected to exhibit stronger local adaptation (Gandon and Nuismer 2009). This is because high gene flow introduces novel alleles into populations, thus increasing a population's genetic diversity and adaptive potential.

Consistent with this theory, several meta-analyses have found that parasites are typically more dispersive and show more local adaptation than their hosts (Greischar and Koskella 2007; Hoeksema and Forde 2008). However, much of the work on host-parasite local adaptation has been focused on metapopulations connected by appreciable gene flow, and relatively few studies have examined vertebrate hosts and their parasites (Lemoine et al. 2012). Although some studies find minimal genetic divergence in *S. solidus* over long distances within continents (Nishimura et al. 2011) and others find significant genetic structure even among lakes within a single geographic region (e.g., Alaskan lakes; Sprehn et al. 2015), it is fair to assume that *S. solidus* is generally more dispersive (via bird terminal hosts) than are lake stickleback. Because we examine populations separated by 7,000 km, our evidence for parasite local adaptation cannot be attributed to differential rates of parasite versus host gene flow between the two continents. However, it is possible that differential gene flow may lead parasites to be more locally adapted than hosts within continents and that exposure to diverse parasite populations could drive hosts to evolve nonspecific resistance.

Host and parasite coevolution is typically seen as a zero-sum game: either the parasites are adapted to the host and infection rates are high or the host is adapted to the parasite and infection rates are low. Our results provide a subtler picture. We see concurrent evidence for both host adaptation (higher resistance in derived lake than in ancestral marine fish, as well as heritable differences between lakes) and parasite adaptation (higher infection of native than of foreign parasites) and also find evidence for both pre- and postinfection differences (i.e., infection success vs. PI in European fish). The net effect still favors the host: at least for low exposure rates (1–3 tapeworms per fish), laboratory infection success rates were below 50% for all four freshwater stickleback, for both foreign and local parasites. Host genotypes that are commonly infected (in nature) were particularly resistant. This last point is nonobvious: one might imagine that highly infected populations are susceptible (hence their high infection). Instead, the data we present indicate that resistance (in the lab) does not negatively covary with infection prevalence in the wild. This implies that geographic variation in parasite prevalence depends most strongly on ecologically dictated exposure rates. Immunity evolves in response to these exposure rate differences, but immunity is insufficient to fully compensate for, or reverse, the effect of exposure. This conclusion runs counter to the previous suggestion that the evolution of resistance to commonly encountered parasites is sufficient to negate among-population differences in exposure rate (Stutz et al. 2014). These contrasting inferences suggest that we still do not sufficiently understand the relative effects of host ecology (exposure) and immunity (resistance) in regulating geographic variation in macroparasite infection rates. However, it is clear that both host ecology and immunity must be studied jointly and are likely to interact to dictate parasite epidemiology.

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APPENDIX

Supplementary Materials

Methods

The methods described in the main text generally explain the procedures performed by our two groups; however, there were a number of inevitable differences in the protocols that each group applied. We believe that these differences should have only minor impacts on how we interpret data from each experiment. But, in the interest of clarity, here we thoroughly describe these methodological differences.

The main text of the article also focuses on individual-based statistics. However, we tested sibling fish and also both males and females, so it is possible that the results for each individual were not independent. To examine whether relatedness or sex can explain variation in our data, we used linear models and binomial or Poisson GLMs. As we found little evidence for family or sex effects, we only describe those results here. We also note that our experimental design had little power to detect family differences and that our primary aim was to detect differences among host and parasite populations.

Canadian Fish Experiments

Before feeding fish with tapeworm-infected copepods, we housed fish in individual tanks for ~16 hours. Each tank was aerated to maintain high oxygen levels in the water. We gave each fish 24 hours to consume copepods and then visually checked the tanks to confirm that all copepods had been eaten. There was some variation in the number of

copepods (between two and five) that were fed to each fish, as some copepods were infected with two tapeworms, but each exposure involved a total of either three or five tapeworms. We did not carefully control the length of time that elapsed between parasite exposure and when we killed hosts. Thus, we did not measure parasite mass but only the presence or absence of infection and the total number of tapeworms in each host.

European Fish Experiments

One day before exposure to infected copepods, fish were housed in 6-L individual tanks. Those tanks were not aerated, to prevent disturbance of the copepod swim. We did not check visually whether each fish had eaten a copepod, but we starved the fish for 1 day before the exposure and gave them 36 hours to consume copepods, which in our experience usually ensures that all copepods have been eaten. Each fish was exposed to a single copepod infected with a single *Schistocephalus solidus* procercoid. All fish were killed and screened for tapeworm 57–60 days after exposure, making the weight of the procercoids directly comparable by calculating each tapeworm parasite index.

Results: Analyses of Within-Population Variation

We sampled between five and 18 fish per family, with each family exposed to between one and three local and foreign tapeworm families (table 1). This resulted in a total of 1–12 families from each stickleback population. We found no significant differences in infection susceptibility among stickleback families from within a given population (LRT: GOS: deviance $[D] = 8.99$, df $= 11$, $P = .62$; ROB: $D = 9.05$, df $= 11$, $P = .62$, SAY: only one family tested; GPS: $D = 0.59$, df $= 2$, $P = .74$; SKO: $D = 5.54$, df $= 2$, $P = .06$). We also found no evidence for sex differences in infection susceptibility within Canadian or European stickleback (LRT: GOS: $D = 0.81$, df $= 1$, $P = .37$; ROB: $D = 2.10$, df $= 1$, $P = .15$; SAY: $D = 0.51$, df $= 1$, $P = .47$; SKO: $D = 1.33$, df $= 1$, $P = .25$; GPS: $D = 0.03$, df $= 1$, $P = .86$).

We similarly tested for variation in infection success among tapeworm families, separately for European or Canadian hosts. With respect to Canadian tapeworms exposed to European fish, a model with tapeworm family as a fixed effect explained infection prevalence better than the null model (LRT: $\chi^2 = 7.99$, df $= 2$, $P = .02$). However, we performed several statistical comparisons using the same tapeworm families, and the family effect does not survive multiple-test correction. Of the three Canadian tapeworm families exposed to European stickleback, one did not infect any European fish, while the other two families infected fish at very low rates. None of the other family-based models explained infection variation better than the corresponding null models (data not shown).

Parasite index (PI) and parasite load (PL) were, respectively, measured in European or Canadian fish. When analyzing the PI data of only infected animals, we found no evidence for family effects in SKO fish ($F_{2,53} = 2.23$, $P = .12$) or in the tapeworm populations (Echo: $F_{1,4} = 0.04$, $P = .86$; Norway: $F_{2,53} = 2.23$, $P = .12$). However, we detected a significant effect of fish family on PI in GPS ($F_{2,36} = 5.99$, $P = .01$), which was driven by a significantly lower PI in one GPS family ($t = -3.22$, $P = 2.7e^{-3}$). Although potentially interesting, we consider this single-family effect a relatively minor result, considering that fish family had little effect in our other analyses, and so it is included only in the supplemental results. We found no effect of sex on PI (SKO: $F_{1,17} = 1.89$, $P = .19$; GPS: $F_{1,37} = 0.57$, $P = .46$). When we examined PL in infected GOS and ROB stickleback, we found no effect of host family, worm family, or sex. Finally, neither worm family (LRT: $\chi^2 = 0.65$, df $= 5$, $P = .99$) nor sex (LRT: $\chi^2 = 0.02$, df $= 1$, $P = .88$) affected PL in SAY stickleback, which lacked family identities.

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3 Chapter 3

Differences in host immune response in reciprocal sympatric and allopatric parasite infections

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3.1 Abstract

Coevolution between hosts and parasites drive reciprocal evolutionary change, yet the underlying genetic mechanisms remain unclear. We used the three-spined sticklebacks (*Gasterosteus aculeatus*) and its specific tapeworm parasite *Schistocephalus solidus* as an experimental model to measure host immune response adequacy. The strong phenotypic differences observed in reciprocal infection experiments using Norwegian and German populations of this host-parasite system suggest different resistance/virulence strategies for the coevolved and non-coevolved systems. To establish the dynamics of host exploitation and gene expression changes in the different host-parasite combinations, we sampled fish at 5, 7 and 9 weeks post-exposure to *S. solidus* for two immunologically-relevant organs (spleen and head-kidneys) at eight immune candidate genes. Here, we show that while the two sympatric combinations reach a similar optimum of relative parasite virulence and host resistance, allopatric combinations reveal either faster or slower host exploitation. Furthermore, we reveal gene expression patterns of antigen recognition, Th1 and Th2 response genes that indicate manipulation of the host immune system by their coevolved parasites once they are ready to infect the next host.

3.2 Introduction

Hosts and parasites impose major reciprocal selection pressures and are engaged in an evolutionary warfare in which they have to constantly counter adapt to each other. In theory this arm race should not lead to escalation as it is constrained by intrinsic costs on both sides. Parasites, especially those that survive for extended period of time in their host and depend on hosts for transmission, need to modulate exploitation to avoid over- or under-exploitation and guaranty transmission to the next host (virulence-transmission trade-off) (Ewald 1993). Hosts have to optimize resources diverted from growth, maintenance and reproduction to be allocated to immune defences (Roberts *et al*. 2004). Combined with habitat-specific parasite communities and pressures, host-parasite coevolution can result in local adaptation, where local genotypes have different optimum level of parasite virulence and host resistance, and perform on average better in their local environment than in a foreign environment (Lively & Dybdahl 2000; Kawecki & Ebert 2004; Thompson 2005; Lazzaro & Little 2009). Local adaptation as a research framework is instrumental in understanding the evolution of host resistance and parasite virulence, as well as disease epidemiology. More generally this can inform us on the basis of adaptation.

Meta-analyses indicate that local adaptation is most likely to occur in specialist parasites (Lajeunesse & Forbes 2002) with high migration rate/gene flow (Gandon & Michalakis 2002; Greischar & Koskella 2007; Hoeksema & Forde 2008) and high virulence combined to high prevalence (Greischar & Koskella 2007). The trophically transmitted cestode *Schistocephalus solidus* presents all these characteristics. It has a three-host complex life cycle but is highly specific and virulent to its secondary intermediate host the three-spined stickleback (*Gasterosteus aculeatus*). Over several weeks, *S. solidus* completes its entire somatic growth in the fish body cavity while having strong deleterious effects on the fish fitness (growth, energetics, concomitant infections; (Barber *et al*. 2008a; Benesh & Kalbe 2016)) and reproductive success (even leading to sexual castration in some case; (Heins & Baker 2008)). Furthermore, the final host insures high dispersal and gene flow, as the tapeworm reproduces sexually into the digestive tract of a fish-eating bird that disperses parasite eggs in its faeces.

S. solidus population show a strong geographical population structure and variation in virulence, potential indications of local adaptation to their host (Sprehn *et al*. 2015; Scharsack *et al*. 2016).

Interestingly, three-spined sticklebacks and *S. solidus* have similar generation time. The fish reproduce annually and are usually exposed to a single wave of infection (Tierney *et al*. 1996). This implies close coevolution and makes the stickleback-*S. solidus* system a very suitable model for experimental studies on reciprocal adaptation in host-parasite interactions. In the first two weeks of infection, *S. solidus* gains up to 10% of its weight per day (Barber & Svensson 2003) and can represent up to 50% of its host body weight by the time its reaches a growth plateau (Arme & Owen 1967). Rapidly reaching an important size might be a strategy to resist elimination by the host innate immune system (Scharsack *et al*. 2007b) and *S. solidus* has to achieve a 50 mg threshold to become infective to the final host (Tierney & Crompton 1992). However, over-exploitation (exploiting the host too much, too fast) could increase host mortality risk and lower the chance of transmission to the next host. On the other side of the interaction, host should limit the immune reaction only to necessary level as to avoid diverting energy away from other important functions (like growth and reproduction) and prevent immunopathology (Schmid-Hempel 2003; Graham *et al*. 2005). This response adjustment must rely on a fine-tuned molecular cross-talk between *S. solidus* and the host immune system, and translate into the selection for an optimum level of parasite virulence and host resistance. *S. solidus* has been shown to evade and manipulate the fish immune response in varying ways at different stages of the infection. While *S. solidus* is able to evade the host's immune detection when it establishes in the fish body cavity, the inhibition of the immune reaction stops once it has reached the infectivity threshold of 50 mg around 6 and 9 weeks in the infection (Scharsack *et al*. 2007b). However, the exact molecular mechanism(s) by which *S. solidus* manipulates its host immune system and how these adaptive strategies may vary between different populations/genotypes remains unknown.

Infection phenotypes (expressed host resistance and parasite virulence) are the outcomes of the complex interactions between host and parasite genotypes (Lambrechts *et al*. 2006). Adaptation to specific parasite genotypes

can be expressed through the selection of specific resistance alleles (genetic sequence variation), but also via modulation of immune gene expression (Barribeau *et al*. 2014; Lenz 2015). In fact, three-spined stickleback populations exhibit both immunogenetic differences and immune gene expression modulations that are driven by parasite communities' divergence (Eizaguirre *et al*. 2011, 2012; Lenz *et al*. 2013). Therefore comparing the expression of immune genes in coevolved and non-coevolved host-parasites combinations can enable us to identify which genes and molecular pathways are implicated in the phenotypic differences observed in *S. solidus* infections.

Using two stickleback/*S. solidus* coevolved populations with different levels of natural parasite prevalence, we performed a fully reciprocal exposure experiment to obtain sympatric and allopatric combinations, and used a candidate gene approach to measure differential adequacy of the host immune response in the different combinations. Kalbe *et al*. (2016) previously found that those two populations have divergent coevolutionary trajectories, which led them nonetheless towards the same relative optimum level of virulence and resistance. To follow host exploitation by the parasite and immune response by the host over time, fish were dissected at three time points post exposure to capture the growth and plateau phase of *S. solidus* infections. At these time points, we measured relative host exploitation (as relative worm weight) and the gene expression of seven candidate genes involved in three key immune functions: antigen recognition, Th1 response, and Th2 response.

3.3 Material and methods

3.3.1 Experimental host and parasite populations

3.3.1.1 Host and parasite populations

We selected two genetically distinct host populations of three-spined sticklebacks (*Gasterosteus aculeatus*) separated by ca. 750 km and presenting two contrasting *Schistocephalus solidus* prevalence: a German brackish lagoon with low prevalence (DE, less than 1% prevalence; Neustädter Binnenwasser, Kremper Au, Neustadt, Germany, 54° 06′ 40″ N, 10° 48′ 50″ E) and a Norwegian lake with high prevalence (NO, ~60% prevalence; Skogseidvatnet, Hordaland, Fusa, Norway, 60° 14' 44" N, 05° 55' 03" E) (Feulner *et al*. 2015; Weber *et al*. 2017).

3.3.1.2 Fish families breeding

For each population, parental fish were caught September 2008, kept in the lab under standardized conditions and randomly paired in February and March 2009 to produce two independent first-generation laboratory-bred fish families. Several sibships per breeding pair were produced and reared in summer conditions (18°C, 16h:8h light:dark photoperiod) for three months.

3.3.1.3 Schistocephalus solidus strains breeding

For each population, four tapeworms collected from infected sticklebacks (caught in October 2005 in the DE population, and in January 2006 in the NO population) were crossed into two independent *S. solidus* families using the *in vitro* system (Smyth 1946; Wedekind 1997). The eggs produced constituted the first generation of two laboratory-bred *S. solidus* lines (DE and NO) and were used to singly expose first-generation laboratory-bred fish from their respective population of origin (see (Kalbe *et al*. 2016) for details on the worm breeding and fish exposure protocols). Four months after exposure, fish were dissected to retrieve the first laboratory-bred generation of the *S. solidus* plerocercoids. Those worms were paired within their population, with each pair consisting of one individual from a different family to prevent inbreeding. This resulted in the second generation of laboratory-bred *S. solidus* families. We used *S. solidus* bred for two generations in controlled conditions to normalize the effect of within-

host parasite competition could have on offsprings' infectivity and virulence as *S. solidus* from the NO population were collected from multiply infected fish contrary to the DE population. The eggs produced were kept at +4°C until use.

3.3.2Experimental exposures

Two allopatric combinations (host and parasite from different origins) and two sympatric combinations (host and parasite from same origin) from both populations were produced by performing full-reciprocal experimental exposure (experimental treatment noted as 'fish origin-(parasite origin)'): (1) Norwegian fish exposed to Norwegian worm NO-(NO); (2) German fish exposed to German worm DE-(DE) (3) Norwegian fish exposed to German worm NO-(DE) and (4) German fish exposed to Norwegian worm DE-(DE) (table 1).

For each population, 10 to 12 fish from each fish families were planned to be exposed in a full factorial design to all four tapeworm families for each time point. However, due to ichthyophthiriosis related fish mortality before and after exposure, some of the combinations could not be carried out resulting in an incomplete fish-worm family design (see table S1). For each population, 5 to 9 control fish were treated similarly to exposed fish but were not fed any copepod.

After each fish was let to feed on a single infected copepod, fish were kept in summer conditions grouped per family at density 6-12 fish per 16L tank and fed *ad libitum* with frozen chironomid until dissection.

3.3.3Dissections

Previous experiments on the same populations showed differences between tapeworm size in sympatric and allopatric combination at 12 weeks post exposure (PE), when the tapeworm should have reached their growth plateau (Benesh & Kalbe 2016; Kalbe *et al*. 2016). Therefore, to catch the kinetic of tapeworm growth, we killed the fish by an overdose of MS22 (tricaine methanesulfonate, 1 mg/ml) at three different time points: five, seven or nine weeks post exposure (PE).

Table 1: Summary table of experimental infection setup and sample sizes.

Combination	*5 weeks +PE*		*7 weeks +PE*		*9 weeks +PE*		*Total*		
	uninfected	infected	uninfected	infected	uninfected	infected	dead	uninfected	infected
Sympatric									
DE-(DE)	21	6	7	3	4	8	-	32	17
NO-(NO)	11	14	20	9	22	9	3	53	32
Allopatric									
DE-(NO)	12	10	14	7	23	6	6	49	23
NO-(DE)	9	8	14	3	9	9	1	32	20
Control	control		control		control		dead	control	
DE	6		5		9		11	20	
NO	8		6		9		13	23	

For each fish we recorded the infection status (uninfected or infected fish, which resulted in three infectious status groups: unexposed control, uninfected and infected fish), standard body length (±0.01 mm) and body weight (±0.1 mg). We immediately sampled, weighted (±0.1 mg) and stored intact in 50 µl of RNA-later (Ambion) two important immunological organs, the spleen and the head-kidneys. In fish, head-kidneys have a similar role as the bone marrow in mammals, assuming functions of haematopoiesis but also, antigen processing, antibody production (IgM) and immune memory (B cells) (Tort, Balasch & Mackenzie 2003; Alvarez-Pellitero 2008). The spleen is with the thymus and kidney one of the fish main lymphoid organs where take place antigen presentation and the initiation of adaptive immune responses (Alvarez-Pellitero 2008). Tissues were allowed to stabilize in RNA-later at room temperature for a day and then transferred to -20°C for 78 to 190 days until RNA extraction.

We calculated the fish condition factor (CF) (following (Frischknecht 1993)) to assess individual condition, the splenosomatic index (SSI, as the ratio of spleen to fish somatic weight) and the head-kidney index (HKI, as the ratio of head-kidney to fish somatic weight) as a measure of immune activation in response to parasite infections (Arnott, Barber & Huntingford 2000; Lefebvre *et al*. 2004). As a measure of *S. solidus* virulence and host exploitation, we calculated the parasite index (PI, as the relative weight of the worm over the somatic weight of the fish; (Arme & Owen 1967)), which correlates with the efficiency of host adaptive immunity to control parasite growth (Kurtz *et al*. 2004). Additionally, as *S. solidus* needs to reach a 50 mg threshold to be infective in the final host (Tierney & Crompton 1992), the weight of the worm could be used to assess successful exploitation of the fish host and a proxy for virulence (Heins, Singer & Baker 1999; Barber *et al*. 2008b).

3.3.4 Quantification of differential gene expression

3.3.4.1 RNA extraction and reverse transcription

We homogenized the whole spleen (0.3 SE±0.3 mg) and head-kidney (0.9 SE±0.5 mg) using a Retsch TissueLyser II mill (2 × 2 min 30 Hz, Qiagen). We extracted total RNA using Total RNA Isolation NucleoSpin® 96RNA (Macheray-Nagel) according to the manufacturer's instructions, including a DNase

treatment step. For each sample, we used 10 µl of RNA template to obtained 20 µl of cDNA with the Omniscript RT Kit (Qiagen). We mixed the RNA template with 2 µl of 10x Buffer RT, 2 µl of dNTP's (0.5 mM/dNTP of final concentration), 2 µl Oligo dT-Primer (3.3 µM final concentration) (Qiagen), 1 µl of Reverse Transcriptase, 0.2 µl of RNase Inhibitor (0.8 units final concentration) (Qiagen) and 2.8 µl of RNase free water and incubated at 37°C for 60 min. Nucleic acid quantification and cDNA purity measurements were performed on 6 randomly selected samples out of each extracted 96-well plate using a Nanodrop-1000 spectrophotometer (Thermo Scientific) with a desired 260/280 absorbance ratio >1.80. The cDNA was stored at -20°C until use (see table S2 for sample sizes).

3.3.4.2 Real-Time quantitative PCR (RT-qPCR) conditions

We measured the expression in the spleen and the head-kidneys of eight candidate genes involved in three important pathways of the immune response: i) antigen recognition and presentation (IgM, MHC-IIß, TLR2), ii) Th1 cellular immunity response (MIF, SOD2, TNFα) and iii) Th2 humoral immunity response (IL-1ß, TGF-ß1; table 2-3). Quantitative real-time PCR amplifications (qPCR) were performed on a LightCycler® 480 Instrument (Roche Applied Science) with a 384-well block. For each primer pair, 4 µl of 1:10 fold diluted cDNA template was mixed with 10 µl of LightCycler® 480 SYBR Green I Master (Roche Applied Science, Mannheim, Baden-Württemberg, Germany), 1 µl (20 ρmol) of each primers and 4 µl of RNase free water, for a final volume of 20 µl. PCR followed by a dissociation analysis were carried out following the conditions described by Hibbeler *et al*. (2008), but limited to 40 PCR cycles (see table S3). All primers were order from Eurofins MWG Operon (Ebersberg, Bavaria, Germany).

3.3.4.3 RT-qPCR plate design

We used four different gene maximization plate designs allowing us to run all the samples (240 head-kidneys; 247 spleens) for two to three genes of interest and the reference gene over seventeen 384-well plates; we ran a total of fourty-nine 384-well plates (design A: 16 plates with MHC-IIß, TLR2, TNFα and UBC; design B: 16 plates with IL-1ß, SOD2, IgM and UBC; design C: 16 plates with MIF, TGF-ß1 and UBC; design D: 1 plates with all genes). Each sample for each gene ran in triplicates on the same plate; samples from the different treatment groups were randomly spread across plates.

Table 2: RT-qPCR primers for the amplification of the target genes and one reference gene.

Gene	Esembl gene ID	Primer sequence 5'-3'	Amplicon length (bp)	Tm (°C)	Chromosome	Locus	Exon spanning	Amplicon length (bp)	Author
IgM	ENSGACG00000012799, ENSGACG00000012769, ENSGACG00000012783 (paralogues)	Fw: AAGGCAGGAGAATGAAACCTTGG	23	61	Group XI	Exon 5	no	175-202	Hibbeler, S. (unpublished)
		Re: CCGAGTGAGCAGACAGGGACTGG	22	66		Exon 4	no		
IL-1β	ENSGACG00000014611	Fw: GCAGTTCGCCGCCACATCTCCAGATCAG	28	71	Group XIII	Exon 3	no	182	Krause, A. (2011)
		Re: CGCAGGGTGCAGGTACGCCGACATGGTC	28	74		Exon 4	no		
MIF	ENSGACG00000017868	Fw: GGCCAAAAGCGACGTGCCCGCGTCTC	26	73	Group IV	Exon 1	no	309	Krause, A. (2011)
		Re: GTAGTGTTGTTCCAGGCCACATTGGCT	27	66		Exon 3	no		
MHC-IIß	ENSGACG00000017967, ENSGACG00000000350, ENSGACG00000000336, ENSGACG00000019051, ENSGACG00000000346, ENSGACG00000000343 (paralogues)	Fw: AACTCCACTGAGCTGAAGGACATC	24	63	Group III, VII, scaffold 131	multiple	no	267-270	Reusch, T. (2003)
		Re: CGTCTCAGAGTGCAGCCTGACGT	23	66		multiple	no		
SOD2	ENSGACG00000009000	Fw: ATGTGACCGCTCAGATTGC	19	57	Group XVIII	Exon 4	no	272	Erin, N. I. (present study)
		Re: CTGGTTAGCACAAGCAGCTACG	22	62		Exon 5	no		
TLR2	ENSGACG00000018669	Fw: CTGACCAGGTACGAAGCCG	19	61	Group VII	Exon 4-5	yes	230	Hibbeler, S. (unpublished)
		Re: CGGAAGGTGATTTTCCTGACC	21	60		Exon 5-6	yes		
TGF-ß1	ENSGACG00000012798	Fw: TGTCCTTCGACGTCACTGAG	20	59	Group I	Exon 5	no	190	M. Ritter
		Re: GGTGGTTGCTTTGTCCTCAT	20	57		Exon 6	no		
TNFα	ENSGACG00000013372	Fw: TACGTTGAGGCAAATCAGCA	20	55	Group XX	Exon 3	no	203	M. Ritter
		Re: AGGACGACTGGCTGTAGACG	20	61		Exon 4	no		
UBC	ENSGACG00000008021	Fw: AGACGGGCATAGCACTTGC	19	59	Group VIII	Exon 4-5	yes	218	Hibbeler, S. (2008)
		Re: CAGGACAAGGAAGGCATCC	19	59		Exon 2-3	yes		

Table 3: Brief description of the target genes. Toll-like receptors (TRLs) recognize a large array of conserved molecular motifs (pathogen-associated molecular patterns or PAMPs) and play an important role in the activation of the innate immunity (Akira, Yamamoto & Takeda 2003). Stimulation of TLRs results in an intracellular signalling pathway that induces an acute inflammatory response and the activation of dendritic cells (Werling & Jungi 2003). It also connects innate and adaptive immunity as TRLs induce the secretion of cytokines and chemokines (secreted signalling proteins), which together with the **MHC** molecule activation lead to the initiation of a specific immune response through lymphocyte activation (Werling & Jungi 2003). While TLRs predominantly recognize microbial antigens, **TLR2** can also be stimulated by helminth antigens (Layland *et al*. 2007; Anthony *et al*. 2007), and in three-spined stickleback, the diversity of MHC-IIß alleles correlates with *S. solidus* burden (Kurtz *et al*. 2004). After antigen recognition, cytokines induce and regulate the nature of the immune response by stimulating the growth, differentiation and activation of different immune effector cells. Early in the immune response, pro-inflammatory cytokines such as **IL-1ß** and **TNFα** stimulate the up-regulation of innate immune factors, pathogens recognition and antigens presentation. During the innate immune response (and during the phagocytosis of opsonized antigens), macrophage and granulocytes produce oxygen free radicals to kill pathogens, the antioxidant **SOD2** protects the host tissues by detoxifying oxygen radicals (Secombes & Fletcher 1992). Later on, anti-inflammatory cytokines such as **TGF-ß1** mediate the response towards an adaptive immune response (Reyes-Cerpa *et al*. 2012; Zou & Secombes 2016). Two main cross-regulating immune pathways are involved in the response to infectious disease: Th1 is a cell-mediated pro-inflammatory response targeting intracellular pathogens and Th2 is a humoral anti-inflammatory (antibody) response targeting extracellular pathogens like helminths (Buchmann 2012). While helminth antigens initiate a polarized Th2 response and the production of specific antibodies by B-cell (mostly **IgM** in fish; Buchmann 2012), helminths are also known to manipulate and modulate the immune response of their host (Maizels *et al*. 2004; Jackson *et al*. 2009; Moreau & Chauvin 2010; Harnett & Harnett 2010). In particular, schistosomes are able to skew the immune response to increase the Th1 response over the Th2 (Hervé *et al*. 2003).

Symbol	Gene name	Molecule type	Producing cells	Receptor cells	Function	Pathway
IgM	Immunoglobulin Mu Chain	Antibody	B-cell	-		Antigen recognition
IL-1β	Interleukin-1 Beta Precursor	Pro-inflammatory cytokine	Macrophage, epithelial cells	T-cell, macrophage	Fever; T-cell and macrophage activation	TH2 pathway
MIF	Macrophage Migration Inhibitory Factor	Pro-inflammatory cytokine	Lymphocyte, monocyte, macrophage, dentrocyte, neutrophil, eosinophil, mastocyte, basophil	Macrophage	Macrophage migration inhibition; macrophage activation; induce steroid resistance	TH1 pathway
MHC-IIß	Major Histocompatibility Complex class IIß	Cell-surface receptor	Antigen-presenting cells (macrophage, dendrocyte, B-cells)	T-cell	T-cell activation	Antigen presentation
SOD2	SuperOxide Dismutase	Antioxidant	-	-	Anti-inflammatory (detoxification of oxygen radicals)	-
TLR2	Toll-Like Receptor 2	Cell-surface receptor	Macrophage, dendrocyte, eosinophil, basophil, mastocyte	-	Induce production of pro-inflammatory cytokines	Antigen recognition
TGF-ß1	Transforming Growth Factor Beta 1	Anti-inflammatory cytokine	Lymphocyte, chondrocyte, monocyte, macrophage, platelets, mastocyte	Lymphocyte, macrophage	Inhibits macrophage, T-cell, and B-cell ; activate fibroblast growth; tissue repair	TH2 pathway
TNFα	Tumor Necrosis Factor Alpha	Pro-inflammatory cytokine	Macrophage, mastocyte, NK cells, T-cells	Macrophage	Endothelial cell activation; induce NO (nitric oxide) production; fever; septic shock	TH1 pathway

One “no template control” (NTC, to detect potential contamination) and one “inter-run calibrator” (IRC) were included on each plate and run in triplicates for each gene (Bustin *et al*. 2009). The IRC was constituted of a pool of 1 µl from each experimental cDNA sample, 1:10 fold diluted. The IRC controlled for technical variation within a given gene ran across different plates (repeatability measure; (Hellemans *et al*. 2007; D’haene & Hellemans 2010)). In total 16371 qPCR reactions were prepared manually (NTC: 555; IRC: 556; HK samples: 7871; SP samples: 7389).

3.3.4.4 Normalized gene expression calculation and analysis

We determined the qPCR efficiency and linear dynamic range of each primer pairs with standard curves from serial dilutions (see Supp. Analysis SA2). All samples were checked using different quality control criteria (see Supp. Analysis SA3) and we used qBase^{+} (Biogazelle, Zwijnaarde, Belgium) to calculate the calibrated normalized relative quantities (CNRQs) from the median of each sample triplicates, as described by Hellemans *et al*. (2007). We used the measured qPCR efficiencies, UBC as a reference gene (see Supp. Analysis SA1 for reference gene choice) and the IRC in the calculations. CNRQs were log transformed for further analysis.

3.3.5 Statistical analysis

All data visualization (‘ggplot2’ package) and statistics were carried out using R 3.2.3 (The R Foundation for Statistical Computing, 2016).

3.3.5.1 Analysis of Phenotypic results

We assessed the effect of the experimental treatment on parasite growth (worm weight) and host exploitation (PI) with linear mixed models (lmer function, lme4 library) and a type-III ANOVA (‘Anova’ function, ‘car’ library) using the factors infection combination (NO-(NO), DE-(DE), NO-(DE), DE-(DE)), number of weeks PE (5, 7 and 9 weeks PE) and their interaction as independent variable. Fish family and worm family were added as random factors in all the models. We used a generalized linear mixed-effect model (‘glmer’ function, ‘lme4’ package) with a binomial family to test for the effect of fish origin and worm origin (NO and DE) on the overall proportion of infected fish after

exposure. We used fish family, worm family and time point as random factors in the model.

We used the same models as previously described to assess the effect of the treatment on infected fish, relative to control fish, on fish condition (CF) and fish health (SSI; HKI). Again, fish family and worm family had an effect and were used as random factors. We used Box-Cox transformations to help meet normality and homoscedasticity assumptions (log transformed CF; log transformed SSI; 4th square root of HKI). We used Tukey's tests for all post hoc comparisons ('lsmeans' function, 'lsmeans' package).

3.3.5.2 Analysis of differential immune gene expression

For the immune gene expression, we focused on infected and control fish. First, we analysed how the gene expression of each candidate gene fluctuated across time to determine if expression remained the same or were up- or down-regulated in the experimental combinations compare to their respective controls. This was performed by normalising the gene expression data by calculating the fold difference to control (fold difference = ($CNRQ_{treatment}$ – mean($CNRQ_{control}$))/ mean($CNRQ_{control}$)) within each time point for the different fish origin ((DE-(DE) and DE-(NO) vs. DE-ctrl; NO-(NO) and NO-(DE) vs. NO-ctrl). As CNRQs represent the quantity of gene transcripts relative to the reference gene (UBC), they are expressed in positive (more transcript for candidate than reference gene) and negative values (less transcript for candidate than reference gene). Because it involves a division, calculating the fold difference could change the sign, and thus direction of gene expression. Therefore, before calculating fold differences, we added the smallest CNRQ value to all of them ($CNRQ_{transformed}$ = CNRQ + 2.1) as a way to conserve the relative difference between all CNRQs while transforming them in positive values. We then used linear mixed-effect models ('lmer' function, 'lme4' package) and type-III ANOVA to test the gene's fold difference to control, for each fish origin for: i) the effect of treatment and across all time points and ii) the effect of the treatment within each time point. Tukey's tests were used for post hoc comparisons ('lsmeans' function, 'lsmeans' package). Fish family and worm family were included as random factors in the models where possible.

We then looked at the variation in overall gene expression profile. After replacing missing CNRQ values with the median of the respective gene, we performed multivariate analysis (PERMANOVA) on Euclidian correlation distance matrices ('adonis' function, 'vegan' package). To limit the bias induced by missing value replacement, the samples missing four or more CNRQs out of the eight candidate genes were excluded from the analysis (control fish: 7/42; infected fish: 6/85). The PERMANOVA was run with 999 permutations on different subsets of the data. The effect of treatment on gene expression was tested both for all the data and separately for each time point (5, 7 and 9 weeks PE) for the following: i) the difference in gene expression between the two control groups (DE-ctrl and NO-ctrl); ii) the effect of infection on gene expression by comparing each experimental combination to its respective control group (DE-(DE) vs. DE-ctrl; DE-(NO) vs. DE-ctrl; NO-(NO) vs. NO-ctrl; NO-(DE) vs. NO-ctrl); iii) the effect of worm origin on gene expression for each fish origin (DE fish: DE-(DE) vs. DE-(NO); NO fish: NO-(NO) vs. NO-(DE)). iv) the effect of fish origin on gene expression for each worm origin (DE worm: DE-(DE) vs. NO-(DE); NO worm: NO-(NO) vs. DE-(NO). To assess the difference in gene expression across time, we also tested the effect of time on gene expression within each experimental combination (DE-(DE), DE-(NO), NO-(NO), NO-(DE)) and control groups (DE-ctrl, NO-ctrl). In all PERMANOVA tests, fish family was included as a block effect where possible (strata option in 'adonis'). This analysis was carried out for different gene subsets corresponding to the complete immune genes set (IgM, IL-1ß, MHC-IIß, MIF, SOD2, TGF-ß1, TLR2, TNFα) and the following functional groups: antigen recognition genes (IgM, MHC-IIß, TLR2), the Th1 response genes (MIF, TNFα), and the Th2 response genes (IL-1ß, SOD2, TGF-ß1, TLR2).

3.4 Results

3.4.1 Parasite infectivity and growth

Thirty-four out of 301 fish died during the experiment and were excluded from the analyses (table 1). We did not find any significant effect of the fish origin (ANOVA, $F_{1,257} = 0.172$, $P = 0.678$) or the worm origin on the proportion of infected fish (ANOVA, $F_{1,257} = 0.003$, $P = 0.957$; see fig. S1). The worm weight and PI were significantly affected by the experimental treatment (ANOVA, worm weight: ANOVA, $F_{4,88} = 78.299$, $P < 0.001$; PI: $F_{4,88} = 47.378$, $P < 0.001$), the time points (worm weight: ANOVA, $F_{3,89} = 83.390$, $P < 0.001$; PI: ANOVA, $F_{3,89} = 56.839$, $P < 0.001$), and the interaction between treatment and time points (worm weight: ANOVA, $F_{12,80} = 38.375$, $P < 0.001$; PI: ANOVA, $F_{12,80} = 25.106$, $P < 0.001$; fig. 1).

3.4.1.1 Worm size variation across time

At 5 weeks PE, the mean worm weight of NO-parasites in both allopatric and sympatric combinations (NO-(NO) and DE-(NO)) was already over the 50 mg threshold (fig. 1a). DE-parasites in sympatric combination (DE-(DE)) passed this threshold later, at 7 weeks PE (fig. 1a). At 9 weeks PE, DE-parasites in allopatric combination NO-(DE) weighted on average 34.6 mg and 77.8% (7 out of 9) of those worms remained under 50 mg (fig. 1a).

Over the complete time frame (comparing 5 and 9 weeks PE), worms in all infection combinations significantly increased in both weight and PI (Tukey's HSD, $P < 0.001$; fig. 1, table 4), except for the DE-parasites in allopatric combination NO-(DE) (worm weight: Tukey's HSD, $P = 0.222$, PI: Tukey's HSD, $P = 0.357$). When looking at the early time frame (5 to 7 weeks PE), only NO-parasites in the allopatric combination DE-(NO) significantly increased in weight (Tukey's HSD, $P = 0.002$), and all combinations (except for NO-(DE)) significantly increased in PI (for all comparisons, Tukey's HSD, $P = <0.050$; table 4). In the later time frame (7 to 5 weeks PE), both NO-parasites in allopatric and sympatric combinations significantly increased in weight (for both, Tukey's HSD, $P < 0.001$; table 4), but none of the combinations significantly increased in PI (for all comparisons, Tukey's HSD, $P = >0.050$; table 4). In contrast, DE-parasites in

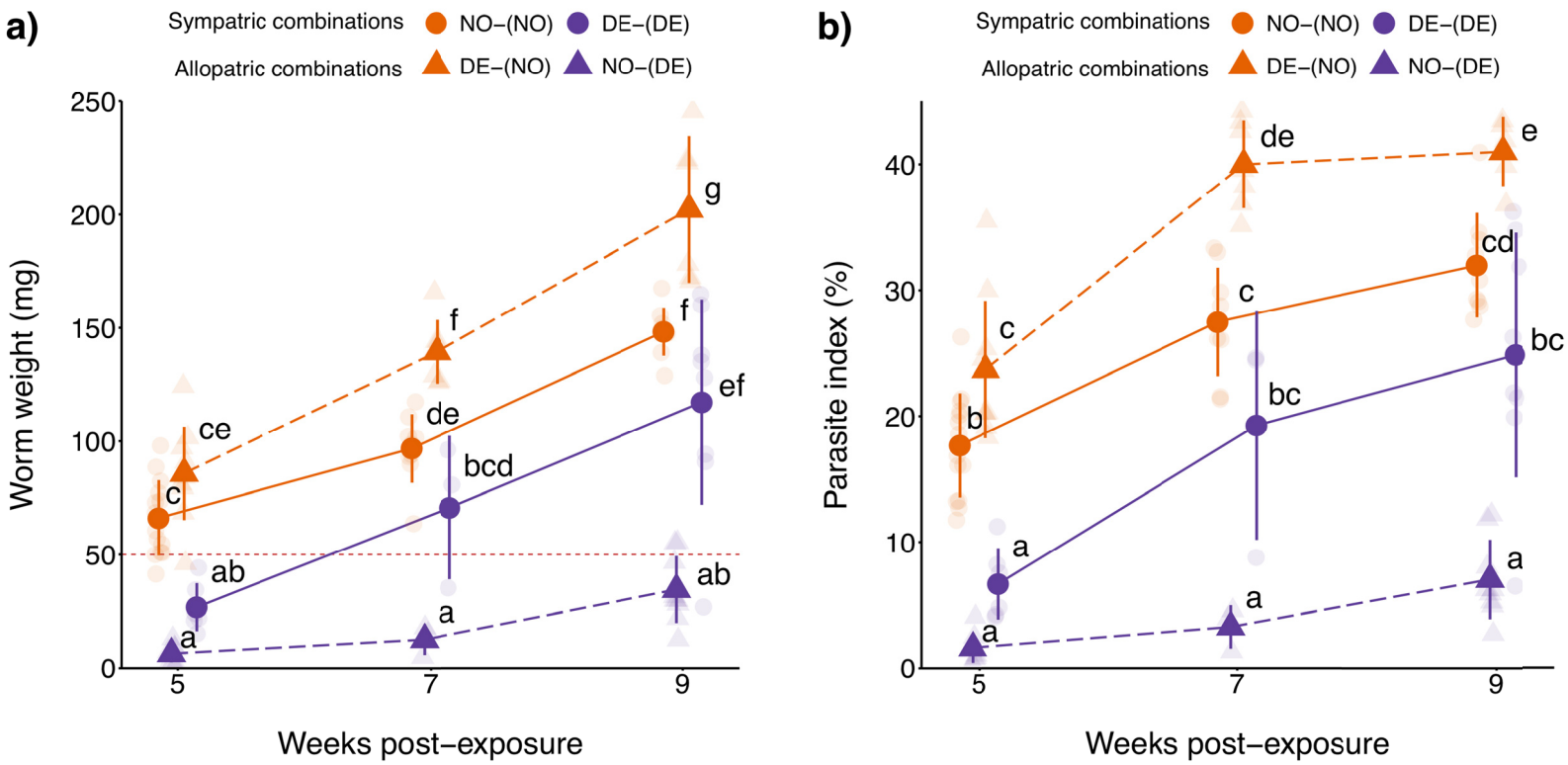


Figure 1

a) Worm weight and **b)** parasite index in the different infection combinations (sympatric NO-(NO), sympatric DE-(DE), allopatric NO-(DE), allopatric DE-(NO)) at the different time points (5, 7 and 9 weeks PE).

allopatric combination NO-(DE) never significantly increase in weight nor PI between time points, or over the entire time frame (Tukey's HSD at 5 to 7, 7 to 9, and 5 to 9 weeks PE respectively; worm weight: P = 1.000, P = 0.928, P = 0.222; PI: P = 1.000, P = 0.979, P = 0.357; table 4).

3.4.1.2 Worm size variation between experimental combinations

Regardless of the time points or combination types, NO-parasites always had a higher mean worm weight and mean PI than DE-parasites (fig. 1). While this differences were significant at 5 weeks PE, there was no significant difference in parasite weight between the two sympatric combinations DE-(DE) and NO-(NO) at 7 or 9 weeks PE (7 weeks PE: Tukey's HSD, P = 0.886; 9 weeks PE: Tukey's HSD, P = 0.463; table 4). A similar pattern was observed for the PI (7 weeks PE: Tukey's HSD, P = 0.669; 9 weeks PE: Tukey's HSD, P = 0.722; table 4). The NO-parasites in allopatric combination DE-(NO) had always significantly higher mean worm weight and mean PI than any other combination (Tukey's HSD, $P < 0.050$), apart from its respective sympatric combination NO-(NO) at 5 and 7 weeks PE (Tukey's HSD, $P > 0.050$; table 4). Conversely, from 7 weeks PE on ends, DE-parasites in allopatric combination NO-(DE) had always significantly lower mean worm weight and mean PI than any other combination (Tukey's HSD, $P < 0.050$; table 4).

3.4.2 Physiological effect of infection on the host

3.4.2.1 Fish weight and condition factor

Within each time point, there was no significant difference in fish somatic weight for any of the experimental combinations (ANOVA, $F_{5,295} = 0.853$, P = 0.973). At 9 weeks PE, all fish increased significantly in somatic weight compare to the previous time points (ANOVA, $F_{2,295} = 56.539$, $P < 0.001$; Tukey's HSD, 5 vs. 9 weeks PE: $P < 0.001$; 7 vs. 9 weeks PE: $P < 0.001$; fig. 2).

The fish condition factor (CF) was significantly affected by the fish origin (ANOVA, $F_{1,133} = 24.132$, $P < 0.001$; fig. 3a), the time point (ANOVA, $F_{2,132} = 7.382$, $P = 0.025$) and the interaction of fish origin, worm origin and time point (ANOVA, $F_{4,130} = 13.869$, $P = 0.008$). After correcting for multiple testing, the pairwise comparison showed that fish condition was stable across time in every experimental combination, apart for DE-ctrl, which significantly increases in

Table 4: Linear mixed effect model on the virulence of *S. solidus* in the different infection combinations across time. Table shows the results of pairwise comparison between the interaction of infection combinations (sympatric NO-(NO), sympatric DE-(DE), allopatric NO-(DE), allopatric DE-(NO)) and time points (5, 7 and 9 weeks PE, respectively noted here as T1, T2 and T3), a) worm weight b) parasite index (PI), significant *P*-values (<0.05) in bold.

a) Worm weight:					
Infection combination x Week PE	**Estimate**	**SE**	**D.F.**	***T*-value**	***P*-value**
DE-(DE) across time					
DE-(DE).T1 - DE-(DE).T2	-43.983	15.029	78.50	-2.927	0.152
DE-(DE).T1 - DE-(DE).T3	-90.492	11.380	77.19	-7.952	**<0.001**
DE-(DE).T2 - DE-(DE).T3	-46.508	14.336	77.98	-3.244	0.070
NO-(NO) across time					
NO-(NO).T1 - NO-(NO).T2	-30.633	9.278	78.52	-3.302	0.060
NO-(NO).T1 - NO-(NO).T3	-82.111	9.432	79.84	-8.706	**<0.001**
NO-(NO).T2 - NO-(NO).T3	-51.478	10.004	77.57	-5.146	**<0.001**
DE-(NO) across time					
DE-(NO).T1 - DE-(NO).T2	-53.603	12.114	62.23	-4.425	**0.002**
DE-(NO).T1 - DE-(NO).T3	-116.443	10.899	77.36	-10.684	**<0.001**
DE-(NO).T2 - DE-(NO).T3	-62.840	12.945	75.92	-4.854	**<0.001**
NO-(DE) across time					
NO-(DE).T1 - NO-(DE).T2	-6.175	14.673	79.74	-0.421	1.000
NO-(DE).T1 - NO-(DE).T3	-28.164	10.236	77.11	-2.751	0.222
NO-(DE).T2 - NO-(DE).T3	-21.989	14.373	79.48	-1.530	0.928
5 weeks PE					
NO-(NO).T1 - NO-(DE).T1	59.775	9.496	18.16	6.295	**<0.001**
NO-(NO).T1 - DE-(NO).T1	-19.640	9.448	7.95	-2.079	0.647
NO-(NO).T1 - DE-(DE).T1	39.417	11.466	9.41	3.438	0.136
NO-(DE).T1 - DE-(NO).T1	-79.415	10.855	10.28	-7.316	**<0.001**
NO-(DE).T1 - DE-(DE).T1	-20.358	12.357	14.47	-1.648	0.865
DE-(NO).T1 - DE-(DE).T1	59.057	11.572	24.28	5.103	**0.001**
7 weeks PE					
NO-(NO).T2 - NO-(DE).T2	84.233	14.521	38.28	5.801	**<0.001**
NO-(NO).T2 - DE-(NO).T2	-42.609	13.024	6.63	-3.271	0.209
NO-(NO).T2 - DE-(DE).T2	26.067	16.249	17.19	1.604	0.886
NO-(DE).T2 - DE-(NO).T2	-126.843	17.260	16.43	-7.349	**<0.001**
NO-(DE).T2 - DE-(DE).T2	-58.167	19.106	28.53	-3.044	0.147
DE-(NO).T2 - DE-(DE).T2	68.676	15.144	38.55	4.535	**0.003**
9 weeks PE					
NO-(NO).T3 - NO-(DE).T3	113.722	10.397	19.92	10.938	**<0.001**
NO-(NO).T3 - DE-(NO).T3	-53.972	12.795	13.58	-4.218	**0.028**
NO-(NO).T3 - DE-(DE).T3	31.036	12.519	6.76	2.479	0.463
NO-(DE).T3 - DE-(NO).T3	-167.694	12.037	14.06	-13.931	**<0.001**

NO-(DE).T3 - DE-(DE).T3	-82.686	11.577	8.83	-7.142	**0.002**
DE-(NO).T3 - DE-(DE).T3	85.008	11.988	26.47	7.091	**<0.001**
5-7 weeks PE					
NO-(NO).T1 - NO-(DE).T2	53.600	14.233	35.75	3.766	**0.025**
NO-(NO).T1 - DE-(NO).T2	-73.243	11.705	6.12	-6.258	**0.013**
NO-(NO).T1 - DE-(DE).T2	-4.567	15.083	17.31	-0.303	1.000
NO-(DE).T1 - NO-(NO).T2	-90.408	10.569	24.45	-8.554	**<0.001**
NO-(DE).T1 - DE-(NO).T2	-133.018	12.915	8.53	-10.300	**<0.001**
NO-(DE).T1 - DE-(DE).T2	-64.342	15.656	22.77	-4.110	**0.017**
DE-(NO).T1 - NO-(NO).T2	-10.993	11.241	8.54	-0.978	0.994
DE-(NO).T1 - NO-(DE).T2	73.240	15.689	20.08	4.668	**0.006**
DE-(NO).T1 - DE-(DE).T2	15.073	15.010	36.97	1.004	0.997
DE-(DE).T1 - NO-(NO).T2	-70.050	12.939	10.07	-5.414	**0.008**
DE-(DE).T1 - NO-(DE).T2	14.183	16.603	23.53	0.854	0.999
DE-(DE).T1 - DE-(NO).T2	-112.659	12.328	29.20	-9.138	**<0.001**
DE-(DE).T1 - NO-(NO).T2	-70.050	12.939	10.07	-5.414	**0.008**
DE-(DE).T1 - NO-(DE).T2	14.183	16.603	23.53	0.854	0.999
DE-(DE).T1 - DE-(NO).T2	-112.659	12.328	29.20	-9.138	**<0.001**
5-9 weeks PE					
NO-(NO).T1 - NO-(DE).T3	31.611	9.222	15.44	3.428	0.097
NO-(NO).T1 - DE-(NO).T3	-136.083	10.930	13.58	-12.450	**<0.001**
NO-(NO).T1 - DE-(DE).T3	-51.075	10.851	5.97	-4.707	0.055
NO-(DE).T1 - NO-(NO).T3	-141.886	10.707	22.91	-13.251	**<0.001**
NO-(DE).T1 - DE-(NO).T3	-195.858	12.175	16.02	-16.087	**<0.001**
NO-(DE).T1 - DE-(DE).T3	-110.850	11.750	10.22	-9.434	**<0.001**
DE-(NO).T1 - NO-(NO).T3	-62.471	11.607	8.69	-5.382	**0.012**
DE-(NO).T1 - NO-(DE).T3	51.251	10.699	8.69	4.790	**0.026**
DE-(NO).T1 - DE-(DE).T3	-31.435	10.910	17.41	-2.881	0.228
DE-(DE).T1 - NO-(NO).T3	-121.528	13.048	9.93	-9.314	**<0.001**
DE-(DE).T1 - NO-(DE).T3	-7.806	12.196	12.77	-0.640	0.999
DE-(DE).T1 - DE-(NO).T3	-175.500	12.617	33.44	-13.909	**<0.001**
7-9 weeks PE					
NO-(NO).T2 - NO-(DE).T3	62.244	10.252	21.29	6.071	**<0.001**
NO-(NO).T2 - DE-(NO).T3	-105.450	12.499	13.33	-8.436	**<0.001**
NO-(NO).T2 - DE-(DE).T3	-20.442	12.403	6.85	-1.648	0.847
NO-(DE).T2 - NO-(NO).T3	-135.711	14.639	36.87	-9.271	**<0.001**
NO-(DE).T2 - DE-(NO).T3	-189.683	16.638	24.82	-11.400	**<0.001**
NO-(DE).T2 - DE-(DE).T3	-104.675	16.133	19.38	-6.488	**<0.001**
DE-(NO).T2 - NO-(NO).T3	-8.868	12.901	6.58	-0.687	0.100
DE-(NO).T2 - NO-(DE).T3	104.854	12.796	7.43	8.194	**0.001**
DE-(NO).T2 - DE-(DE).T3	22.168	11.558	21.93	1.918	0.737
DE-(DE).T2 - NO-(NO).T3	-77.544	16.342	17.00	-4.745	**0.007**
DE-(DE).T2 - NO-(DE).T3	36.178	15.518	21.01	2.331	0.486
DE-(DE).T2 - DE-(NO).T3	-131.517	15.754	43.91	-8.348	**<0.001**

b) Parasite index: Infection combination x Week PE	Estimate	SE	D.F.	*T*-value	*P*-value
DE-(DE) across time					
DE-(DE).T1 - DE-(DE).T2	-0.123	0.032	77.49	-3.877	**0.011**
DE-(DE).T1 - DE-(DE).T3	-0.181	0.024	76.63	-7.539	**<0.001**
DE-(DE).T2 - DE-(DE).T3	-0.058	0.030	77.13	-1.909	0.750
NO-(NO) across time					
NO-(NO).T1 - NO-(NO).T2	-0.096	0.019	78.01	-4.982	**<0.001**
NO-(NO).T1 - NO-(NO).T3	-0.136	0.019	77.90	-7.059	**<0.001**
NO-(NO).T2 - NO-(NO).T3	-0.040	0.021	76.69	-1.919	0.744
DE-(NO) across time					
DE-(NO).T1 - DE-(NO).T2	-0.142	0.024	75.49	-5.935	**<0.001**
DE-(NO).T1 - DE-(NO).T3	-0.179	0.023	76.76	-7.789	**<0.001**
DE-(NO).T2 - DE-(NO).T3	-0.037	0.026	78.50	-1.420	0.956
NO-(DE) across time					
NO-(DE).T1 - NO-(DE).T2	-0.015	0.031	77.93	-0.497	1.000
NO-(DE).T1 - NO-(DE).T3	-0.054	0.021	76.56	-2.497	0.357
NO-(DE).T2 - NO-(DE).T3	-0.039	0.030	77.70	-1.283	0.979
5 weeks PE					
NO-(NO).T1 - NO-(DE).T1	0.162	0.032	3.75	5.108	0.085
NO-(NO).T1 - DE-(NO).T1	-0.067	0.021	6.62	-3.192	0.227
NO-(NO).T1 - DE-(DE).T1	0.116	0.036	4.42	3.241	0.270
NO-(DE).T1 - DE-(NO).T1	-0.229	0.034	4.24	-6.726	**0.026**
NO-(DE).T1 - DE-(DE).T1	-0.046	0.027	12.61	-1.694	0.843
DE-(NO).T1 - DE-(DE).T1	0.183	0.035	4.96	5.286	**0.047**
7 weeks PE					
NO-(NO).T2 - NO-(DE).T2	0.243	0.040	8.33	6.082	**0.006**
NO-(NO).T2 - DE-(NO).T2	-0.113	0.028	8.06	-4.031	0.074
NO-(NO).T2 - DE-(DE).T2	0.089	0.044	7.76	2.035	0.669
NO-(DE).T2 - DE-(NO).T2	-0.356	0.044	8.48	-8.051	**0.001**
NO-(DE).T2 - DE-(DE).T2	-0.154	0.040	30.05	-3.809	**0.026**
DE-(NO).T2 - DE-(DE).T2	0.202	0.041	9.02	4.927	**0.020**
9 weeks PE					
NO-(NO).T3 - NO-(DE).T3	0.245	0.033	4.19	7.418	**0.018**
NO-(NO).T3 - DE-(NO).T3	-0.109	0.027	12.70	-4.102	**0.038**
NO-(NO).T3 - DE-(DE).T3	0.072	0.037	4.40	1.926	0.722
NO-(DE).T3 - DE-(NO).T3	-0.355	0.036	5.04	-9.867	**0.003**
NO-(DE).T3 - DE-(DE).T3	-0.173	0.026	8.67	-6.758	**0.003**
DE-(NO).T3 - DE-(DE).T3	0.181	0.035	5.40	5.098	**0.046**
5-7 weeks PE					
NO-(NO).T1 - NO-(DE).T2	0.147	0.039	7.59	3.779	0.107
NO-(NO).T1 - DE-(NO).T2	-0.209	0.025	6.96	-8.207	**0.002**
NO-(NO).T1 - DE-(DE).T2	-0.007	0.042	7.22	-0.168	1.000
NO-(DE).T1 - NO-(NO).T2	-0.259	0.033	4.37	-7.741	**0.014**
NO-(DE).T1 - DE-(NO).T2	-0.371	0.037	4.81	-9.976	**0.003**

NO-(DE).T1 - DE-(DE).T2	-0.169	0.034	21.70	-4.952	**0.003**
DE-(NO).T1 - NO-(NO).T2	-0.029	0.024	8.34	-1.227	0.970
DE-(NO).T1 - NO-(DE).T2	0.214	0.041	7.97	5.199	**0.019**
DE-(NO).T1 - DE-(DE).T2	0.060	0.040	8.23	1.484	0.911
DE-(DE).T1 - NO-(NO).T2	-0.212	0.038	4.96	-5.597	**0.037**
DE-(DE).T1 - NO-(DE).T2	0.031	0.035	24.30	0.881	0.999
DE-(DE).T1 - DE-(NO).T2	-0.325	0.036	5.70	-9.049	**0.003**
5-9 weeks PE					
NO-(NO).T1 - NO-(DE).T3	0.108	0.031	3.57	3.447	0.262
NO-(NO).T1 - DE-(NO).T3	-0.246	0.024	10.62	-10.199	**<0.001**
NO-(NO).T1 - DE-(DE).T3	-0.065	0.035	3.86	-1.846	0.754
NO-(DE).T1 - NO-(NO).T3	-0.299	0.033	4.39	-8.957	**0.008**
NO-(DE).T1 - DE-(NO).T3	-0.408	0.036	5.24	-11.294	**0.001**
NO-(DE).T1 - DE-(DE).T3	-0.227	0.026	9.67	-8.708	**0.001**
DE-(NO).T1 - NO-(NO).T3	-0.069	0.024	8.76	-2.905	0.277
DE-(NO).T1 - NO-(DE).T3	0.175	0.034	4.06	5.185	0.071
DE-(NO).T1 - DE-(DE).T3	0.002	0.034	4.34	0.067	1.000
DE-(DE).T1 - NO-(NO).T3	-0.253	0.038	4.99	-6.671	**0.017**
DE-(DE).T1 - NO-(DE).T3	-0.008	0.027	11.49	-0.289	1.000
DE-(DE).T1 - DE-(NO).T3	-0.362	0.036	6.09	-9.952	**0.001**
7-9 weeks PE					
NO-(NO).T2 - NO-(DE).T3	0.205	0.033	4.17	6.191	**0.037**
NO-(NO).T2 - DE-(NO).T3	-0.150	0.026	12.26	-5.635	**0.003**
NO-(NO).T2 - DE-(DE).T3	0.031	0.037	4.37	0.842	0.996
NO-(DE).T2 - NO-(NO).T3	-0.284	0.040	8.36	-7.090	**0.002**
NO-(DE).T2 - DE-(NO).T3	-0.393	0.043	9.21	-9.149	**0.001**
NO-(DE).T2 - DE-(DE).T3	-0.212	0.034	20.61	-6.213	**0.001**
DE-(NO).T2 - NO-(NO).T3	0.072	0.028	8.01	2.604	0.397
DE-(NO).T2 - NO-(DE).T3	0.317	0.037	4.62	8.570	**0.008**
DE-(NO).T2 - DE-(DE).T3	0.144	0.035	5.01	4.128	0.117
DE-(DE).T2 - NO-(NO).T3	-0.129	0.044	7.82	-2.959	0.271
DE-(DE).T2 - NO-(DE).T3	0.115	0.034	20.33	3.413	0.083
DE-(DE).T2 - DE-(NO).T3	-0.239	0.042	9.56	-5.707	**0.007**

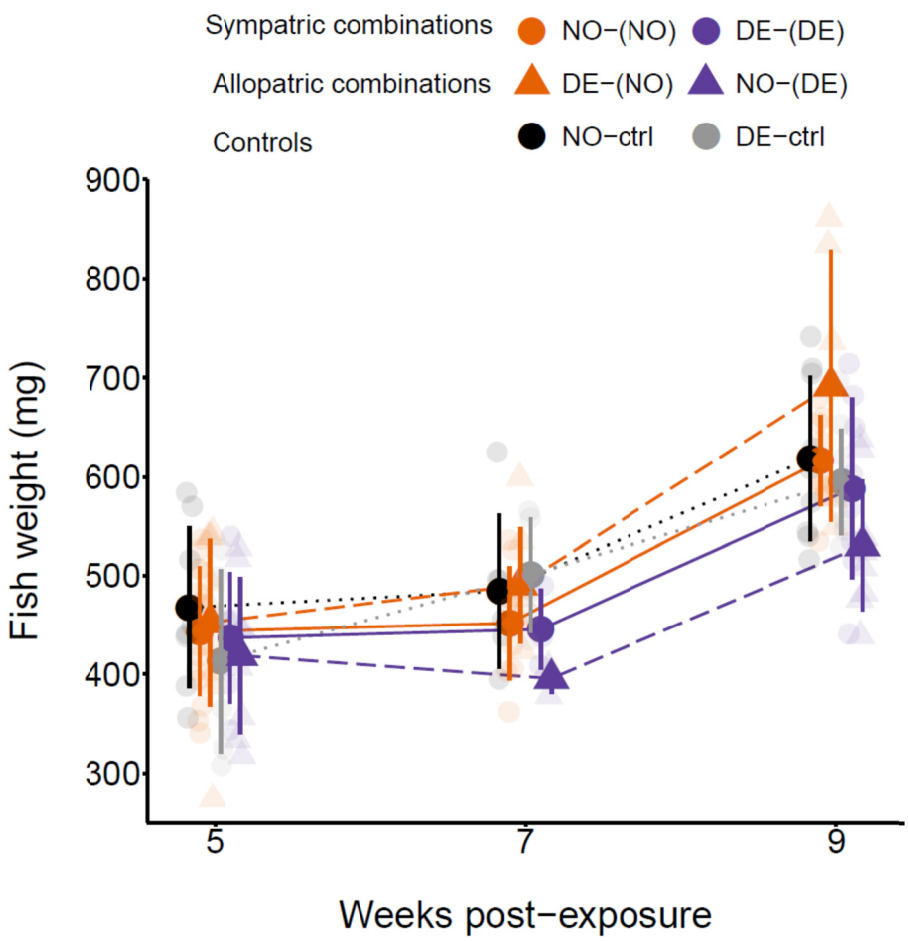


Figure 2
Fish somatic weight (in mg) in the different experimental combination (sympatric NO-(NO), sympatric DE-(DE), allopatric NO-(DE), allopatric DE-(NO), controls DE-ctrl and NO-ctrl) across the different time points (5, 7 and 9 weeks PE).

condition between 5 and 9 weeks PE (Tukey's HSD, $P > 0.004$; fig. 3a, see table S4). On average DE control fish had higher condition than NO control fish and this difference was significant at 7 and 9 weeks PE (Tukey's HSD, 7 weeks PE: $P > 0.001$; 9 weeks PE: $P = 0.001$; fig. 3a, see table S4). This difference was explained by the fact that NO control fish had consistently higher standard length than DE controls (on average NO: 36.7 SE ±0.9 mm, DE: 33.7 SE ±1.1 mm). Within fish population, fish condition in the allopatric and sympatric infection combinations was not significantly different at any given time point and did not differ from the respective controls (see table S4).

3.4.2.2 Splenosomatic index (SSI) and Head-kidney index (HKI)

The splenosomatic index (SSI) differed significantly between fish origin (ANOVA, $F_{1,130} = 3.913$, $P = 0.048$) and worm origin (ANOVA, $F_{2,129} = 7.521$, $P = 0.023$). The Head-kidney index was significantly affected by worm origin (ANOVA, $F_{2,131} = 6.720$, $P = 0.035$), time point (ANOVA, $F_{2,131} = 6.889$, $P = 0.032$) and the interaction of fish origin, worm origin and time point (ANOVA, $F_{4,129} = 11.111$, $P = 0.025$). But we could not detect any significant difference in SSI or HKI between experimental combinations and the respective controls at any time points with pairwise comparisons (Tukey's HSD, $P > 0.050$; fig. 3b and c, see table S4).

3.4.3 Host immune gene expression

For the experimental combination NO-(NO) at time point 7 weeks PE, gene expression data was not available for fish family NO-F2 (see table S1). Consequently, we had an incomplete design at 7 weeks PE for the gene expression data, with only one fish family exposed to up to two worm families for each experimental treatment (DE-(DE), DE-(NO), NO-(NO), NO-(DE), DE-ctrl, NO-ctrl). Therefore, we only used the worm family as a random factor in the model analysing gene expression data at 7 weeks PE. Similarly when comparing the gene expression profile of fish infected with DE worms at 5 weeks PE, we only had samples from one fish family for each of the family origin infected with one worm family (DE-F1 infected with DE-B; NO-F2 infected with DE-A) and did not use the worm family as a random factor.

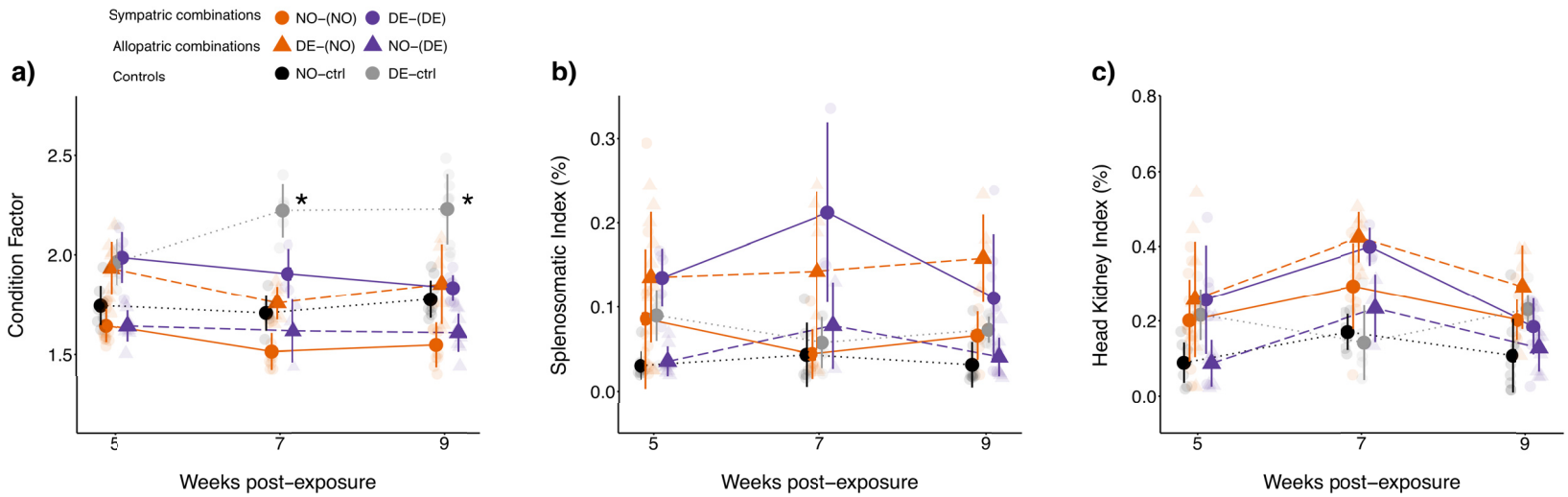


Figure 3

a) Fish condition factor, **b)** splenosomatic index (%) and **c)** head kidney index (%) in the different experimental combination (sympatric NO-(NO), sympatric DE-(DE), allopatric NO-(DE), allopatric DE-(NO), controls DE-ctrl and NO-ctrl) across the different time points (5, 7 and 9 weeks PE).

The poor quality of qPCR amplification in the spleen samples rendered the analysis of gene expression in this tissue impossible. Similarly, the TNFα gene had to be excluded from the analysis for the head-kidney samples (for more details, see Supp. Analysis SA2). Therefore, we will focus subsequently on the analysis of differential gene expression of seven candidate genes (IgM, IL-1ß, MHC-IIß, MIF, SOD2, TGF-ß1, TLR2) in the head-kidney for the different treatment groups.

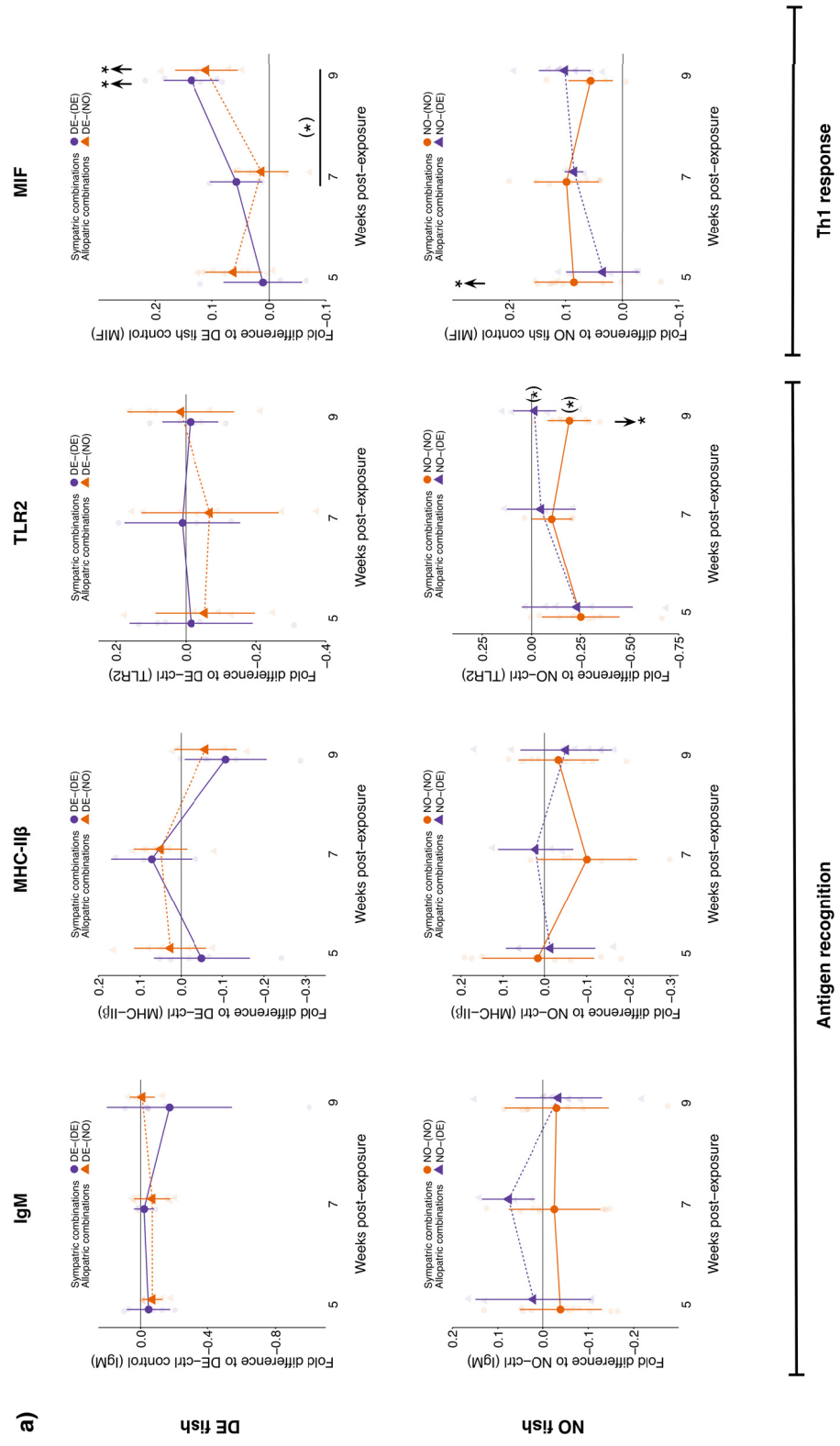
3.4.3.1 Antigen recognition genes

There was no significant variation between infected and control fish in IgM and MHC-IIß expression for all combinations (see table S5-S7, fig. 4). TLR2 expression was on average lower in NO infected fish compared to controls. At 9 weeks post exposure, this difference was significant for the sympatric combination NO-(NO) (ANOVA, $F_{2,19}$ = 10.694, P = 0.005), showing a down-regulation of TLR2 compare to both the control (Tukey's HSD, P = 0.018) and allopatric combination NO-(DE) (Tukey's HSD, P = 0.012; see table S5-S7, fig. 4). TLR2 expression was not significantly different from controls in DE infected fish.

3.4.3.2 Th2 response genes

From 7 weeks PE onwards, IL-1ß expression was on average lower in DE infected fish than in controls and significantly down-regulated in allopatric combination DE-(NO) (ANOVA, 7 weeks PE: $F_{2,13}$ = 14.144, P <0.001; 9 weeks PE: $F_{2,19}$ = 12.557, P = 0.002; see table S5-S7, fig. 4). At 9 weeks PE, IL-1ß expression was significantly down-regulated in allopatric combination DE-(NO) compare to both controls (Tukey's HSD, P = 0.004) and the sympatric combination DE-(DE) (Tukey's HSD, P = 0.005; see table S5-S7, fig. 4). In NO infected fish, IL-1ß expression was significantly higher in the allopatric combination NO-(DE) compare to sympatric combination NO-(NO) (ANOVA, $F_{2,21}$ = 6.744, P = 0.034; Tukey's HSD, P = 0.028; see table S5-S7, fig. 4).

Overall, there was a significant effect of the treatment (allopatric, sympatric, control) on TGF-ß1 expression in NO fish (ANOVA, $F_{2,19}$ = 10.490, P = 0.005; see table S5-S7, fig. 4). The allopatric combination NO-(DE) expressed significantly more TGF-ß1 than the sympatric combination NO-(NO) at 5 weeks PE (ANOVA, $F_{2,19}$ = 6.229, P = 0.044; Tukey's HSD, P = 0.046; see table S5-S7,


a)
DE fish
NO fish
IgM
MHC-IIß
TLR2
MIF
Sympatric combinations
Allopatric combinations
DE–(DE)
DE–(NO)
NO–(NO)
NO–(DE)
Weeks post–exposure
Fold difference to DE–ctrl control (IgM)
Fold difference to DE–ctrl (MHC–IIβ)
Fold difference to DE–ctrl (TLR2)
Fold difference to DE fish control (MIF)
Fold difference to NO–ctrl (IgM)
Fold difference to NO–ctrl (MHC–IIβ)
Fold difference to NO–ctrl (TLR2)
Fold difference to NO fish control (MIF)
Antigen recognition
Th1 response

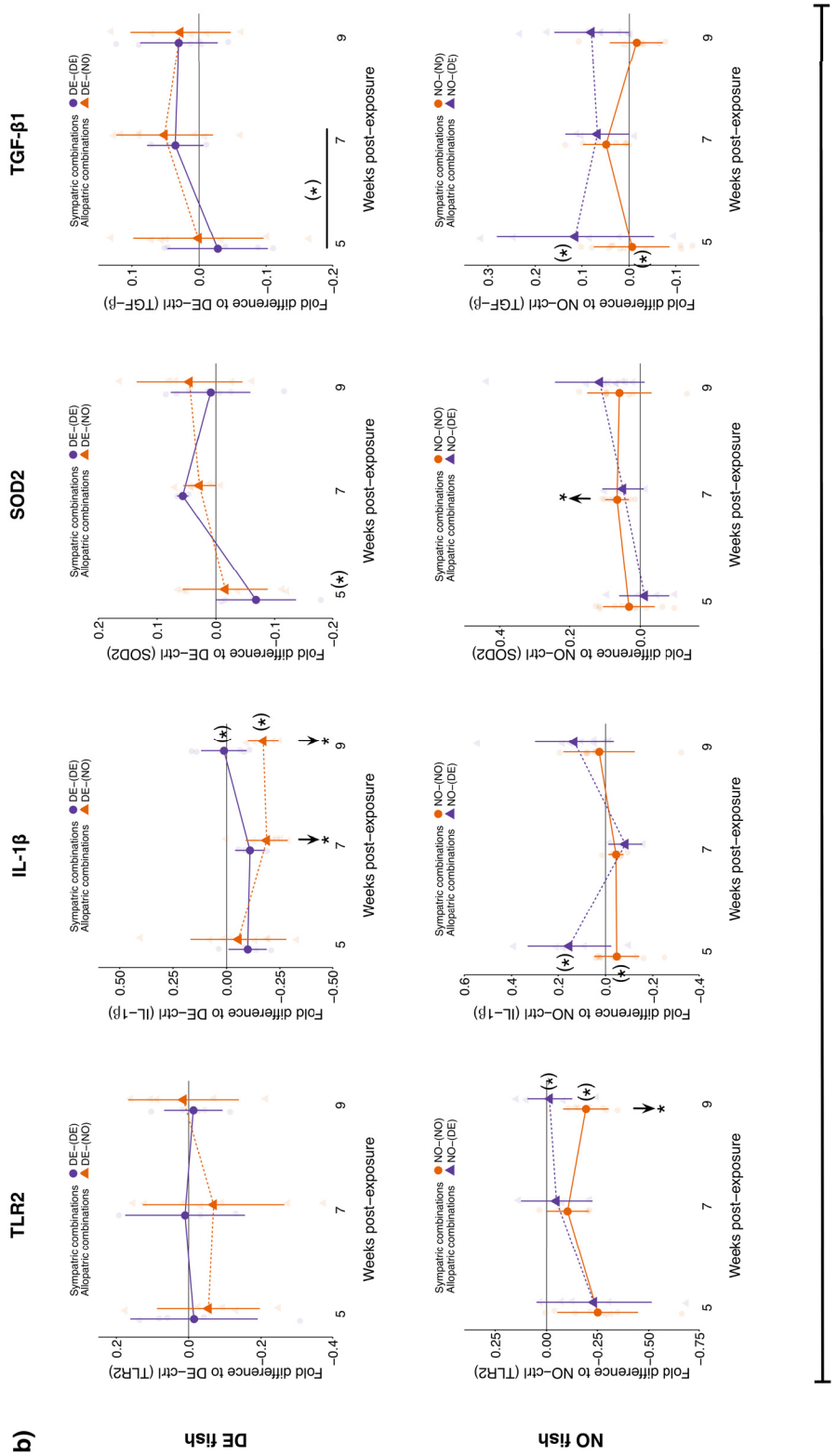

b)
DE fish
NO fish
TLR2
IL-1β
SOD2
TGF-β1
Th2 response
Sympatric combinations
Allopatric combinations
DE–(DE)
DE–(NO)
NO–(NO)
NO–(DE)
Weeks post–exposure
Fold difference to DE–ctrl (TLR2)
Fold difference to DE–ctrl (IL–1β)
Fold difference to DE–ctrl (SOD2)
Fold difference to DE–ctrl (TGF–β)
Fold difference to NO–ctrl (TLR2)
Fold difference to NO–ctrl (IL–1β)
Fold difference to NO–ctrl (SOD2)
Fold difference to NO–ctrl (TGF–β)

Figure 4

Gene expression fold differences to controls for seven immune genes in German (DE-(DE), DE-(NO)) and Norwegian (NO-(NO), NO-(DE)) infected fish: **a)** Antigen recognition (IgM, MHC-IIß, TLR2) and Th2 response gene (MIF); **b)** Th2 response genes (TLR2, IL-1ß, SOD2, TGF-ß1). (→*) indicates significant a up or down regulation compare to control (*P*-values <0.05); (*) indicates significant difference between sympatric and allopatric combinations or between time points (*P*-values <0.05).

fig. 4). However, this was not significantly different from the control group NO-ctrl (Tukey's HSD, P = 0.082). In DE fish, TGF-β1 expression was significantly lower at 5 weeks PE compared to 7 weeks PE (ANOVA, $F_{2,49}$ = 10.235, P = 0.006; Tukey's HSD, P = 0.004; see table S5-S7, fig. 4).

The SOD2 expression of sympatric combination NO-(NO) was significantly up-regulated compare to the controls at 7 weeks PE (ANOVA, $F_{2,13}$ = 10.500, P = 0.005; Tukey's HSD, P = 0.004; see table S5-S7, fig. 4). In DE fish, SOD2 expression was significantly lower at 5 weeks PE compare to the two later time points (ANOVA, $F_{2,13}$ = 11.264, P = 0.003; 5 vs. 7 weeks PE: Tukey's HSD, P = 0.008; 5 vs. 9 weeks PE: Tukey's HSD, P = 0.018; see table S5-S7, fig. 4).

3.4.3.3 Th1 response gene

Both allopatric DE-(NO) and sympatric DE-(DE) combinations exhibited a significant up-regulation of MIF expression at 9 weeks PE compare to the controls (ANOVA, $F_{2,15}$ = 36.832, P >0.001; DE-(DE): Tukey's HSD, P >0.001; DE-(NO): Tukey's HSD, P >0.001), and to 7 weeks PE (ANOVA, $F_{2,49}$ = 9.931, P = 0.007; Tukey's HSD, P = 0.005; see table S5-S7, fig. 4). In NO infected fish, MIF expression was up-regulated compare to controls in NO-(NO) at 5 weeks PE (ANOVA, $F_{2,21}$ = 6.486, P = 0.039; Tukey's HSD, P = 0.034; see table S5-S7, fig. 4).

3.4.3.4 Differential gene expression profiles between treatment groups

Since the TNFα gene had to be excluded from the analysis (for more details, see Supp. Analysis SA2), we could not perform the analysis for differential gene expression of the Th1 response gene subset.

There was no significant difference between the expression profiles of the two control groups (DE-ctrl, NO-ctrl) at any time points for immune genes, antigen recognition genes, or Th2 response genes (refer to Supp. table S8-S9 for all PERMANOVA results, fig. S2).

Overall time points and for all the tested functional groups, the sympatric DE-(DE) fish were not significantly different from the DE controls, whereas sympatric NO-(NO) fish profile were significantly affected by infection compare to NO controls (see table S8-S9; fig. S2). However, this was not translated into

significant differences between NO-(NO) and NO-ctrl profiles at any of the individual time points (Tukey's HSD, P >0.050).

The gene expression profile of Th2 response genes was affected by infection in the two allopatric combinations compared to their respective controls; at 5 weeks PE, for NO-(DE) fish (PERMANOVA, $F_{1,33}$ = 3.500, *P* = 0.019) and at 9 weeks PE for DE-(NO) fish (PERMANOVA, $F_{1,9}$ = 3.071, *P* = 0.016; see table S8-S9, fig. S2).

3.5 Discussion

A theoretical outcome of host-parasite coevolution is optimal virulence, whereby in different coevolved populations, host and their parasites achieve equal levels of infection despite different parasite virulence and host resistance (Anderson & May 1982). Here, we tested whether the phenotype of optimal virulence in different coevolved populations can be explained by underlying molecular patterns. We used two sympatric populations of three-spined stickleback/*Schistocephalus solidus*. Performing fully crossed exposure experiment, we reveal that optimal virulence is mediated via the immune response by both the host, and the parasite.

3.5.1 Pattern of optimal virulence despite asymmetric pressure

Norwegian parasites (NO) consistently grew bigger and faster compared to German parasites (DE). However, by the end of the experiment there was no significant difference between the two sympatric combinations for parasite index (PI) when the worms reached an exploitation ceiling. This is the expected pattern of convergent optimal state in both coevolved populations (Barber & Svensson 2003). The level of host exploitation in sympatric combinations was intermediate to the one observed in allopatric combinations. While this suggests a genetic basis to this pattern, asymmetry is observed: the NO-parasites in the allopatric combination DE-(NO) achieved the highest level of host exploitation, whereas DE-parasites in allopatric combinations NO-(DE) grew slower and had the smallest level of host exploitation. Specifically, even after nine weeks of infection, the DE-worms rarely reached the infective threshold weight of 50 mg in the NO-fish. This suggests the maladaptation of DE-hosts in controlling the growth of the NO-parasites (over-exploitation of the host), while NO-hosts have a higher control over the DE-worm growth (under-exploitation of the host). All these results are consistent with previous reciprocal infection experiments using the same host-parasite pairs (Kalbe *et al*. 2016) and show that while the more resistant NO-hosts have more virulent NO-parasites, less resistant DE-hosts have less virulent DE-parasites. We can therefore conclude that despite different coevolutionary trajectories, optimal virulence is possible.

3.5.2 Impact of infection on fish condition

NO and DE control fish showed different body conditions likely indicative of population-specific characteristics resulting from divergent adaptation to marine (DE) and lacustrine environments (NO) (Leinonen *et al*. 2006). Infection did not alter conditions of the fish with regards to their respective controls. Infected fish showed growth compensation, a well-known phenomenon in *S. solidus* infected sticklebacks when fed *ad libitum* (Arnott *et al*. 2000; Wright, Wootton & Barber 2007). In nature, due to competition for resources, but also additional selection pressure (concomitant infections, predation, *etc*.) growth and condition would be expected to differ between the different infection combinations and uninfected fish (Barber & Ruxton 1998). Specifically, the severity of infection increases with increasing parasite index (MacNab *et al*. 2009; Heins *et al*. 2010a; Barber, Walker & Svensson 2011), thus the large size reached by the NO-worm in DE-fish would most likely have a significant impact on the host's fitness, competitive abilities and predation risk (Milinski 1985). As a matter of fact, experimentally infected DE-fish transplanted in natural conditions had higher mortality rates when infected with NO-worms compare to DE-worms, highlighting the cost of harbouring a faster growing worm (Benesh & Kalbe 2016).

3.5.3 Host immune gene expression

3.5.3.1 Antigen recognition gene expression

Antigen recognition genes are crucial for the initiation of a targeted immune response. Among the antigen recognition genes we tested, only TLR2 showed differential expression, as it was down-regulated at 9 weeks PE in the sympatric combination NO-(NO), compared to the allopatric combination DE-(NO) and their NO control. A down regulation of an antigen recognition gene likely represents a physiological manipulation of the fish immunity by the highly virulent NO tapeworm. The fact that this manipulation occurs after 9 weeks post-infection suggests that the NO-parasite has evolved the capacity to manipulate its host when it reaches infectious stage for the next host.

In general, immunodepression can facilitate inter- and intraspecific concomitant infections, which by draining host energy and, in some cases exacerbating host manipulation, could increase the chance of transmission to the final host (Cox 2001; Hafer & Milinski 2016a). Specifically, host immune manipulation could be the molecular mechanism behind the findings that both DE- and NO-fish infected with NO-tapeworms acquire more trematode infections than DE-infected fish when experimentally or naturally exposed to *Diplostomum pseudospathaceum* (Benesh & Kalbe 2016; Piecyk & Ritter in prep).

Apart from parasite manipulation, +5 weeks PE is probably too late to detect differential regulation of genes involved in the mounting of an immune response against *S. solidus*. In fact, Scharsack *et al*. (2007b) used host and parasite from the same DE population and observed a fluctuation of monocytes proliferation between 1 and 4 weeks PE, possibly indicating cycles of detection by the host followed by immune evasion by the parasite, potentially through successive shading of surface antigens (Hammerschmidt & Kurtz 2005a). Investigating immune responses at earlier time points could give better insights into the recognition of the *S. solidus* parasite by the host immune system.

3.5.3.2 Pro-inflammatory response (Th1) gene expression

We observed a significant up-regulation of MIF gene in the sympatric combinations NO-(NO) and DE-(DE), immediately after worms had reached 50 mg (at 5 and 9 weeks PE, respectively). As MIF stimulates macrophages activation, this represents indirect evidence of the increased leucocytes respiratory burst activity via parasite immune evasion (Chung & Secombes 1988). This would correlate with findings of Robertson *et al*. (2016) who identified that the expression of Th1 response genes increased with the PI of wild infected stickleback. However, once the worm has reached its infectivity threshold, an inflammatory reaction would not be sufficient at removing the infection and would probably instead be more costly and damaging to the host as the release of large amount of antigen could lead to immunopathology (Meeusen 1999; Buchmann 2012). Therefore, an increase in Th1 response gene might be sign of parasite manipulation; some helminths manipulate the host immune response by producing homologues of host cytokines such as TGF-β and MIF. Parasite-MIF then can induce the production of endogenous MIF by the host, potentially

driving the immune response away from Th2 towards Th1 response (Maizels *et al*. 2004; Prieto-Lafuente *et al*. 2009). There is evidence that secretory/excretory products from 7-week old *S. solidus* plerocercoids are able to activate head-kidney leucocytes respiratory burst *in vitro* (Scharsack *et al*. 2013). In the allopatric DE-(NO), we also observed the MIF gene up-regulation at the same time as the sympatric DE-(DE) fish at 9 weeks PE. However, parasites in the DE-(NO) combination had reached the infectivity threshold long before, prior to 5 weeks PE. Thus if the NO-worm was responsible for the manipulation we would expect to observe the MIF up-regulation the latest at 5 weeks PE like their sympatric homologues, unless there exists an evolved mechanism decoupling infectivity and manipulation (Schmid-Hempel 2008).

3.5.3.3 Anti-inflammatory response (Th2) gene expression

In NO-(NO) fish, the up-regulation of the inflammatory cytokine MIF was followed at 7 weeks PE by an up-regulation of the antioxidant SOD2, a potential mechanism to restrain the oxidative stress of inflammation. We did not see this increase in the DE-(DE) combination but cannot exclude the possibility that this could develop after 9 weeks PE. Within the first days of the infection, the inflammatory response is likely to play an important role in eliminating *S. solidus* prior to definitive establishment in the body cavity (Wedekind & Little 2004; Scharsack *et al*. 2007b). However, in chronic helminthic infections, immune inflammation has to be contained to limit the damaged to host tissue. Helminth antigens normally trigger the early production of IL-4 cytokine, which induce a polarized T helper (Th) 2 response and cross-regulates the inflammatory Th1 response (Kreider *et al*. 2007; Buchmann 2012). We thus expected that the genes involved in the anti-inflammatory and wound healing Th2 response would be up-regulated in the more resistant NO host. At 5 weeks PE, the Th2 response gene profile of NO-(DE) was significantly different from NO-ctrl and both IL-1β and TGF-β1 were up-regulated compared to the sympatric combination NO-(NO). This indicates that while the NO worms have evolved ways to manipulate and avoid detection by the immune system of their sympatric NO host, the host is able to develop an appropriate Th2 immune response against the allopatric DE worm, and thus dramatically contain its growth. This pattern is absent from the DE fish and IL-1β was down-regulated compare to DE-ctrl in the allopatric combination

DE-(NO) starting from 7 weeks PE. This suggests stronger immune manipulation of the DE fish by the more virulent NO worm, potentially skewing the immune response towards Th1 by inhibiting Th2.

3.5.3.4 Gene expression profile in allopatric and sympatric combinations

The expression profiles (for all immune genes, antigen recognition and Th2 response) of the two control groups DE-ctrl and NO-ctrl did not significantly differ from each other, therefore, potential differences observed between gene expression profiles of the experimental combinations could be attributed to the treatment.

We revealed a significant difference of the Th2 response gene profiles compared to their respective controls for the allopatric combinations NO-(DE) fish, at 5 weeks PE, and DE-(NO) fish, at 9 weeks PE. These differences in gene expression profiles were most likely driven by the down-regulation of IL-1ß and TGF-ß1. Except for IL-1ß down-regulation at 9 weeks PE, DE-host immune gene expression curves and profiles were very similar in sympatric DE-(DE) and allopatric DE-(NO) infections. This disconnection between parasite virulence level and DE-host reaction suggest that DE-fish have evolved a stereotypical response to *S. solidus* infections. This means that DE-fish are able to recognize the NO parasite but to adjust their immune response to the worm higher virulence. The NO-worm in DE-(NO) does not seem to self-regulate host-exploitation until reaching an exploitation ceiling, when DE-fish display important growth compensation between 7 and 9 weeks PE. Therefore, the down-regulation of IL-1ß at 7 and 9 weeks PE in DE-(NO) fish might indicate a trade-off between growth and immune function.

3.5.4 Conclusion

While we expected chronic *S. solidus* infection to influence immune gene expression profiles in some ways, sympatric treatments did not significantly deviate from the controls in their immune genes expression profiles. Thus we could not interpret differences between sympatric and allopatric combinations as an effect of the treatment at the level of the gene expression profiles. Overall, it is difficult to determine if our findings are part of the immune

response initiated by the host or signs of parasite manipulation; and how they affect the host-parasite interaction to lead to the final phenotypic differences in host exploitation. Investigating time points, prior to 5 weeks PE, before the NO-(NO) and DE-(NO) combinations reach their 50 mg threshold, could give more insights into the immune mechanisms underpinning infection and leading to the differences in PI. For example, antigen recognition genes like MHC-IIß could be involved at earlier time points as specific antibody productions might take around 2-3 weeks in fish (Rijkers, Frederix-Wolters & van Muiswinkel 1980).

Additionally, the variability due to the incomplete family design and the small sample size within time point make it difficult to detect difference in gene expression signals. Lastly, candidate gene approaches have limits. Other genes might be more relevant as for instance, Robertson *et al*. (2016) identified a correlation between the expression levels of the immunosuppressive FoxP3a gene and the PI of *S. solidus* infection in wild populations. They were also able to measure immune expression in the spleen, which might highlight technical problems in our qPCR assay. With many critical steps, gene expression studies are extremely sensitive to the accumulation of technical errors that can affect accuracy and sensitivity of the final results (Derveaux, Vandesompele & Hellemans 2010; Baker 2011). In particular, the primer and qPCR conditions developed by Robertson *et al*. (2016) seemed better optimized to measure gene expression in the spleen tissues and could be tested on HK samples; and their use of more than one reference gene in normalization might be especially critical in the comparison of gene expression from whole tissue constituted of many different cell subpopulations (Tricarico *et al*. 2002; Bustin *et al*. 2005).

In conclusion, we were able to replicate the results found by Kalbe et al. (2016), demonstrating the higher resistance and virulence of the Norwegian host-parasite population over the German one, and to further describe the kinetic of allopatric and sympatric infections. We detected differential expression, especially in allopatric combinations, which hints towards a role for the Th2 response pathway in the phenotypic differences. While we revealed some mechanisms correlated with optimal virulence, we cannot exclude that cross interactions between Th responses and various pathways are the target of manipulation and resistance.

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4 Chapter 4

Intraspecific within-host parasite competition alters virulence in a fish-cestode system

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4.1 Abstract

In nature, multiple infections with different strains or species of parasites are the rule rather than the exception. They can influence the expression and evolution of virulence by generating within-host conflicts. Yet, it remains unknown whether competition favours increased or reduced virulence. The close adaptation of the tapeworm *Schistocephalus solidus* to its stickleback host (*Gasterosteus aculeatus*) makes it an ideal model system to study the effect of intraspecific parasite competition on virulence and host fitness. Using replicated high- and low-virulence parasite strains, we measured the effects of direct competition (sharing the same host) on the expression of overall and individual virulence. In mixed infections, low virulence parasites benefited from sharing a host with a more virulent strain, yet strain-specific differences persisted. Our results reveal a genetic and plastic expression of virulence, best explained by the production of both common and strain-specific goods.

4.2 Introduction

Understanding the determinants of disease virulence has become a major topic in epidemiology and evolutionary ecology (Galvani 2003). Host-parasite interactions play an important role in the evolution of parasite virulence through the coevolutionary arms race known as Red Queen dynamics (Van Valen 1973). As both parasite virulence and host resistance are costly and are the results of life history trade-offs, highly virulent parasites may not always be the outcome of coevolution (Frank 1996). For instance, host over-exploitation can feedback negatively on host density and decrease parasite transmission rates (Ewald 1993). Hence, theory predicts selection on an optimal intermediate level of virulence through local adaptation (Anderson & May 1982). However, virulence polymorphism can be observed in natural populations as different parasite strains can show different level of virulence. This suggests that multiple equilibriums are possible. Host heterogeneity is often used to explain this pattern (Regoes, Nowak & Bonhoeffer 2000), but parasite-parasite interactions could also play a role and affect virulence evolution.

In natural ecosystems, hosts are rarely infected by only one parasite but rather harbour multiple parasites from different species or even genera (Petney & Andrews 1998). However, testing experimentally how intra- and interspecific parasite-parasite interactions can contribute to the evolution of virulence remains a challenge (Rigaud *et al*. 2010; Alizon, de Roode & Michalakis 2013). From a theoretical point of view, under multiple infections, the host can be regarded as a finite pool of resources that each parasite may transform into goods - goods being defined as both tangible (i.e. nutrients) and intangible (i.e. protection from the immune system) benefits that a parasite actively gains through host exploitation and manipulation (Bashey 2015). Production of these goods is costly for the host, but necessary for the parasite to grow and ultimately for its fitness.

While within-host parasite interactions are now receiving more attention (Restif & Graham 2015), it is difficult to predict how competition will influence overall virulence expression and evolution (Alizon *et al*. 2013). In direct competition, individual parasites have to share limited host resources, which

reduces individual fitness and results in a fitness advantage for more virulent strains (Frank 1996; de Roode *et al*. 2005). Therefore, competition can favour the evolution of increased virulence (van Baalen & Sabelis 1995; Davies *et al*. 2002; Brown, Hochberg & Grenfell 2002; Bell *et al*. 2006). However, others studies show that competition can also drive evolution towards lower virulence (Gower & Webster 2005; Choisy & de Roode 2010; Abkallo *et al*. 2015). Specifically, in the case of the production of public goods, goods can be commonly exploited by co-infecting parasites. In this context, cheaters, i.e. parasites that do not pay the costs of host manipulation but still benefiting from public goods, could invade the system and tilt evolution towards lower virulence as avirulent parasites would still accomplish their life-cycle and contribute to the gene pool (Leggett *et al*. 2014). Within-host competition for host resources has the potential to influence virulence evolution in either direction, and could explain how parasite populations maintain genetic polymorphism in virulence (Rigaud *et al*. 2010).

To date, most experimental studies involve macro- and micro-parasites that reproduce (often clonally) within the host and can readily be selected by within-host competition (Gower & Webster 2005; Mideo 2009; Abkallo *et al*. 2015). But many parasites multiply/reproduce mainly in their final host, utilizing intermediate hosts to maximize growth and fitness (Benesh, Chubb & Parker 2013). It is inside their intermediate hosts that they might more directly compete for host resources, space or host manipulation, and therefore more significantly interact with their co-infecting competitors (Cézilly, Perrot-Minnot & Rigaud 2014). Co-infections in intermediate hosts are therefore a good model to explore the consequences of multiple infections for individual parasite virulence.

In this study, we tested how parasites with different intrinsic levels of virulence individually performed when competing against each other. We used replicated strains of high virulence and low virulence from the cestode *Schistocephalus solidus*, which spends most of its complex life cycle in the body cavity of its specific intermediate host, the three-spined stickleback (*Gasterosteus aculeatus*). Immune suppression and evasion are well-known effects of chronic helminthic infections (Maizels *et al*. 2004). In *S. solidus* there

is evidence for immune manipulation mediated via excretory/secretory products; the intensity of this modulation has been shown in vitro to be strain-specific (Scharsack *et al*. 2004, 2013; Franke *et al*. 2014). Furthermore, in cestodes, exchanges with the host and food intake require transport through the outer tegument. Modification of the body-cavity environment by increasing available nutrients and immune suppression could be an important factor of interaction between co-infecting strains/species (Pedersen & Fenton 2007). Additionally, *S. solidus* size in the stickleback provides an excellent proxy for virulence and fitness. Indeed, it positively correlates with both the damage caused to the fish host, and with infectivity and reproductive success in the final bird host (Wedekind *et al*. 1998; Schärer *et al*. 2001; Lüscher & Wedekind 2002; Bagamian, Heins & Baker 2004; Milinski 2006).

We specifically compared the performance and phenotype of individual parasites when competing with strains of similar level of virulence or with strains of different virulence. We hypothesize that under heterologous infection (high virulence + low virulence), low virulence worms should perform better in co-infection than under single infection. If this is the case, it would indicate there are benefits from sharing goods with virulent strains which could explain the maintenance of virulence polymorphism.

4.3 Material and methods

4.3.1 Experimental model

As a model parasite we used *Schistocephalus solidus*, a trophically transmitted pseudophyllidean cestode with a complex life cycle, which presents three main advantages. First, individual parasite performance can be easily measured in this system. While *S. solidus* reproduces sexually in piscivorous birds and cyclopoid copepods serve as first intermediate host, it is specific only to its second intermediate host, the three-spined stickleback (*Gasterosteus aculeatus*). For several weeks the parasite completes the majority of its growth in the body cavity of its stickleback host, starting by developing from the procercoid into the plerocercoid stage. The plerocercoid stage experiences a particularly long-term interaction with the host's immune system and potential co-infecting parasites (Barber & Scharsack 2010). Second, virulence is a heritable trait in *S. solidus* as strains originating from different natural populations express consistent virulence levels and can be bred and maintained in the laboratory (Kalbe *et al*. 2016). Third, helminths like *S. solidus*, which reproduce in the intestine and disperse through the faeces of migratory birds, are likely to experience gene flow amongst geographically distant populations, as predicted by meta-population functioning (Louhi *et al*. 2010). The probability of strains with different virulence levels competing within the same host population is likely and enables investigating interactions between *S. solidus* strains with contrasting virulence in mixed populations.

To test for within-host parasite-parasite interactions, we specifically chose two *S. solidus* populations with contrasting epidemiology of infection resulting in dramatic differences in virulence. Previous experiments have shown that one Norwegian *S. solidus* strain (high prevalence >40% and common multiple infections; Skogseidvatnet, 60° 14' 38" N, 05° 54' 51" E) has a consistently relatively higher virulence compared to one German strain (low prevalence <1% and low to no occurrence of multiple infections; Neustädter Binnenwasser, 54° 06' 41" N, 10° 48' 33" E), in both German and Norwegian fish (Kalbe *et al*. 2016). Henceforth, German and Norwegian *S. solidus* are respectively classified as the low virulence strain (Lv) and high virulence strain (Hv).

4.3.2 Experimental exposure

We produced six lab-bred families of *S. solidus* from each virulence strain Lv and Hv following the *in vitro* system developed by Smyth (1946) and modified by Wedekind (Wedekind 1997). We used laboratory-cultured copepods (*Macrocyclops albidus*), each infected with a single 16-day old *S. solidus* procercoid, to expose experimentally each individual host. We exposed six lab-bred families of three-spined sticklebacks, originating from the lake Großer Plöner See (Germany, 54°09'21"N, 10°25'50"E), where *S. solidus* infection is extremely rare (for details of the exposure protocol see Scharsack *et al.* (2007b)).

We designed three co-infection treatments, including simultaneous double exposure to two high virulent parasites (Hv+Hv), two low virulent parasites (Lv+Lv) or a heterologous combination of one Hv and one Lv parasite (Hv+Lv, fig. 1). As control groups, single exposure to one high virulent parasite (Hv), one low virulent parasite (Lv) or to none (control) were performed (fig. 1). The experiment consisted of two independent trials; for each trial, we used three different fish families and three different parasite families of high and low virulence. To exclude confounding effects through genetic relatedness of the parasites, we combined two independent *S. solidus* families for homologous double infections; in heterologous double infections the two parasites were unrelated by nature. In total this resulted in six independent parasite family combinations for each double-exposure treatment.

4.3.3 Dissection protocol

Eight weeks post-exposure, fish were killed with an overdose of MS222 (tricaine methanesulfonate, 1 mg/ml). We recorded sex, standard length, body weight, and weights of key internal organs (head kidneys, spleen, liver, gonads and the body kidney). If the fish was infected with *S. solidus*, we recorded the number of plerocercoids and individual parasite weights.

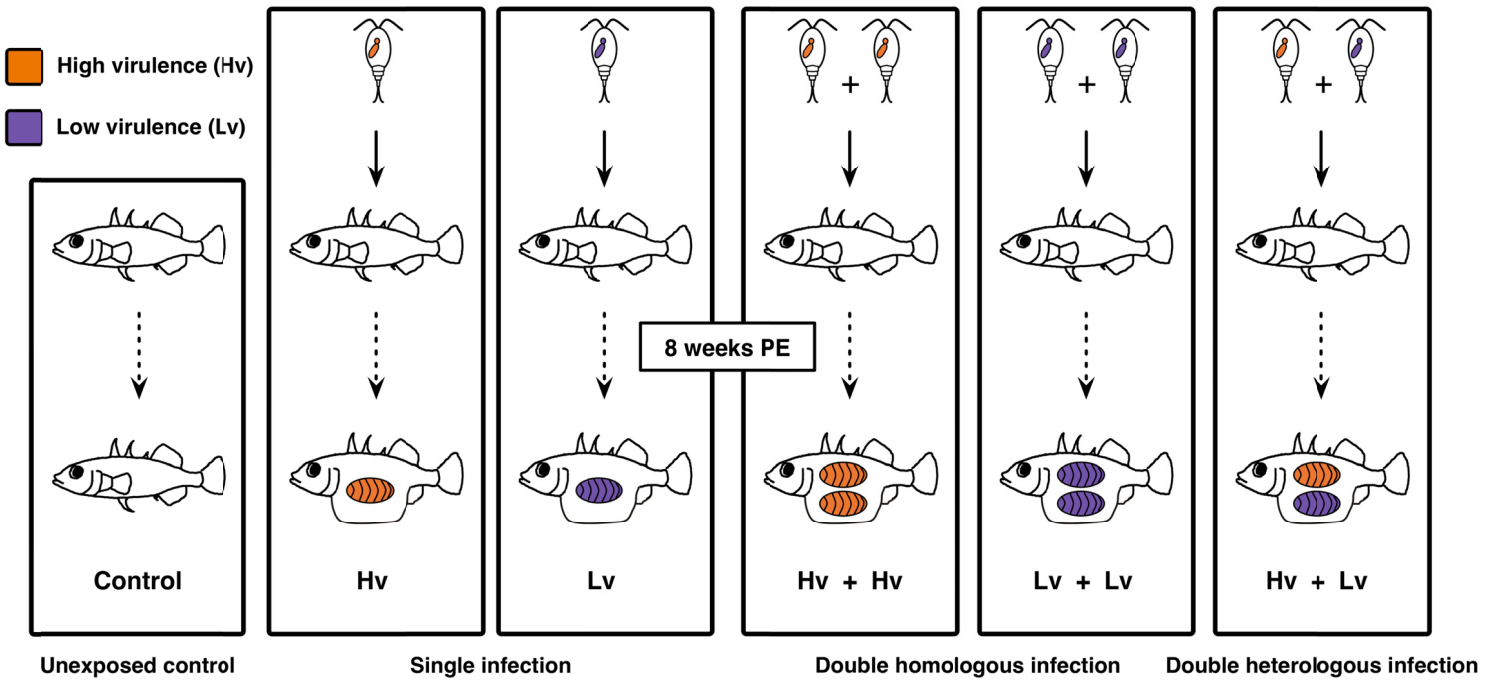

Figure 1

Experimental design: fish were exposed to one copepod infected with an Hv or Lv parasite, two copepods of a single virulence type (Hv+Hv and Lv+Lv), or two copepods with parasites of different virulence (Hv +Lv). An unexposed fish group was included as a control. Fish were dissected eight weeks post exposure (PE).

We calculated the following indices using the fish somatic weight to reduce the effect of differences in fish sexual maturation: i) the total parasite index (tPI) as a measure of parasite virulence (tPI = (total parasite weight [g] / fish somatic weight [g])*100); ii) the fish condition factor (CF) after Frischknecht (1993) ; iii) the hepatosomatic index (HSI) as an indication of fish energy reserves (Chellappa *et al*. 1995); iv) the splenosomatic index (SSI) and the head kidney index (HKI) as enlarged spleen and head kidney can be attributed to an elevated immune response to parasite infections (Press & Evensen 1999; Lefebvre *et al*. 2004); v) the relative parasite weight or discrete parasite index (dPI) describes the relationship between the weight of one individual parasite and the fish somatic weight, discriminating between two parasites in the same fish (dPI = individual parasite weight [g] / fish somatic weight [g])*100).

4.3.4 Microsatellite genotyping

To determine the strain and family of each successfully infecting parasite, we collected a tissue sample of each parasite for genotyping. We extracted DNA using the DNeasy® 96 Blood & Tissue Kit (Qiagen, Germany) following the manufacturer's protocol. We amplified five microsatellite loci (SsCTA22, SsCAB6, SsCTB24, SsCA25, SsCA58) (see Binz *et al*. (2000) for details) and conducted fragment analysis with GeneMarker 1.95 (SoftGenetics).

4.3.5Statistical Analyses

All statistics were carried out using R 2.12.2 (R Development Core Team, 2014). For all mixed effect models, the dissection date was included as a random factor to account for variation due to time difference. Fish sex was also included as a random factor due to known differences between male and female sticklebacks, in particular in infection rates (Reimchen 2001).

All analyses included fish family as a random effect. We first performed a combined analysis, including both trials and all fish families, to test for differences among treatments for the tested variables. Next, we tested each trial independently and the effect of fish family to confirm that the experimental results were consistent through both trials and between replicated

fish families. Worm family variation is accounted for in the treatment. While we found some variation between trials and among fish families, we observed overall similar treatment effects in the two experimental trials and in all fish families for the different tested variables and therefore report results from the combined analyses (see Supp. Analysis SA1 and SA2).

We used a linear mixed effect model (lmer function, lme4 library) and a type-III ANOVA (Anova function, car library) to assess how total parasite index (tPI) discrete parasite index (dPI), condition factor (CF), hepatosomatic index (HSI), splenosomatic index (SSI) and head kidney index (HKI) differed among infection treatments (Lv single homologous, Hv single homologous, Lv+Lv double homologous, Hv+Hv double homologous or Hv+Lv double heterologous). For the infection rate, we used general linear mixed-effect model with binomial (parasite presence/absence) or poisson family (number of successfully established parasite). Analyses of fish indices also included the unexposed fish control group. Response variables were transformed to achieve normal distribution, if necessary (log transformation for HSI, square root transformation for SSI and HKI). We used Tukey's tests for all post hoc comparisons (lsmeans function, lsmeans library).

4.4 Results

4.4.1 Infection rates

The infection rate did not vary between fish exposed to a single Lv or Hv parasite (Lv or Hv, $F_{1,546}$ = 1.239, P = 0.266) nor did it vary among double-exposure treatments (Lv+Lv, Hv+Hv or Hv+Lv, $F_{2,927}$ = 1.389, P = 0.499) (fig. S4). There was also no significant difference among double-exposure treatments in the number of successfully established parasites (0, 1 or 2) ($F_{2,927}$ = 0.212, P = 0.899).

4.4.2 Total parasite index (overall virulence)

The total parasite index tPI, differed significantly among infection types (ANOVA, $F_{4,321}$ = 1397.980, p < 0.001) (fig. 2). The tPI varied significantly among all treatment pairs (Tukey's HSD, p < 0.001), except between single and double infections of low virulence parasites (Lv vs. Lv+Lv, Tukey's HSD, P = 0.904). Hv+Hv double infections showed the highest tPI, followed by Hv single infections. Single Lv infections had the lowest tPI. The heterologous Hv+Lv double-infection had an intermediate tPI (fig. 2).

4.4.3 Discrete parasite index (individual virulence)

There was a significant interaction between infection type and parasite virulence strain on the individual parasite index (dPI) (ANOVA, $F_{5,452}$ = 1563.470, p < 0.001), with all infection types significantly differing from each other (Tukey's HSD, p < 0.010) (fig. 3).

As expected from the breeding of the parasite strains, Hv showed significantly higher dPI than Lv in single parasite infected groups (Tukey's HSD, p < 0.001). The dPI of single infections (Hv and Lv) was significantly higher than that of the respective homologous double infections (Hv+Hv and Lv+Lv, Tukey's HSD, p < 0.001). This suggests some costs of multiple infections for individual parasites, independent of their level of virulence.

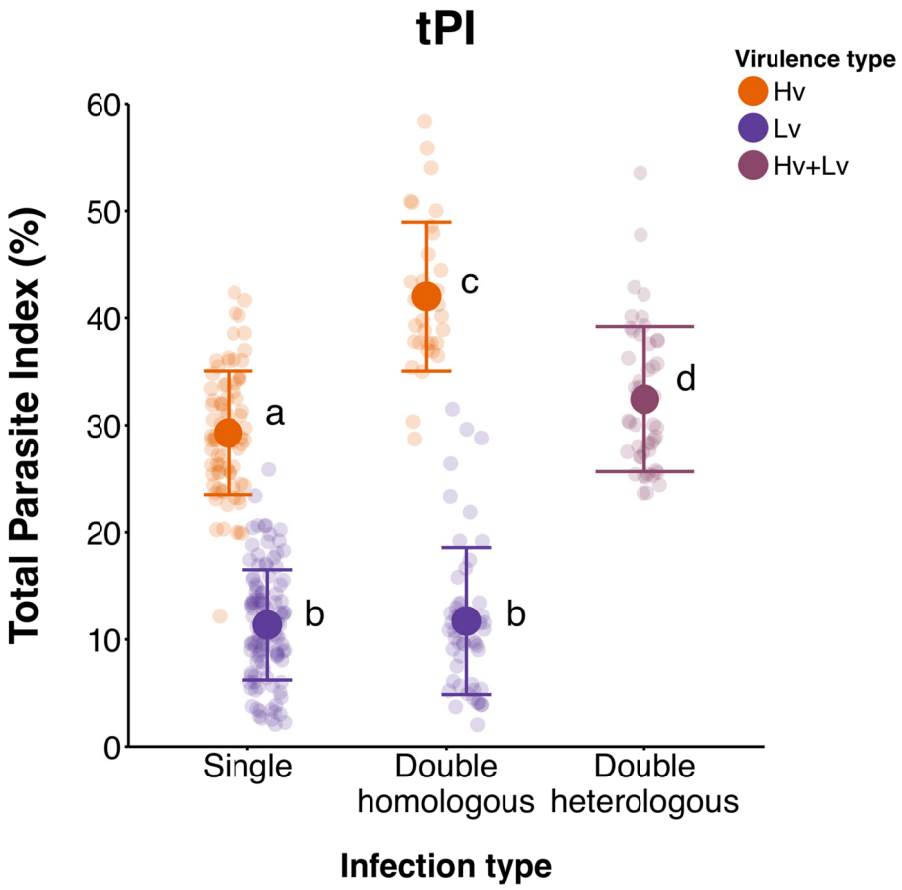

Figure 2

Total parasite index (tPI ±SD) in the different treatment groups.

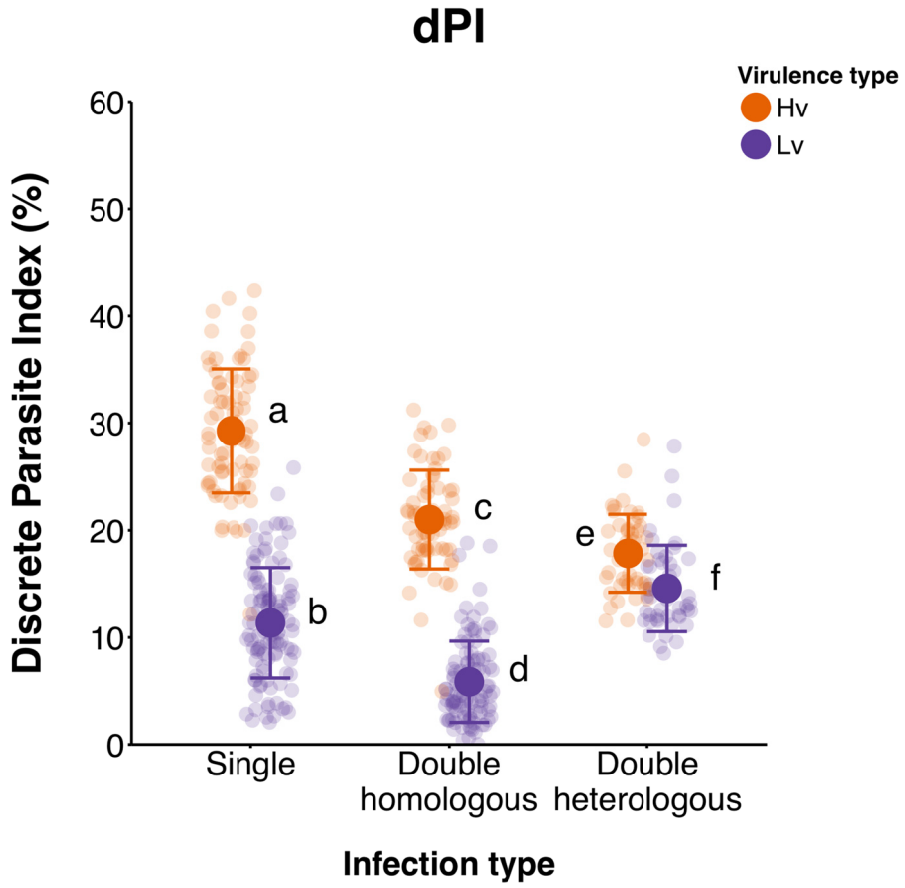

Figure 3

Virulence of individual parasites, measured as mean discrete parasite index (dPI ±SD), in the different treatment groups.

In heterologous double infections (Hv+Lv), Hv parasites had significantly higher dPIs than Lv co-infecting competitors (Tukey's HSD, $P = 0.003$). Interestingly, dPI for Hv was significantly lower in Hv+Lv treatments than in Hv single infections (Tukey's HSD, $p < 0.001$), or even in Hv homologous double infection (Tukey's HSD, $P = 0.007$). Conversely, Lv parasites in heterologous infections had significantly higher dPI than in Lv+Lv homologous double infections (Tukey's HSD, $P < 0.001$), or Lv single infections (Tukey's HSD, $p < 0.001$). Slight variations across experimental trials were observed but overall patterns remained qualitatively similar (see fig. S2.1 and table S2.2): i.e. Hv consistently performed worse in heterologous infections than in single infections, while Lv did better in heterologous compared to single infections.

In summary, Lv parasites gained from sharing their hosts with a more virulent Hv parasite, growing significantly bigger than with no competitor or in double homologous infections.

4.4.4Fish condition and immunological traits

Fish body condition factor (CF) varied with experimental treatment (ANOVA, $F_{5,392} = 53.313$, $p < 0.001$). This effect was mainly driven by fish groups infected with Hv parasites (Hv, Hv+Hv, or Hv+Lv treatments), which significantly differed from the unexposed control group (Tukey's HSD, $p < 0.001$). Furthermore, fish infected with two Hv parasites had the lowest CF of all treatment groups (Tukey's HSD, $p < 0.010$).

There was a significant effect of experimental treatment on the hepatosomatic index (HSI; ANOVA, $F_{5,392} = 108.050$, $p < 0.001$). Fish from all infection types (Lv, Hv, Lv+Lv, Hv+Hv, Hv+Lv) had a significantly lower HSI than the unexposed control fish (Tukey's HSD, $p < 0.001$) (fig. 4b) further highlighting the costs of parasitism. Even in Lv single infections, which had the smallest parasite burden, HSI was significantly decreased compared to control fish (Tukey's HSD, $p < 0.001$). The HSI of homologous single or double infections were not significantly different from each other (respectively, Hv and Hv+Hv: Tukey's HSD, $P = 0.966$; Lv and Lv+Lv: Tukey's HSD, $P = 0.997$) or from the heterologous double infection (respectively, Hv and Hv+Lv: Tukey's HSD, $P = 0.574$; Lv and

Hv+Lv: Tukey's HSD, $P = 0.828$). The HSI of Hv single infections was significantly lower than in Lv single or Lv+Lv double infections (respectively, Tukey's HSD, $P = 0.007$ and $P = 0.017$).

There was a significant effect of infection treatment on the splenosomatic index (SSI; $F_{5,392} = 29.119$, $p < 0.001$). The SSI was significantly higher than in unexposed control fish when only one Hv parasite was present, i.e. in the single Hv (Tukey's HSD, $p < 0.01$) and double heterologous Hv+Lv infections (Tukey's HSD, $p < 0.01$).

There was a significant effect of the treatment on the headkidney index (HKI; ANOVA, $F_{5,392} = 60.262$, $p < 0.001$). The HKI was significantly higher for all infected groups than in the unexposed control group (Tukey's HSD, $p < 0.010$) (fig. 4d), suggesting an up regulation of fish immunity upon infection. Even though there were striking differences in parasite burden among infection types, differences in HKI were observable only between single Hv infected fish and single Lv infected fish, with lower HKI in single Hv infection (Tukey's HSD, $P = 0.003$).

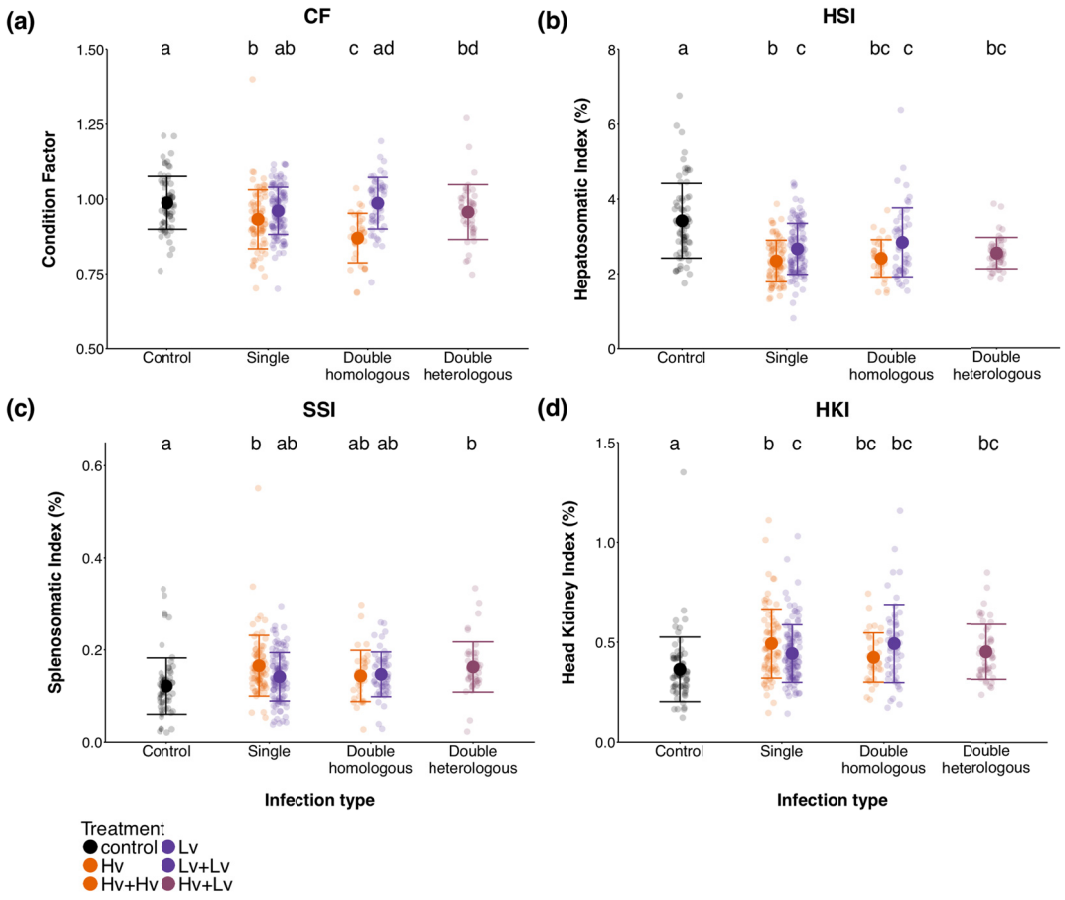

Figure 4

Variation in fish condition and immune parameters in the different treatment groups: **a)** mean condition factor (CF ±SD) **b)** mean hepatosomatic index (HSI ±SD) **c)** mean splenosomatic index (SSI ±SD) and **d)** mean head kidney index (HKI ±SD). Letters above indicate which groups are significantly different (Tukey's HSD).

4.5 Discussion

In natural populations, variation in epidemiology, such as the frequency of multiple infections, seems to select for different evolutionary trajectories and host exploitation strategies (Rigaud *et al*. 2010; Alizon *et al*. 2013). While the evolution of parasite and disease virulence is generally seen as the result of host-parasite coevolution, our results show that virulence in *Schistocephalus solidus* is also shaped by intraspecific interactions among co-infecting parasites mediated by the production of different types of goods (see Box 1 and fig. 5). This may highlight a trade-off between survival, through manipulation of the host immune system, and growth, through competitive ability to exploit the host resources (Rigaud *et al*. 2010).

4.5.1 Different strains, different host costs

Fish infected with only Lv parasites (Lv or Lv+Lv) did not suffer reduced CF, suggesting that more host resources are available than can be exploited by the parasite. Conversely, the high virulence of Hv parasites was costly for the host in both single and double homologous infections. These results are consistent with the expected levels of virulence and highlight how host exploitation is largely determined by parasite intrinsic characteristics.

HSI, a marker of fish metabolic condition, decreased upon infection independently of the parasite type, compared to unexposed control fish. In general, Hv parasites (Hv and Hv+Hv) were associated with lower HSI than Lv parasites (Lv and Lv+Lv), once more validating the increased host costs of Hv strains. Furthermore, in heterologous combinations, the reduction of HSI appears intermediate to the costs displayed under Hv and Lv exposures: the presence of the low virulence parasite diminishes the cost of the Hv parasite to the fish. This hints at a compromise between host exploitation and intrinsic resource acquisition capacity in mixed infection.

When considering activation of the host immune system (SSI and HKI), we found that the greatest difference was between heterologous infections and unexposed control fish. Strain-specific differences were detected only for HKI in

single infections. Altogether, these indices suggest that upon infection a general response is triggered in the host, independent of the virulence type. Lack of specific response is likely due to the use of a host population naïve to the experimental parasite strains.

4.5.2 Differences in strain virulence

In single infections, the replicated Hv and Lv parasite strains showed the expected pattern of virulence where Hv worms grew bigger than Lv worms. As size is a proxy for virulence, this confirms that virulence has a strong genetic basis in *S. solidus*.

Quite logically, the number of infecting parasites also affected parasite growth as shown by the reduced individual parasite index in homologous infections (Hv+Hv and Lv+Lv) compared to single infections. Yet, the total parasite burden in Hv+Hv infection was higher than that of Hv in single infections, indicating that more host resources could be exploited jointly. But this effect was not seen with Lv parasites suggesting that those parasites reached their maximum capacity of exploitation. Whether Lv parasites are unable to efficiently exploit the host, or show self-limitation to avoid over-exploitation is unclear. Nonetheless, our results confirm that our two strains differ in virulence and in intrinsic exploitation ceiling.

4.5.3 Virulence is a heritable and plastic trait

Our results revealed that heterologous double infections displayed an intermediate level of parasite burden, in-between Hv and Lv homologous combinations. Strikingly, this intermediate level was achieved not only by a decrease in Hv parasite growth due to double infection, but also by increased growth of Lv parasites, above that achieved by Lv worms in single infections. This clearly demonstrates that in *S. solidus*, while there is a clear heritability in virulence, this trait is also context-dependent or plastic as it varies with co-infection (fig. 3).

4.5.4 Virulence is a combination of common and strain-specific goods

The most parsimonious explanation for the observed intermediate total parasite burden in heterologous combinations is the sharing of public goods produced from host resources. In *S. solidus*, public goods are produced via excretory/secretory products that modify the immediate environment of the parasite, i.e. the fish body cavity (Hewitson, Grainger & Maizels 2009). From the results of our experiment, we can infer that two categories of public goods are produced: common and strain-specific goods. Common goods could include both nutrients made available in the body cavity and resources gained from the manipulation of the innate (unspecific) immunity (Scharsack *et al.* 2004, 2007b; Mideo 2009). On the other hand, strain-specific goods are those goods available upon a specific, adaptive immune response, acting against specific parasite strains (Kurtz *et al.* 2004; Mideo 2009).

In this context, we hypothesize that production of public goods, whether common or strain-specific, is proportional to the worm's virulence. Through exploitation of common goods in a heterologous infection, an Lv parasite sharing a host with an Hv parasite can increase in virulence (i.e. size), while the Hv parasite decreases in virulence (see Box 1 and fig. 5b), and they jointly reach an intermediate ***total*** parasite index. However, common goods alone are not sufficient to explain the maintained ***individual*** differences between Hv and Lv in mixed infections (see Box 1 and fig. 5b). This difference indicates the existence of strain-specific goods that are solely available to a certain strain likely linked to the manipulation of the adaptive immune system (see Box 1 and fig. 5d; Brown *et al.* 2002; Dionisio & Gordo 2006; Leggett *et al.* 2014).

The combination of common and strain-specific goods could represent a trade-off between cooperation and competition with possible mates. *S. solidus* is a simultaneous hermaphrodite that favours outcrossing when possible (Lüscher & Wedekind 2002), whereby increasing fitness in the offspring (Wedekind *et al.* 1998; Milinski 2006). Therefore, in multiple infection, while common goods help any co-infecting parasite, including potential mating partner (Schärer *et al.* 2001), strain-specific goods could be the mechanism through which *S. solidus*

favours related individuals to increase inclusive fitness (Jäger & Schjørring 2006).

4.5.5 The case of apparent competition

To focus on parasite-parasite interactions, we used a naïve host population with regards to *S. solidus*. Nonetheless, the stickleback population is geographically closer to the host population of the Lv than the Hv strain. If the fish hosts were locally adapted to Lv parasites, this strain could appear to be less virulent than Hv parasites while in fact, it would be the host that was more resistant to this strain. This phenomenon, known as 'apparent competition' (Bashey 2015), could elicit a more adequate response to both Lv and Hv parasites due to the presence of an Lv parasite in heterologous infection. While this would explain why Hv is less virulent in mixed infections, this would not explain why Lv parasites are more competent/virulent when they share their host with a Hv parasite. This pattern is confirmed by the general and identical immune response of the fish upon any type of infection. Overall, our results demonstrate that in addition to the known parasite-host interaction effects, parasite-parasite interactions can influence the outcome of infections and therefore virulence.

4.5.6 Parasite-parasite interactions mediated via the host immune system

The observed experimental patterns can also be interpreted in the light of the ecological context in which both strains evolved. The Hv strain evolved high virulence in a population with high infection prevalence and frequent multiple infections. Its stickleback host population has already evolved a high level of resistance, particularly compared to the host population of the Lv strain (Kalbe *et al.* 2016). Therefore, two Hv parasites sharing the cost of immune manipulation might give each individual parasite more opportunities to compete and jointly better produce and exploit goods. On the other hand, because the Lv strain is at low prevalence in the host population, it rarely encounters competitors and does not face host resistance. Consequently Lv parasites exploit host resources less efficiently but at a lower cost. Together, our results link

ecological conditions with the evolution of virulence through both host-parasite and parasite-parasite interactions.

4.5.7Conclusion

Our results show that intraspecific competition can directly alter the virulence of a parasite as low virulent parasites can benefit from the presence of high virulence co-infecting parasites. Depending on the frequency of co-infection, decrease of virulence of Hv worms in heterologous infections could allow for virulent genotypes to be maintained and possibly invade a Lv population. On the other hand, the Lv parasite's "free-loading" in the Hv parasite population allows for the maintenance of diversity within the parasite population and reduces risks of an arms-race escalation. The sharing of goods thus increases viability of parasite populations within their hosts by maintaining polymorphism at virulence genes.

Overall, our results revealed not only the genetic inheritance of virulence but also its context-dependent expression. Similar to variation in tolerance levels observed in host populations (Råberg, Sim & Read 2007), we demonstrate that parasite virulence is also plastic and its expression depends on the coexistence with other individual parasites. Our results have important implications for our understanding of the evolution of virulence in particular, and for host-parasite interactions in general.

Box 1 Virulence models of co-infecting parasites

The effects of different virulence models including intrinsic factors, production of common goods (available to all parasites), and production of strain-specific goods (available to only a specific strain) on the discrete parasite index of High virulence (Hv) and Low virulence (Lv) parasites in double homologous infection (Hv+Hv and Lv+Lv) or heterologous infection (Hv+Lv).

a) Intrinsic virulence

If virulence is an intrinsic factor, the parasite index is solely determined by the virulence type (Hv or Lv) and is independent of the virulence level of a co-infecting parasite (fig. 5a).

b) Common goods

If virulence is determined by the production of common goods proportional to the intrinsic virulence level (black dots), all the goods produced by the co-infecting parasites are available and equally shared by both. In this case, the parasite index of heterologous co-infecting parasites is the same and is intermediate between indices of homologous infections (fig. 5b).

c) Strain-specific goods

If virulence is determined by the production of strain-specific goods proportional to the intrinsic virulence level (colored dots), the resources produced by a virulence type are only available for this specific strain and cannot be used by a heterologous co-infecting parasite. This mimics the effect of the intrinsic virulence model **a)**; the parasite index is independent of the virulence level of the co-infecting parasite (fig. 5c).

d) Common goods + Strain-specific goods

If virulence is a combination of the production of both common goods (black dots) and strain-specific goods (colored dots) proportional to intrinsic virulence levels, a limited proportion of the resources produced by a virulence type are available to a heterologous co-infecting parasite. In this case, an Lv parasite will benefit from sharing a host with an Hv parasite which is producing more common goods. The two parasites will reach an intermediate total parasite index, but the Hv parasite will show a higher discrete parasite index than its co-infecting Lv parasite, due to higher production of strain-specific goods. These predictions correspond to the results of our experiment (fig. 5d).

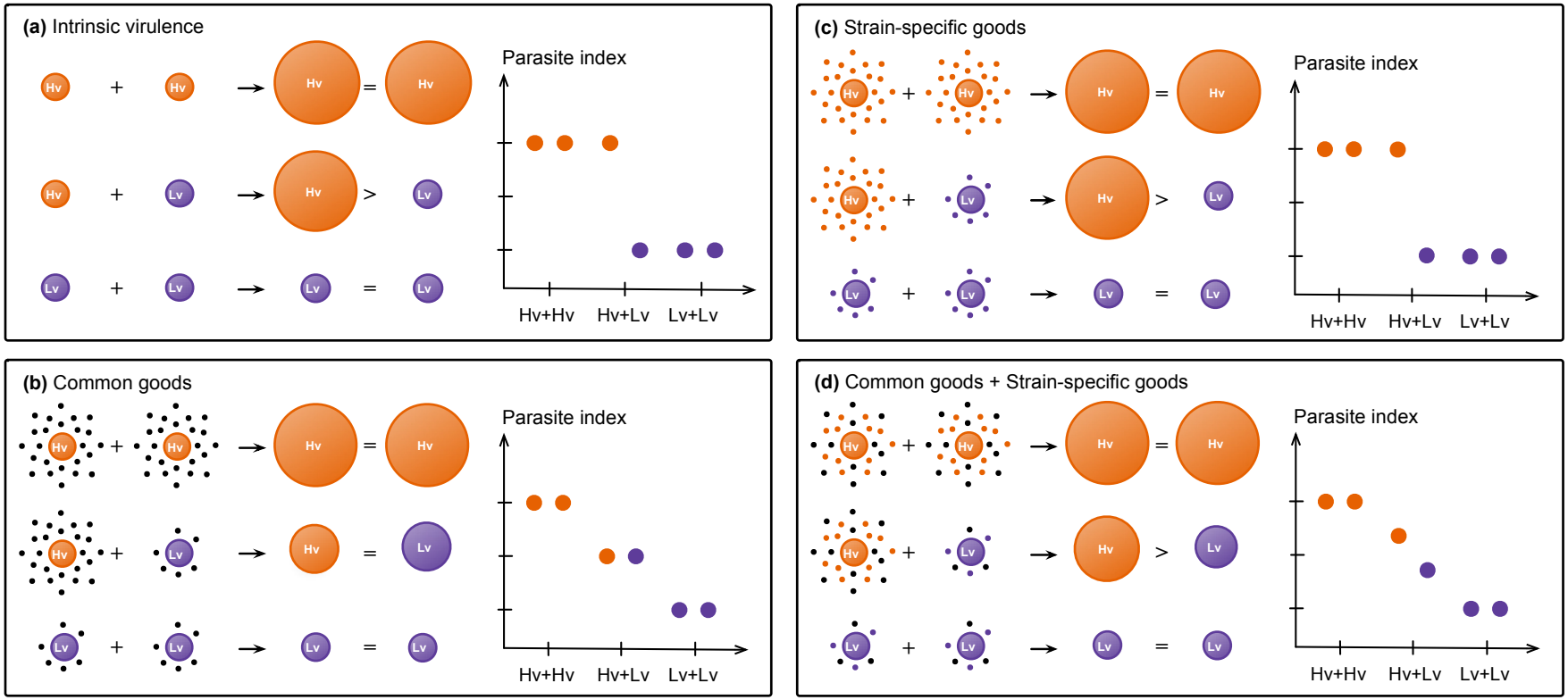

Figure 5

Schematic representation and predictions for the different virulence models of co-infecting parasites **a)** intrinsic virulence, **b)** common goods, **c)** strain-specific goods, **d)** common goods + strain-specific goods.

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Conclusion & Perspectives

In this thesis, I explored how host and parasite genotype interactions (G x G) at different ecological levels (zooming in from community, to population, to the individual) can influence the evolutionary trajectory of parasite virulence and host resistance. During my doctoral research, I tested i) how the parasite community as a whole can shape host immunocompetence and restrict gene flow between host populations; ii) how spatial variation in parasite prevalence and host susceptibility correlates, and to which extent they can depend on ecological or coevolutionary processes; iii) how local adaptation to a specific parasite species in different populations can lead to optimal virulence and resistance levels; and finally iv) how within-host parasite interactions can influence the evolution of virulence away from this optimal level?

In ***Chapter 1***, I presented the first well-documented case of a macroparasite-free three-spined stickleback population. Using this natural system of a non-coevolved river population flowing into a parasitized lake population, I experimentally confirmed the theoretical predictions that reduced parasite-mediated selection leads to lower resistance to infections. Divergent parasite-mediated selection also resulted in a limited gene flow between non-coevolved and coevolved populations, highlighting the fundamental role of parasites in the ecological divergence and reproductive isolation of organisms. This system offers a great opportunity to test for parasite-mediated sexual selection and reduced immigrant viability in nature and in the laboratory.

One step further would be to confirm in the wild the deleterious effect of a diverse parasite community on first-generation migrants and descendants of the non-coevolved host population. One way to do this would be to use transplant experiments to expose to both parasite-free and parasite-rich environments, pure and hybrid lab-bred fish from the two populations. Measuring fish relative parasite susceptibility and condition in the two environments would give the ultimate evidence that relaxed parasite selection prevent the river fish to establish in the parasite-rich environment downstream. Additionally, a formal proof of sexual reproductive isolation between non-coevolved and coevolved population could be to assess in behavioural mate

choice experiment the preference of lake fish for their own kind over first-generation migrant and hybrid river fish after exposure to the parasite-rich environment.

In this chapter, I focused on neutral genetic markers to measure the isolation between non-coevolved and coevolved host population. However, if parasite-mediated selection is responsible for the genetic divergence observed between the river and lake populations, clear predictions can also be formulated concerning the relative diversity of adaptive markers. For instance, the pleotropic MHC genes would be good candidates as they are involved in both parasite resistance and mate choice and seem to be under divergent selection in different stickleback populations exposed to contrasting parasite communities (Eizaguirre *et al*. 2011, 2012). Hence I would suggest measuring the allelic diversity of MHC genes to confirm that relaxed parasite selection in the river population result in a lower MHC allelic diversity compare to lake fish.

Finally, our findings highlights that given the right environmental conditions (such as lack of intermediate hosts), macroparasite-free three-spined stickleback populations can exist. It would be interesting to screen wild three-spined populations (or other model systems) looking for other macroparasite-free populations that are connected to parasite-rich populations. If these specific evolutionary and ecological settings exist in different natural systems, it would allow to verify the reproducibility of the effect of relaxed parasite selection, as well as disentangling the potential confounding factors at play in the present system; namely genetic drift or bottleneck versus relaxed parasite-mediated selection.

In ***Chapter 1***, I introduced how contrasting heterogeneous environments and divergent parasite communities can select for different host genotypes. In ***Chapter 2***, I focused on the pressure of selection imposed by one specific parasite species, *S. solidus*. The reciprocal experimental infections of stickleback and *S. solidus* population from same and different continents revealed that resistance to *S. solidus* is a recently derived trait, to which the parasite has locally counter adapted. Susceptible marine stickleback populations (used as a proxy for the ancestral marine populations prior to freshwater habitat colonization) have low infection prevalence while lake populations are

commonly infected despite having developed global resistance to both local and foreign *S. solidus* strains. The pattern of infections of the different populations does not exactly match the classic theoretical models, potentially highlighting the complexity of the interactions in this system, which might implicates multiple genes. The variation in parasite prevalence observed in freshwater stickleback populations under similar exposure risk reflects the diversity of the defence mechanisms developed against *S. solidus*. While some populations control parasite growth, others seem to be able to resist infection by encysting tapeworms before they establish (Weber *et al.* 2016). This model system could help understand under which conditions host preferentially develop tolerance or resistance strategies to infection.

In ***Chapter 3***, I showed that two host-parasite population pairs of three-spined stickleback and *S. solidus* show signs of local adaptation and have reached the same relative level of optimal host resistance and parasite virulence following different coevolutionary histories. This system is a promising model to identify the genetic basis of virulence and resistance in the *S. solidus* infections and could be applied to study the genetic interplay between host and parasite during coevolution. I used a candidate gene approach to identify the variation in host immune response involved infection phenotypic differences. As I was unable to identify clear genetic markers accounting for the phenotypic differences between allopatric and sympatric infection combinations, I recommend using earlier time points in the infection to identify the specifics of the immune response to *S. solidus*. Further analyses looking at *S. solidus* gene expression are in progress and will help identify the specific molecular cross-talk between host and parasite, the so-called interactome (Biron & Loxdale 2013). As demonstrated by the identification of candidate mimicry proteins (Hebert *et al.* 2015), the study of the recently sequenced genome of *S. solidus* could uncover how this parasite manipulates its host and which phenotypical changes are attributable to the host or the parasite. Screening other stickleback and *S. solidus* populations for the optimal resistance-virulence level could also determine if this is a generalized specificity of this system.

In ***Chapter 4*** I approached a higher level of complexity in host-parasite interactions, the effect of within-host parasite interactions on virulence

expression. In nature host are rarely infected with one individual parasite or one parasite species, and parasite interactions within the host have the potential to favour increased virulence through competition, cooperation and phenotypic plasticity of individual parasite. Here I explored intraspecific interactions and found that virulence is a plastic trait in *S. solidus* which is influenced by the co-infecting parasite strain. Specifically, a high virulent strain in single or homologous infections, showed lower host exploitation when in heterologous infection, and vice versa for the low virulence strain. I interpreted these variations in virulence expression as the results of common and strain-specific goods production. To determine if these observations are due to individual parasites directly interacting with each other, or via the host immune system, gene expression studies of the specimens are in progress. Additionally, to study further the heritability of virulence and unravel the genetic of virulence expression in the stickleback/*S. solidus* system, crossing between high and low virulent strains have been performed (Ritter, Kalbe & Henrich 2017). Within-host interactions of different genotype could alter virulence expression and move coevolution away from optimal levels.

To truly understand the mechanism and selective pressure of host-parasite interactions, more studies of inter- and intra-specific within-host parasite interaction are needed. In the case of manipulative parasites like *S. solidus*, different age, different virulence or even different species of co-infecting parasites can raise conflict and have important implications on how successful at manipulating the host and completing the life cycle individual parasite can be (Hafer & Milinski 2016b).

To conclude, my work presented a comprehensive multidisciplinary approach to better understand host-parasite interactions in my model system. I showed that complex inter- and intraspecific interaction at different ecological scales have the potential to influence the host-parasite interactions between the three-spined stickleback and its tapeworm *S. solidus*. First, I demonstrated that the parasite community as whole shapes the host immunocompetence. Then I showed that both ecological processes and coevolutionary history affect resistance and virulence patterns of host-parasite populations. I looked in more details in the interactions between host and parasites genotypes through the

modulation of immune gene expressions in locally adapted pairs. And Finally, I showed that using the public goods frameworks to understand within-host parasite interactions was a promising way to interpret phenotypic plasticity and maintenance of polymorphism in virulence.

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Appendix

Appendix – Chapter 1

Supplementary table S1

Sample sizes for each sampling site and survey year. Sample size of fish from each population sampled for DNA, identified as RA immigrants or RA-L admixed individuals, screened for parasites and that had their inner organs weighed during the field surveys. Most of the fish (N = 274) were dissected in Norway shortly after collection, while the rest were transferred alive (N = 49) or frozen (N = 97) to the Max Planck Institute for Evolutionary Biology (Plön, Germany) before dissection.

Population	Abbr.	Year	DNA	RA immigrants	RA-L admixed	Screened for parasites*	Inner organs weighed*
Orraelva river ('River Above' waterfall)	RA	2009	40	-	-	8	8
		2010	35	-	-	15	15
		2012	43	-	-	16	32
		2013	15	-	-	10	10
		all	***133***	***-***	***-***	***49***	***65***
Orraelva river ('River Below' waterfall)	RB	2009	49	3	5	4	4
		2010	24	3	3	21	21
		2012	116	19	4	112	112
		2013	174	9	4	144	170
		all	***363***	***34***	***16***	***281***	***307***
Skogseidvatnet lake	L	2009	47	-	-	8	8
		2010	52	-	-	15	15
		2012	43	-	1	31	31
		2013	25	-	-	20	20
		all	***167***	***-***	***1***	***74***	***74***
	Total	***all***	***663***			***404***	***446***

*****, RA-L admixed individuals excluded

Supplementary table S2

Summary of experimental infections with *Diplostomum pseudospathaceum*. Sample size of the different experimental groups per genetic type (Skogseidvatnet 'River Above' RAxRA, 'River Above' maternal hybrid RAxL, lake maternal hybrid LxRA, lake LxL) and per laboratory-bred fish family.

Genetic type	Fish family		Exposed	Uninfected	Infected
	Male	Female			
RAxRA	RA-M1	RA-F1	10	-	10
	RA-M2	RA-F2	12	-	12
	RA-M3	RA-F3	12	-	12
	RA-M4	RA-F4	12	-	12
	RA-M5	RA-F5	12	-	12
	all		***58***	-	***58***
LxL	L-M1	L-F1	12	-	12
	L-M3	L-F3	11	-	11
	L-M4	L-F4	9	-	9
	L-M5	L-F5	12	-	12
	L-M6	L-F6	12	-	12
	all		***56***	-	***56***
LxRA	L-M1	RA-F1	12	-	12
	L-M2	RA-F2	12	-	12
	L-M3	RA-F3	11	-	11
	L-M4	RA-F4	12	-	12
	all		***47***	***-***	***47***
RAxL	RA-M1	L-F1	12	-	12
	RA-M3	L-F3	11	-	11
	RA-M4	L-F4	13	-	13
	RA-M5	L-F5	12	-	12
	all		***48***	-	***48***

Supplementary table S3

Summary of experimental infections with *Schistocephalus solidus*. Sample size of the different experimental groups per genetic type (Skogseidvatnet 'River Above' RAxRA and lake LxL) and per laboratory-bred fish family.

Genetic	Fish family		Unexposed control	Exposed	Dead			Exposure results	
	Male	Female			Unexposed control	Uninfected	Infected	Uninfected	Infected
RAxRA	RA-M3	RA-F3	10	30	-	7	3	17	13
	RA-M4	RA-F4	9	30	1	5	5	15	15
	RA-M5	RA-F5	10	29	2	2	2	18	11
	all		***29***	***89***	***3***	***14***	***10***	***50***	***39***
LxL	L-M1	L-F1	8	32	-	-	-	23	9
	L-M3	L-F3	9	29	-	-	-	22	7
	L-M9	L-F9	10	30	-	-	-	27	3
	all		***27***	***91***	***-***	***-***	***-***	***72***	***19***

Supplementary table S4

Summary of genetic diversity measures for each sampling sites at nine microsatellite loci. Sample size (N), allelic diversity (A), private allele number (private), observed heterozygosity (Ho), expected heterozygosity (Hs) and *P*-value from Hardy-Weinberg exact test (HW) for heterozygote deficits across all years for each sampling site ('River Above' RA, 'River Below' RB and lake L).

Locus	RA							RB							L						
	N	A	Private	Ho	Hs	F_{IS}	HW	N	A	Private	Ho	Hs	F_{IS}	HW	N	A	Private	Ho	Hs	F_{IS}	HW
Gac4170	132	2	-	0.545	0.469	-0.1647	0.0685	361	14	2	0.784	0.810	+0.0323	0.0387	167	12	-	0.832	0.841	+0.0103	0.1211
Gac1125	133	2	-	0.481	0.502	+0.0409	0.7291	362	23	1	0.809	0.845	+0.0417	0.0368	167	23	1	0.874	0.881	+0.0073	0.7475
Gac5196	133	5	-	0.549	0.505	-0.0881	0.0638	362	16	2	0.793	0.820	+0.0333	0.3172	167	13	-	0.898	0.863	-0.0410	0.7024
Gac1097	130	3	-	0.531	0.489	-0.0850	0.5117	357	22	-	0.829	0.856	+0.0317	0.2479	161	24	2	0.894	0.896	+0.0020	0.9200
Gac7033	133	5	-	0.604	0.604	+0.0044	0.1153	361	20	3	0.712	0.834	+0.1467	**0.0000***	160	19	2	0.769	0.822	+0.0645	0.4869
STN32	133	2	-	0.368	0.371	+0.0062	1.0000	362	24	4	0.724	0.792	+0.0868	**0.0000***	163	21	1	0.810	0.847	+0.0443	0.0678
STN18	133	2	-	0.474	0.447	-0.0600	0.5603	363	12	-	0.667	0.676	+0.0140	0.0558	167	12	-	0.731	0.687	-0.0639	0.1234
STN75	133	2	-	0.023	0.093	+0.7590	**0.0000***	359	15	2	0.591	0.737	+0.1991	0.0029	167	14	1	0.689	0.731	+0.0575	0.0131
STN84	131	3	-	0.664	0.663	-0.0012	0.2983	352	24	3	0.699	0.696	-0.0035	0.4702	162	24	3	0.679	0.718	+0.0538	0.2230
Mean	132	3	-	0.471	0.460	0.0457		360	19	2	0.734	0.785	0.0647		164	18	1	0.797	0.809	0.0149	

In bold with asterisk *, significant

Supplementary table S5

Loci departing from Hardy-Weinberg equilibrium and locus pairs showing linkage disequilibrium for each sampling sites and survey year at nine microsatellite loci. The results are shown for analysis including or excluding the RA immigrant individuals' data (significant difference at $P < 0.050$ after Bonferroni correction). When the putative RA immigrant genotypes were excluded from the RB dataset, only RA2009 deviated from Hardy-Weinberg equilibrium for *STN75* and L2012 showed signs of linkage for three pairs of loci. Thus the migration of the differentiated RA fish into the RB site is most likely responsible for loci showing linkage disequilibrium in the populations below the waterfall.

Population	***Abbreviation***	***Year***	***Hardy-Weinberg equilibrium***		***Linkage disequilibrium***	
			with RA immigrants	without RA immigrants	with RA immigrants	without RA immigrants
Orraelva river ("River Above" waterfall)	RA	2009	STN75	STN75	-	-
		2010	-	-	-	-
		2012	-	-	-	-
		2013	-	-	-	-
		all	STN75	STN75	-	-
Orraelva river ("River Below" waterfall)	RB	2009	-	-	-	-
		2010	-	-	Gac4170-Gac1097; STN32-STN75	-
		2012	STN32	-	Gac1097-STN32; Gac4170-STN75	-
		2013	SNT32, STN75	-	Gac1097-STN18	-
		all	Gac7033, STN32	-	-	Gac7033-Gac1125; Gac1125-STN18

Skogseidvatnet lake	L	2009	-	-	-	-
		2010	-	-	Gac1097-Gac5196; Gac1125-Gac7033; Gac1125-STN18; Gac4170-STN32; Gac5196-STN84	-
		2012	-	-	Gac7033- STN75	Gac1125- Gac5196; Gac7033- STN18; Gac7033- STN84
		2013	-	-	-	-
		all	-	-	-	-

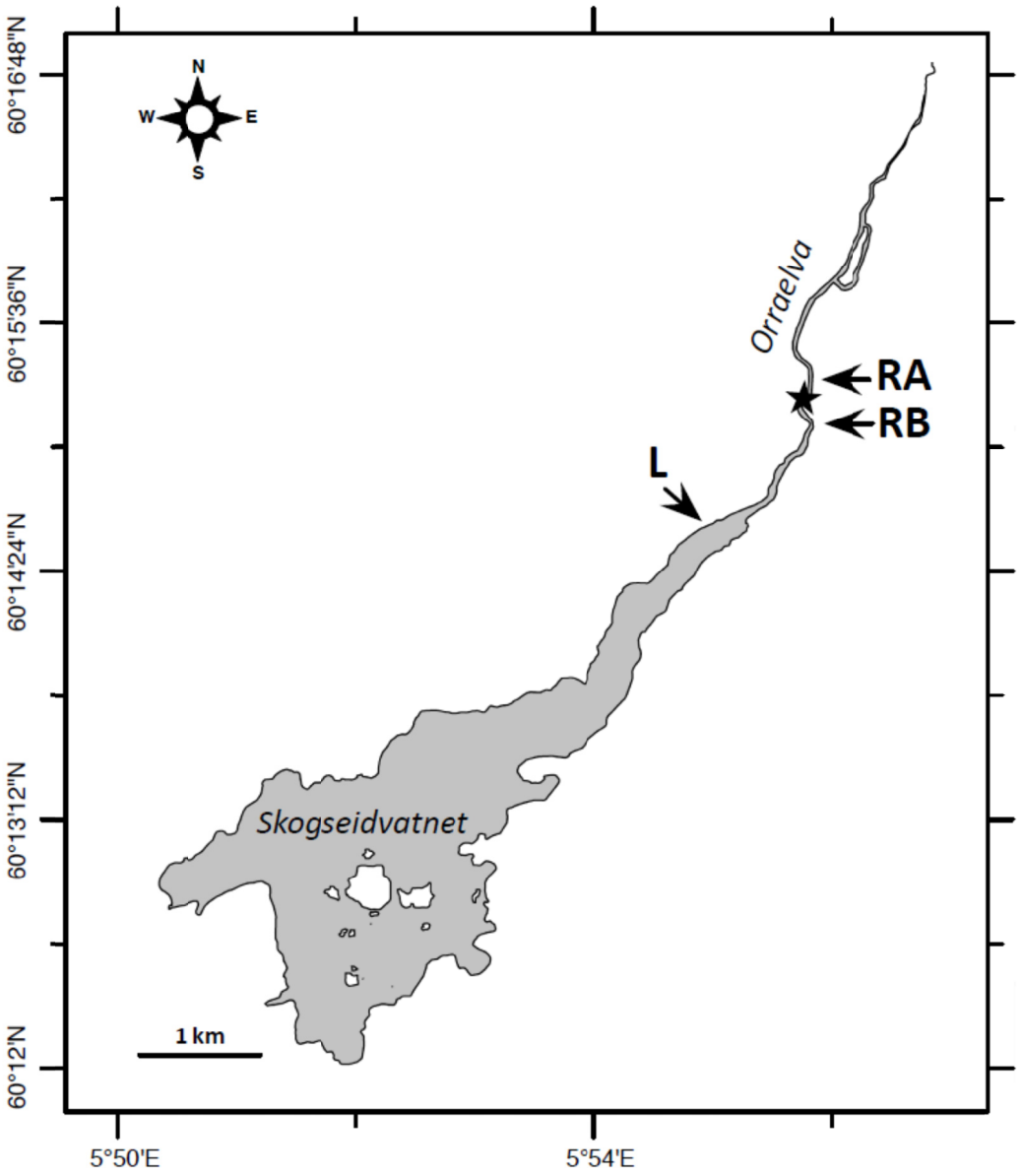
Supplementary table S6

Parasite prevalence, mean intensity and maximum intensity for each sampling sites and survey year. Prevalence (P), mean intensity (MI) and maximum intensity (Max.) of parasites in 1+ year *G. aculeatus* from the three Skogseidvatnet sampling sites ('River Above' (RA), 'River Below' (RB) and lake (L)) in Hordaland, Fusa, Western Norway (RA-L admixed individuals are excluded; see Table S1 for sample sizes). Developmental stage and location in the host are given in parentheses: M, metacercaria; C, cyst; A, adult; Pl, plerocercoid; L, larva; vh, eye vitreous humour; el, eye lenses; at, all tissues; i, intestine; sb, swimbladder; gb, gall bladder; cc, coelomic cavity.

			RA			RB			L		
Species	**Class**	**Year**	**P (%)**	**MI**	**Max.**	**P (%)**	**MI**	**Max.**	**P (%)**	**MI**	**Max.**
Apatemon sp. (C; vh)	Digenea	2009	-	-	-	75.0	1.7	3	37.5	4.7	10
		2010	-	-	-	38.1	2.7	6	86.7	2.5	7
		2012	-	-	-	31.2	1.8	5	80.6	4.5	18
		2013	-	-	-	44.4	2.1	10	60.0	4.5	31
		all	-	-	-	***39.1***	***2.0***	***10***	***71.6***	***4.0***	***31***
Diplostomum sp. (M; vh)	Digenea	2009	-	-	-	75.0	5.3	8	50.0	17.7	47
		2010	-	-	-	52.4	4.5	11	13.3	4.5	6
		2012	-	-	-	56.2	6.8	44	83.9	19.6	272
		2013	-	-	-	57.6	11.1	194	95.0	17.6	196
		all	-	-	-	***56.9***	***8.8***	***194***	***68.9***	***18.1***	***272***
Diplostomum sp. (M; el)	Digenea	2009	-	-	-	25.0	1.0	1	-	-	-
		2010	-	-	-	-	-	-	26.7	1.7	3
		2012	-	-	-	4.5	1.0	1	9.7	1.3	2
		2013	-	-	-	-	-	-	-	-	-
		all	-	-	-	***2.1***	***1.0***	***1***	***9.4***	***1.6***	***3***
Strigeinae gen. sp. (M; vh)	Digenea	2009	-	-	-	-	-	-	62.5	14.2	34
		2010	-	-	-	19.0	1.0	1	100.0	19.9	65
		2012	-	-	-	3.6	1.2	2	25.8	3.0	8
		2013	-	-	-	5.5	2.4	6	10.0	2.0	3
		all	-	-	-	***5.7***	***1.7***	***6***	***40.5***	***13.2***	***65***
Diphyllobothrium sp. (Pl; cc)	Cestoda	2009	-	-	-	25.0	5.0	5	37.5	18.0	45

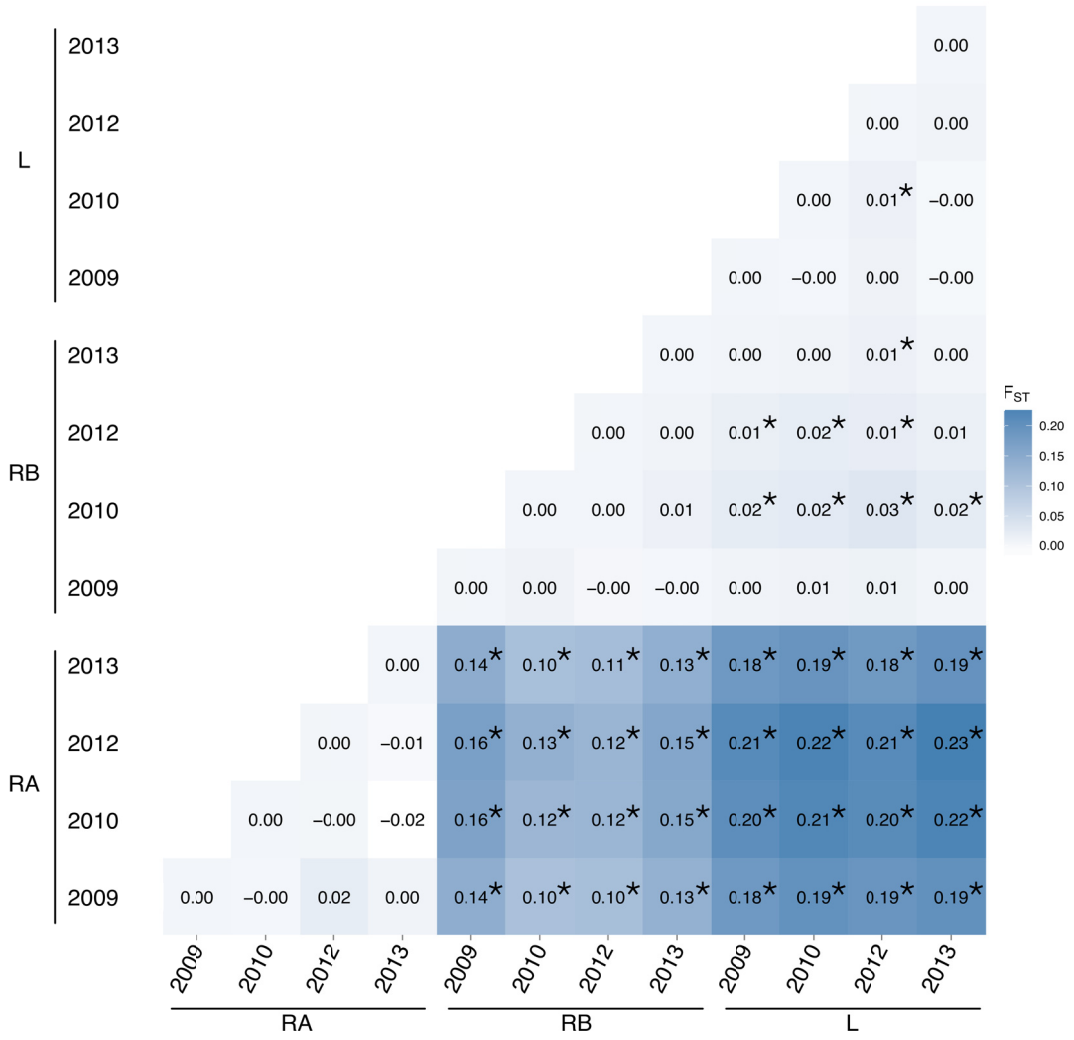
		2010	-	-	-	38.1	7.1	14	80.0	18.7	44
		2012	-	-	-	25.9	4.9	40	58.1	14.7	45
		2013	-	-	-	29.9	8.3	49	90.0	15.2	61
		all	-	-	-	***28.8***	***6.9***	***49***	***68.9***	***16.0***	***61***
Eubothrium (Pl, i)	Cestoda	2009	-	-	-	-	-	-	-	-	-
		2010	-	-	-	4.8	1.0	1	-	-	-
		2012	-	-	-	8.0	2.7	10	22.6	15.4	64
		2013	-	-	-	9.7	46.7	292	30.0	14.0	47
		all	-	-	-	***8.5***	***28.3***	***292***	***17.6***	***14.8***	***64***
Schistocephalus solidus (Pl; cc)	Cestoda	2009	-	-	-	-	-	-	37.5	1.0	1
		2010	-	-	-	19.0	1.0	1	80.0	1.1	2
		2012	-	-	-	9.8	1.8	4	38.7	4.1	15
		2013	-	-	-	14.6	1.1	3	95.0	1.7	3
		all	-	-	-	***12.8***	***1.3***	***4***	***62.2***	***2.1***	***15***
Proteocephalus sp. (Pl, A; i)	Cestoda	2009	-	-	-	-	-	-	12.5	1.0	1
		2010	-	-	-	-	-	-	-	-	-
		2012	-	-	-	-	-	-	-	-	-
		2013	-	-	-	0.7	1.0	1	5.0	1.0	1
		all	-	-	-	***0.3***	***1.0***	***1***	***2.7***	***1.0***	***1***
Contracaecum sp. (L; cc, at)	Nematoda	2009	-	-	-	-	-	-	12.5	3.0	3
		2010	-	-	-	-	-	-	-	-	-
		2012	-	-	-	0.9	1.0	1	-	-	-
		2013	-	-	-	-	-	-	-	-	-
		all	-	-	-	***0.3***	***1.0***	***1***	***1.7***	***3.0***	***3***
Eustrongylides sp. (L; cc, at)	Nematoda	2009	-	-	-	-	-	-	-	-	-
		2010	-	-	-	-	-	-	-	-	-
		2012	-	-	-	-	-	-	6.25	1.0	1
		2013	-	-	-	0.7	1.0	1	-	-	-
		all	-	-	-	***0.3***	***1.0***	***1***	***1.7***	***1.0***	***1***

-, not found.

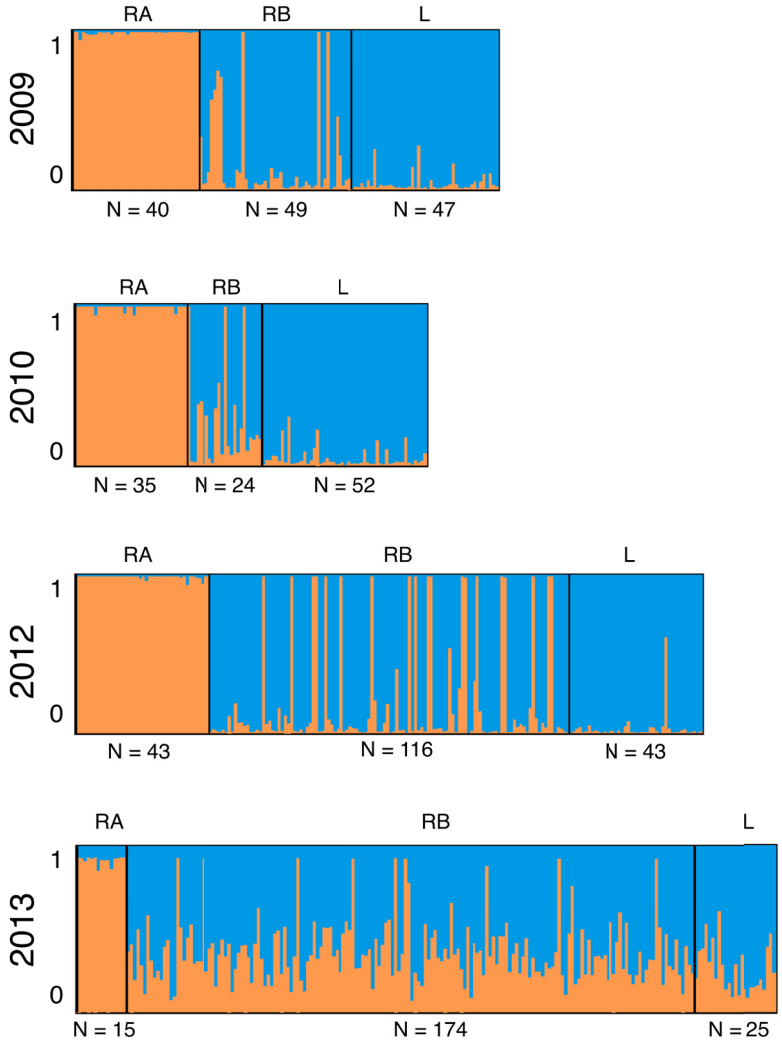


Supplementary figure S1

Map showing sampling locations in the Skogseidvatnet system (Western Norway). The waterfall separating the Orrealva River upstream the Skogseidvatnet Lake is represented by a star shape. The three sampling sites “River Above” (RA), “River Below” (RB) and Skogseidvatnet Lake (L) are denoted by arrow. The map is modified from Google Earth.

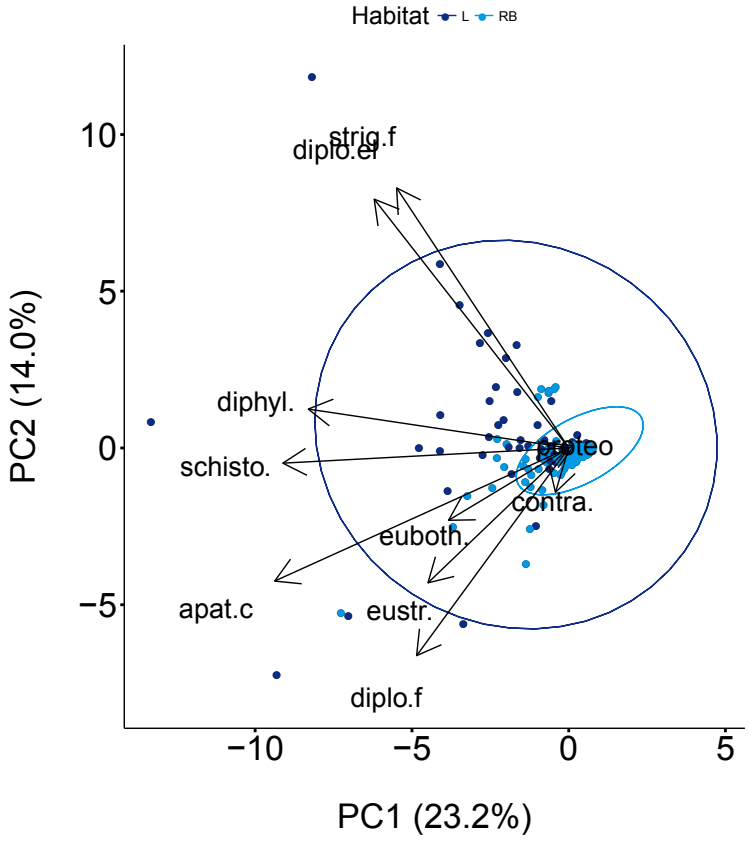


Supplementary figure S2
Heatmap of pairwise F_{ST} values between each sampling sites (RA, RB, L) across four survey years (2009, 2010, 2012, 2013). Asterisk indicates significant difference at $P < 0.050$ after Bonferroni correction.



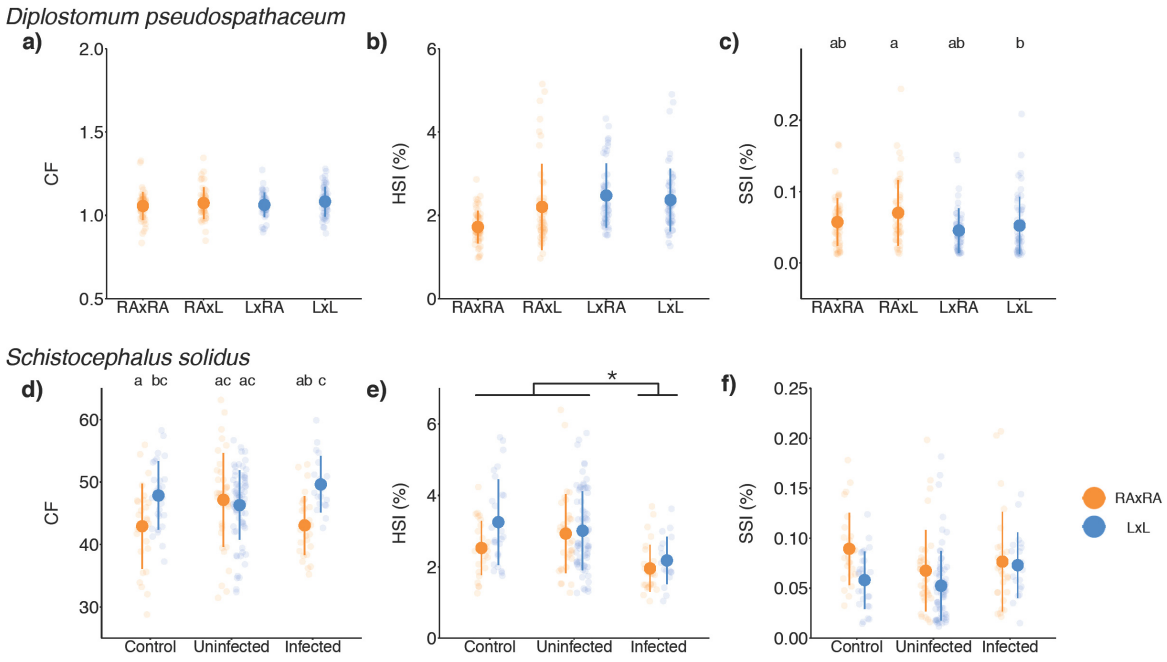
Supplementary figure S3

Estimated population structure for each sampling site and survey year. Results of Bayesian population structure for microsatellite data for three sampling sites (RA, RB, L) for four survey years (2009, 2010, 2012, 2013) indicating two parent populations (K = 2) of three-spined sticklebacks, shown in orange (population above the waterfall RA) and blue (population below the waterfall L). Individual probabilities of assignment are shown on the y-axis and are grouped by parent populations calculated by the admixture program in STRUCTURE 2.3.4.



Supplementary figure S4

Principal Component Analysis (PCA) of the abundance of 10 parasites species in the 'River Below' (RB) and lake (L) habitats. A similarity percentage test (simper function in R, vegan library) revealed that the infections with *Diplostomum sp.* (31.1% of the variation between groups), *Diphyllobothrium sp.* (30.3% of the variation) *and Strigeinae gen. sp.* (17.8% of the variation), explained cumulatively 79.2% of the difference between the two populations' parasite communities (RA-L admixed individuals are excluded; see Table S1 for sample sizes). Abbreviations: Apat.c: *Apatemon sp.* cysts; contra.: *Contracaecum sp.*; diphyl.: *Diphyllobothrium sp.*; diplo.f: *Diplostomum sp.* free; diplo.el: *Diplostomum sp.* eyes lens; euboth.: *Eubothrium*; eustr.: *Eustrongylides sp.*; proteo.: *Protocephalus sp.*; schisto.: *Schistocephalus solidus*; strig.f: *Strigeinae gen. sp.* free.



Supplementary figure S5

Fish condition parameters in experimental infections for each fish genetic type ('River Above' RAxRA, 'River Above' maternal hybrid RAxL, lake maternal hybrid LxRA, lake LxL):

- *Diplostomum pseudospathaceum exposures:* **a)** mean fish condition factor CF (±SD) **b)** mean hepatosomatic index HSI (±SD) **c)** mean splenosomatic index SSI (±SD) (RAxRA N = 58, RAxL N = 47, LxRA N = 48, LxL N = 56);

- *Schistocephalus solidus exposures*: **d)** mean fish condition factor (±SD) **e)** mean hepatosomatic index (±SD) **f)** mean splenosomatic index (±SD) (four extreme values were excluded) (Control fish: RAxRA N = 26, LxL N = 27; uninfected fish: RAxRA N = 36, LxL N = 72; infected fish: RAxRA N = 29, LxL N = 19).

Means annotated with different letters or asterisks are significantly different (Tukey's HSD, $P < 0.050$).

Appendix – Chapter 3

Supplementary table S1

Detailed table of experimental infection setup and sample sizes. Allopatric and sympatric exposure of four three-spined stickleback families (Norwegian fish families: NO-09M001xF001, NO-09M006xF006; German fish families: NST-09M001xF001, NST-09M006xF006) to four *S. solidus* families (Norwegian worm families: NO-A, NO-B; German worm families: NST-A, NST-B).

Combination	Fish family	Worm family	5 weeks +PE		7 weeks +PE		9 weeks +PE		Total	
			uninfected	infected	uninfected	infected	uninfected	infected	uninfected	infected
Sympatric										
DE-(DE)	NST-09M001xF001	NST-A	-	-	-	-	-	-	-	-
		NST-B	5	5	7	3	3	7	15	15
	NST-09M006xF006	NST-A	16	1	-	-	1	1	17	2
		NST-B	-	-	-	-	-	-	-	-
Total			***21***	***6***	***7***	***3***	***4***	***8***	***32***	***17***
NO-(NO)	NO-09M001xF001	NO-A	-	3	4	3	6	2	10	8
		NO-B	4	7	6	5	5	6	15	18
	NO-09M006xF006	NO-A	7	4	10	1	11	1	28	6
		NO-B	-	-	-	-	-	-	-	-
			11	***14***	***20***	***9***	***22***	***9***	***53***	***32***
Allopatric										
DE-(NO)	NST-09M001xF001	NO-A	-	3	5	2	7	2	12	***7***
		NO-B	7	3	5	5	7	2	19	10
	NST-09M006xF006	NO-A	5	4	4	-	9	2	18	6
		NO-B	-	-	-	-	-	-	-	-
Total			***12***	***10***	***14***	***7***	***23***	***6***	***49***	***23***

NO-(DE)	NO-09M001xF001	NST-A	-	-	-	-	-	-	-	-
		NST-B	5	6	8	3	4	7	17	16
	NO-09M006xF006	NST-A	4	2	6	-	5	2	15	4
		NST-B	-	-	-	-	-	-	-	-
Total			***9***	***8***	***14***	***3***	***9***	***9***	***32***	***20***
Controls										
DE	NST-09M001xF001	-	-		5		6		11	
	NST-09M006xF006	-	6		-		3		9	
Total			***6***		***5***		***9***		***20***	
NO	NO-09M001xF001	-	2		6		6		14	
	NO-09M006xF006	-	6		-		3		9	
Total			***8***		***6***		***9***		***23***	

Supplementary table S2

Summary of samples used in RT-qPCR assay for the different tissues (HK: head-kidneys; SP: spleen) and experimental conditions.

Combination	5 weeks +PE				7 weeks +PE				9 weeks +PE				Total	
	uninfected		infected		uninfected		infected		uninfected		infected			
	HK	SP	HK	SP	HK	SP	HK	SP	HK	SP	HK	SP	HK	SP
Sympatric														
DE-(DE)	11	11	6	6	5	5	3	3	4	4	8	8	37	37
NO-(NO)	7	9	12	11	14	14	8	8	16	16	8	8	65	66
Allopatric														
DE-(NO)	10	10	10	10	10	10	7	7	15	15	6	6	58	58
NO-(DE)	7	9	5	8	5	5	3	3	9	9	9	9	38	43
Control	HK		SP		HK		SP		HK		SP		HK	SP
DE	6		6		5		5		8		9		19	20
NO	8		8		6		6		9		9		23	23

Supplementary table S3

RT-qPCR thermocycling parameters performed on a LightCycler® 480 Instrument (Roche Applied Science).

Program name	Target (°C)	Acquisition mode	Hold (mm:ss)	Ramp rate (°C/sec)	Acquisitions (per °C)	Cycles	Analysis mode
Incubation	95	None	10:00	4.4	-	1	None
Down	94	None	1:00	2.2	-	1	None
PCR	94	None	0:20	4.4	-	40	Quantification
	68	Single	1:00	2.2	-		
Melting	95	None	1:00	4.4	-	1	Melting curves
	60	None	0:30	1.5	-		
	95	Continuous		0.06	10		
Cool	37	None	0:10	1.5	-	1	None

Supplementary table S4

Linear mixed effect model on the fish indices in the different experimental treatments across time. Tables show the results of pairwise comparison between the interaction of experimental treatments (sympatric NO-(NO), sympatric DE-(DE), allopatric NO-(DE), allopatric DE-(NO), NO-ctrl, DE-ctrl) and time points (5, 7 and 9 weeks PE, respectively noted here as T1, T2 and T3), significant *P*-values (<0.05) in bold for **a)** fish weight; **b)** condition factor (CF); **c)** splenosomatic index (SSI); **d)** head-kidney index (HKI).

a) Fish weight:					
Treatments x Week PE	**Estimate**	**SE**	**D.F.**	***T*-value**	***P*-value**
DE-ctrl across time					
DE-ctrl.T1 - DE-ctrl.T2	0.014	0.017	47.07	0.856	1.000
DE-ctrl.T1 - DE-ctrl.T3	-0.007	0.013	77.36	-0.516	1.000
DE-ctrl.T2 - DE-ctrl.T3	-0.021	0.013	113.87	-1.637	0.974
NO-ctrl across time					
NO-ctrl.T1 - NO-ctrl.T2	-0.024	0.014	82.95	-1.704	0.960
NO-ctrl.T1 - NO-ctrl.T3	-0.006	0.011	111.36	-0.558	1.000
NO-ctrl.T2 - NO-ctrl.T3	0.017	0.012	114.33	1.413	0.994
DE-(DE) across time					
DE-(DE).T1 - DE-(DE).T2	-0.037	0.016	113.78	-2.318	0.669
DE-(DE).T1 - DE-(DE).T3	0.017	0.012	112.50	1.446	0.992
DE-(DE).T2 - DE-(DE).T3	0.055	0.015	113.26	3.578	**0.049**
NO-(NO) across time					
NO-(NO).T1 - NO-(NO).T2	-0.024	0.010	114.07	-2.469	0.559
NO-(NO).T1 - NO-(NO).T3	-0.003	0.010	114.58	-0.292	1.000
NO-(NO).T2 - NO-(NO).T3	0.021	0.011	112.71	2.025	0.850
DE-(NO) across time					
DE-(NO).T1 - DE-(NO).T2	-0.046	0.012	113.34	-3.879	**0.019**
DE-(NO).T1 - DE-(NO).T3	-0.009	0.012	112.58	-0.749	1.000
DE-(NO).T2 - DE-(NO).T3	0.038	0.013	115.74	2.882	0.279
NO-(DE) across time					
NO-(DE).T1 - NO-(DE).T2	-0.039	0.015	114.62	-2.551	0.497
NO-(DE).T1 - NO-(DE).T3	-0.010	0.011	112.40	-0.930	1.000
NO-(DE).T2 - NO-(DE).T3	0.029	0.015	114.30	1.933	0.893
5 weeks PE					
NO-ctrl.T1 - DE-ctrl.T1	-0.031	0.014	16.68	-2.195	0.734
NO-(NO).T1 - NO-ctrl.T1	0.030	0.014	4.37	2.139	0.741
NO-(DE).T1 - NO-ctrl.T1	0.004	0.015	5.63	0.244	1.000
DE-(DE).T1 - DE-ctrl.T1	0.016	0.017	8.41	0.931	1.000
DE-(NO).T1 - DE-ctrl.T1	0.011	0.016	6.23	0.721	1.000
NO-(NO).T1 - DE-(NO).T1	-0.012	0.011	9.89	-1.146	0.997
NO-(NO).T1 - NO-(DE).T1	0.026	0.013	6.71	2.069	0.778
NO-(NO).T1 - DE-(DE).T1	-0.017	0.015	7.54	-1.164	0.995
NO-(DE).T1 - DE-(DE).T1	-0.043	0.014	18.77	-3.193	0.213

DE-(NO).T1 - NO-(DE).T1	0.038	0.014	7.09	2.727	0.478
DE-(NO).T1 - DE-(DE).T1	-0.005	0.014	9.57	-0.350	1.000
7 weeks PE					
NO-ctrl.T2 - DE-ctrl.T2	0.007	0.016	21.31	0.451	1.000
NO-(NO).T2 - NO-ctrl.T2	0.030	0.015	6.18	2.071	0.775
NO-(DE).T2 - NO-ctrl.T2	0.019	0.019	12.39	1.025	0.999
DE-(DE).T2 - DE-ctrl.T2	0.068	0.019	13.55	3.487	0.156
DE-(NO).T2 - DE-ctrl.T2	0.072	0.016	8.12	4.552	0.062
NO-(NO).T2 - DE-(NO).T2	-0.034	0.014	13.17	-2.525	0.548
NO-(NO).T2 - NO-(DE).T2	0.011	0.017	17.84	0.631	1.000
NO-(NO).T2 - DE-(DE).T2	-0.030	0.019	14.68	-1.574	0.965
NO-(DE).T2 - DE-(DE).T2	-0.041	0.020	47.50	-2.056	0.827
DE-(NO).T2 - NO-(DE).T2	0.045	0.020	16.26	2.322	0.662
DE-(NO).T2 - DE-(DE).T2	0.004	0.018	19.45	0.243	1.000
9 weeks PE					
NO-ctrl.T3 - DE-ctrl.T3	-0.031	0.012	13.62	-2.647	0.481
NO-(NO).T3 - NO-ctrl.T3	0.026	0.014	4.75	1.895	0.837
NO-(DE).T3 - NO-ctrl.T3	0.007	0.014	4.64	0.523	1.000
DE-(DE).T3 - DE-ctrl.T3	-0.008	0.015	4.96	-0.556	1.000
DE-(NO).T3 - DE-ctrl.T3	0.013	0.015	6.17	0.891	1.000
NO-(NO).T3 - DE-(NO).T3	-0.018	0.013	18.52	-1.350	0.992
NO-(NO).T3 - NO-(DE).T3	0.019	0.013	7.54	1.425	0.975
NO-(NO).T3 - DE-(DE).T3	0.003	0.015	7.11	0.198	1.000
NO-(DE).T3 - DE-(DE).T3	-0.016	0.013	13.29	-1.244	0.995
DE-(NO).T3 - NO-(DE).T3	0.037	0.015	9.04	2.443	0.601
DE-(NO).T3 - DE-(DE).T3	0.021	0.015	10.60	1.438	0.979

b) Condition factor: Treatments x Week PE	Estimate	SE	D.F.	*T*-value	*P*-value
DE-ctrl across time					
DE-ctrl.T1 - DE-ctrl.T2	-0.291	0.081	61.06	-3.596	0.057
DE-ctrl.T1 - DE-ctrl.T3	-0.287	0.065	89.12	-4.388	**0.004**
DE-ctrl.T2 - DE-ctrl.T3	0.004	0.063	115.32	0.058	1.000
NO-ctrl across time					
NO-ctrl.T1 - NO-ctrl.T2	0.017	0.067	95.89	0.252	1.000
NO-ctrl.T1 - NO-ctrl.T3	-0.044	0.056	114.44	-0.778	1.000
NO-ctrl.T2 - NO-ctrl.T3	-0.060	0.059	115.56	-1.019	1.000
DE-(DE) across time					
DE-(DE).T1 - DE-(DE).T2	0.075	0.078	114.40	0.959	1.000
DE-(DE).T1 - DE-(DE).T3	0.154	0.059	113.35	2.600	0.462
DE-(DE).T2 - DE-(DE).T3	0.079	0.074	113.97	1.059	1.000
NO-(NO) across time					
NO-(NO).T1 - NO-(NO).T2	0.123	0.047	114.52	2.596	0.465
NO-(NO).T1 - NO-(NO).T3	0.081	0.047	114.55	1.709	0.961
NO-(NO).T2 - NO-(NO).T3	-0.042	0.052	113.48	-0.806	1.000
DE-(NO) across time					
DE-(NO).T1 - DE-(NO).T2	0.130	0.058	115.68	2.250	0.716
DE-(NO).T1 - DE-(NO).T3	0.076	0.056	113.43	1.345	0.997
DE-(NO).T2 - DE-(NO).T3	-0.054	0.064	116.32	-0.855	1.000
NO-(DE) across time					
NO-(DE).T1 - NO-(DE).T2	0.014	0.075	115.07	0.183	1.000
NO-(DE).T1 - NO-(DE).T3	0.033	0.053	113.27	0.619	1.000
NO-(DE).T2 - NO-(DE).T3	0.019	0.074	114.79	0.258	1.000
5 weeks PE					
NO-ctrl.T1 - DE-ctrl.T1	-0.211	0.070	14.72	-2.995	0.308
NO-(NO).T1 - NO-ctrl.T1	-0.086	0.078	3.50	-1.111	0.991
NO-(DE).T1 - NO-ctrl.T1	-0.087	0.082	4.36	-1.056	0.996
DE-(DE).T1 - DE-ctrl.T1	0.053	0.094	6.24	0.571	1.000
DE-(NO).T1 - DE-ctrl.T1	-0.029	0.086	4.77	-0.336	1.000
NO-(NO).T1 - NO-(DE).T1	>0.001	0.069	4.90	0.002	1.000
NO-(NO).T1 - DE-(NO).T1	-0.269	0.055	7.97	-4.864	**0.045**
NO-(NO).T1 - DE-(DE).T1	-0.351	0.081	6.05	-4.320	0.114
NO-(DE).T1 - DE-(NO).T1	-0.269	0.078	5.73	-3.445	0.266
NO-(DE).T1 - DE-(DE).T1	-0.351	0.069	15.73	-5.118	**0.008**
DE-(NO).T1 - DE-(DE).T1	-0.082	0.077	6.89	-1.077	0.997
7 weeks PE					
NO-ctrl.T2 - DE-ctrl.T2	-0.519	0.078	19.45	-6.606	**>0.001**
NO-(NO).T2 - NO-ctrl.T2	-0.192	0.082	4.60	-2.354	0.654
NO-(DE).T2 - NO-ctrl.T2	-0.083	0.101	8.72	-0.828	1.000
DE-(DE).T2 - DE-ctrl.T2	-0.312	0.103	9.42	-3.032	0.332
DE-(NO).T2 - DE-ctrl.T2	-0.450	0.086	5.74	-5.201	0.056
NO-(NO).T2 - NO-(DE).T2	-0.109	0.091	12.36	-1.193	0.997

NO-(NO).T2 - DE-(NO).T2	-0.261	0.068	12.14	-3.810	0.104
NO-(NO).T2 - DE-(DE).T2	-0.399	0.100	11.40	-3.982	0.086
NO-(DE).T2 - DE-(NO).T2	-0.152	0.102	12.48	-1.490	0.975
NO-(DE).T2 - DE-(DE).T2	-0.290	0.099	42.70	-2.940	0.274
DE-(NO).T2 - DE-(DE).T2	-0.138	0.094	13.49	-1.474	0.979
9 weeks PE					
NO-ctrl.T3 - DE-ctrl.T3	-0.455	0.060	11.13	-7.536	**0.001**
NO-(NO).T3 - NO-ctrl.T3	-0.211	0.078	3.77	-2.710	0.530
NO-(DE).T3 - NO-ctrl.T3	-0.163	0.079	3.77	-2.071	0.765
DE-(DE).T3 - DE-ctrl.T3	-0.387	0.081	4.04	-4.757	0.129
DE-(NO).T3 - DE-ctrl.T3	-0.392	0.082	4.60	-4.795	0.107
NO-(NO).T3 - NO-(DE).T3	-0.048	0.072	5.61	-0.664	1.000
NO-(NO).T3 - DE-(NO).T3	-0.274	0.068	15.41	-4.046	0.056
NO-(NO).T3 - DE-(DE).T3	-0.279	0.084	6.08	-3.328	0.287
NO-(DE).T3 - DE-(NO).T3	-0.226	0.083	6.98	-2.733	0.477
NO-(DE).T3 - DE-(DE).T3	-0.231	0.064	11.57	-3.583	0.150
DE-(NO).T3 - DE-(DE).T3	-0.005	0.079	7.58	-0.061	1.000

c) SSI: Treatments x Week PE	Estimate	SE	D.F.	*T*-value	*P*-value
DE-ctrl across time					
DE-ctrl.T1 - DE-ctrl.T2	0.499	0.493	20.78	1.012	0.999
DE-ctrl.T1 - DE-ctrl.T3	0.151	0.399	46.79	0.379	1.000
DE-ctrl.T2 - DE-ctrl.T3	-0.347	0.385	109.01	-0.902	1.000
NO-ctrl across time					
NO-ctrl.T1 - NO-ctrl.T2	-0.153	0.443	36.20	-0.346	1.000
NO-ctrl.T1 - NO-ctrl.T3	0.114	0.355	91.19	0.322	1.000
NO-ctrl.T2 - NO-ctrl.T3	0.268	0.371	107.61	0.722	1.000
DE-(DE) across time					
DE-(DE).T1 - DE-(DE).T2	-0.410	0.477	112.68	-0.859	1.000
DE-(DE).T1 - DE-(DE).T3	0.448	0.373	110.90	1.202	0.999
DE-(DE).T2 - DE-(DE).T3	0.858	0.465	112.32	1.846	0.925
NO-(NO) across time					
NO-(NO).T1 - NO-(NO).T2	0.211	0.309	113.09	0.684	1.000
NO-(NO).T1 - NO-(NO).T3	0.014	0.318	111.28	0.045	1.000
NO-(NO).T2 - NO-(NO).T3	-0.197	0.318	111.68	-0.619	1.000
DE-(NO) across time					
DE-(NO).T1 - DE-(NO).T2	0.048	0.379	85.18	0.127	1.000
DE-(NO).T1 - DE-(NO).T3	-0.342	0.347	111.24	-0.985	1.000
DE-(NO).T2 - DE-(NO).T3	-0.390	0.408	104.76	-0.956	1.000
NO-(DE) across time					
NO-(DE).T1 - NO-(DE).T2	-0.686	0.464	113.95	-1.476	0.991
NO-(DE).T1 - NO-(DE).T3	-0.085	0.326	110.96	-0.260	1.000
NO-(DE).T2 - NO-(DE).T3	0.601	0.455	113.74	1.320	0.997
5 weeks PE					
NO-ctrl.T1 - DE-ctrl.T1	-1.083	0.397	20.04	-2.728	0.420
NO-(NO).T1 - NO-ctrl.T1	0.765	0.339	12.03	2.257	0.697
NO-(DE).T1 - NO-ctrl.T1	0.143	0.379	14.58	0.378	1.000
DE-(DE).T1 - DE-ctrl.T1	0.452	0.457	16.65	0.990	0.999
DE-(NO).T1 - DE-ctrl.T1	0.249	0.392	16.38	0.634	1.000
NO-(NO).T1 - DE-(NO).T1	-0.567	0.297	18.45	-1.913	0.874
NO-(NO).T1 - NO-(DE).T1	0.621	0.313	19.68	1.982	0.846
NO-(NO).T1 - DE-(DE).T1	-0.771	0.362	14.83	-2.130	0.767
NO-(DE).T1 - DE-(DE).T1	-1.392	0.384	27.51	-3.627	0.079
DE-(NO).T1 - NO-(DE).T1	1.189	0.342	13.78	3.476	0.157
DE-(NO).T1 - DE-(DE).T1	-0.203	0.367	24.24	-0.554	1.000
7 weeks PE					
NO-ctrl.T2 - DE-ctrl.T2	-0.431	0.462	20.78	-0.934	1.000
NO-(NO).T2 - NO-ctrl.T2	0.400	0.356	19.99	1.124	0.999
NO-(DE).T2 - NO-ctrl.T2	0.676	0.487	32.63	1.386	0.993
DE-(DE).T2 - DE-ctrl.T2	1.361	0.503	35.91	2.708	0.410
DE-(NO).T2 - DE-ctrl.T2	0.699	0.400	25.81	1.748	0.937
NO-(NO).T2 - DE-(NO).T2	-0.731	0.393	13.09	-1.859	0.886

NO-(NO).T2 - NO-(DE).T2	-0.276	0.463	41.62	-0.596	1.000
NO-(NO).T2 - DE-(DE).T2	-1.392	0.501	25.23	-2.781	0.380
NO-(DE).T2 - DE-(DE).T2	-1.116	0.590	50.71	-1.893	0.901
DE-(NO).T2 - NO-(DE).T2	0.455	0.530	24.56	0.858	1.000
DE-(NO).T2 - DE-(DE).T2	-0.661	0.483	42.20	-1.370	0.994
9 weeks PE					
NO-ctrl.T3 - DE-ctrl.T3	-1.046	0.324	24.08	-3.227	0.185
NO-(NO).T3 - NO-ctrl.T3	0.865	0.330	12.80	2.621	0.497
NO-(DE).T3 - NO-ctrl.T3	0.342	0.324	11.29	1.057	0.999
DE-(DE).T3 - DE-ctrl.T3	0.155	0.351	13.04	0.442	1.000
DE-(NO).T3 - DE-ctrl.T3	0.742	0.358	18.81	2.070	0.803
NO-(NO).T3 - DE-(NO).T3	-0.923	0.398	25.72	-2.319	0.665
NO-(NO).T3 - NO-(DE).T3	0.523	0.330	19.87	1.582	0.969
NO-(NO).T3 - DE-(DE).T3	-0.337	0.390	11.88	-0.862	1.000
NO-(DE).T3 - DE-(DE).T3	-0.859	0.365	20.23	-2.353	0.643
DE-(NO).T3 - NO-(DE).T3	1.446	0.378	19.26	3.824	0.068
DE-(NO).T3 - DE-(DE).T3	0.587	0.389	30.34	1.506	0.983

d) HKI: Treatments x Week PE	Estimate	SE	D.F.	*T*-value	*P*-value
DE-ctrl across time					
DE-ctrl.T1 - DE-ctrl.T2	0.014	0.017	47.07	0.856	1.000
DE-ctrl.T1 - DE-ctrl.T3	-0.007	0.013	77.36	-0.516	1.000
DE-ctrl.T2 - DE-ctrl.T3	-0.021	0.013	113.87	-1.637	0.974
NO-ctrl across time					
NO-ctrl.T1 - NO-ctrl.T2	-0.024	0.014	82.95	-1.704	0.960
NO-ctrl.T1 - NO-ctrl.T3	-0.006	0.011	111.36	-0.558	1.000
NO-ctrl.T2 - NO-ctrl.T3	0.017	0.012	114.33	1.413	0.994
DE-(DE) across time					
DE-(DE).T1 - DE-(DE).T2	-0.037	0.016	113.78	-2.318	0.669
DE-(DE).T1 - DE-(DE).T3	0.017	0.012	112.50	1.446	0.992
DE-(DE).T2 - DE-(DE).T3	0.055	0.015	113.26	3.578	**0.049**
NO-(NO) across time					
NO-(NO).T1 - NO-(NO).T2	-0.024	0.010	114.07	-2.469	0.559
NO-(NO).T1 - NO-(NO).T3	-0.003	0.010	114.58	-0.292	1.000
NO-(NO).T2 - NO-(NO).T3	0.021	0.011	112.71	2.025	0.850
DE-(NO) across time					
DE-(NO).T1 - DE-(NO).T2	-0.046	0.012	113.34	-3.879	**0.019**
DE-(NO).T1 - DE-(NO).T3	-0.009	0.012	112.58	-0.749	1.000
DE-(NO).T2 - DE-(NO).T3	0.038	0.013	115.74	2.882	0.279
NO-(DE) across time					
NO-(DE).T1 - NO-(DE).T2	-0.039	0.015	114.62	-2.551	0.497
NO-(DE).T1 - NO-(DE).T3	-0.010	0.011	112.40	-0.930	1.000
NO-(DE).T2 - NO-(DE).T3	0.029	0.015	114.30	1.933	0.893
5 weeks PE					
NO-ctrl.T1 - DE-ctrl.T1	-0.031	0.014	16.68	-2.195	0.734
NO-(NO).T1 - NO-ctrl.T1	0.030	0.014	4.37	2.139	0.741
NO-(DE).T1 - NO-ctrl.T1	0.004	0.015	5.63	0.244	1.000
DE-(DE).T1 - DE-ctrl.T1	0.016	0.017	8.41	0.931	1.000
DE-(NO).T1 - DE-ctrl.T1	0.011	0.016	6.23	0.721	1.000
NO-(NO).T1 - DE-(NO).T1	-0.012	0.011	9.89	-1.146	0.997
NO-(NO).T1 - NO-(DE).T1	0.026	0.013	6.71	2.069	0.778
NO-(NO).T1 - DE-(DE).T1	-0.017	0.015	7.54	-1.164	0.995
NO-(NO).T1 - DE-(NO).T1	-0.012	0.011	9.89	-1.146	0.997
NO-(DE).T1 - DE-(DE).T1	-0.043	0.014	18.77	-3.193	0.213
DE-(NO).T1 - NO-(DE).T1	0.038	0.014	7.09	2.727	0.478
DE-(NO).T1 - DE-(DE).T1	-0.005	0.014	9.57	-0.350	1.000
7 weeks PE					
NO-ctrl.T2 - DE-ctrl.T2	0.007	0.016	21.31	0.451	1.000
NO-(NO).T2 - NO-ctrl.T2	0.030	0.015	6.18	2.071	0.775
NO-(DE).T2 - NO-ctrl.T2	0.019	0.019	12.39	1.025	0.999
DE-(DE).T2 - DE-ctrl.T2	0.068	0.019	13.55	3.487	0.156
DE-(NO).T2 - DE-ctrl.T2	0.072	0.016	8.12	4.552	0.062

NO-(NO).T2 - DE-(NO).T2	-0.034	0.014	13.17	-2.525	0.548
NO-(NO).T2 - NO-(DE).T2	0.011	0.017	17.84	0.631	1.000
NO-(NO).T2 - DE-(DE).T2	-0.030	0.019	14.68	-1.574	0.965
NO-(DE).T2 - DE-(DE).T2	-0.041	0.020	47.50	-2.056	0.827
DE-(NO).T2 - NO-(DE).T2	0.045	0.020	16.26	2.322	0.662
DE-(NO).T2 - DE-(DE).T2	0.004	0.018	19.45	0.243	1.000
9 weeks PE					
NO-ctrl.T3 - DE-ctrl.T3	-0.031	0.012	13.62	-2.647	0.481
NO-(NO).T3 - NO-ctrl.T3	0.026	0.014	4.75	1.895	0.837
NO-(DE).T3 - NO-ctrl.T3	0.007	0.014	4.64	0.523	1.000
DE-(DE).T3 - DE-ctrl.T3	-0.008	0.015	4.96	-0.556	1.000
DE-(NO).T3 - DE-ctrl.T3	0.013	0.015	6.17	0.891	1.000
NO-(NO).T3 - DE-(NO).T3	-0.018	0.013	18.52	-1.350	0.992
NO-(NO).T3 - NO-(DE).T3	0.019	0.013	7.54	1.425	0.975
NO-(NO).T3 - DE-(DE).T3	0.003	0.015	7.11	0.198	1.000
NO-(DE).T3 - DE-(DE).T3	-0.016	0.013	13.29	-1.244	0.995
DE-(NO).T3 - NO-(DE).T3	0.037	0.015	9.04	2.443	0.601
DE-(NO).T3 - DE-(DE).T3	0.021	0.015	10.60	1.438	0.979

Supplementary table S5

Linear model on the gene expression fold difference to control fish in the different experimental treatments overall and within time point. Tables show the results of the type-III ANOVA for linear models considering the effect of experimental treatments (DE-(DE), DE-(NO), DE-ctrl or NO-(NO), NO-(DE), NO-ctrl), time point (5, 7 and 9 weeks PE) and their interaction on the gene expression difference to control for **a)** DE infected and control fish (CF); **b)** NO infected and control fish; significant *P*-values (<0.05) in bold.

a) Infected and control, DE	Overall			5 weeks PE			7 weeks PE			9 weeks PE		
	F	D.F.	*P*-value	F	D.F.	*P*-value	F	D.F.	*P*-value	F	D.F.	*P*-value
IgM												
Treatment	0.616	2	0.735	2.590	2	0.274	0.150	2	0.928	2.067	2	0.356
Time point	0.597	4	0.742	-	-	-	-	-	-	-	-	-
Treatment x Time point	3.543	4	0.471	-	-	-	-	-	-	-	-	-
IL-1ß												
Treatment	0.802	2	0.670	0.052	2	0.974	14.144	2	**>0.001**	12.557	2	**0.002**
Time point	0.549	2	0.760	-	-	-	-	-	-	-	-	-
Treatment x Time point	8.083	4	0.088	-	-	-	-	-	-	-	-	-
MHC-IIß												
Treatment	1.654	2	0.437	0.419	2	0.811	1.751	2	0.417	5.681	2	0.058
Time point	5.742	4	0.057	-	-	-	-	-	-	-	-	-
Treatment x Time point	5.677	4	0.224	-	-	-	-	-	-	-	-	-
MIF												
Treatment	5.823	2	0.054	2.105	2	0.349	2.713	2	0.257	36.832	2	**>0.001**
Time point	9.931	2	**0.007**	-	-	-	-	-	-	-	-	-
Treatment x Time point	10.823	4	**0.029**	-	-	-	-	-	-	-	-	-
SOD2												
Treatment	2.478	2	0.290	1.570	2	0.456	4.125	2	0.127	0.839	2	0.657
Time point	11.264	2	**0.003**	-	-	-	-	-	-	-	-	-
Treatment x Time point	2.587	4	0.629	-	-	-	-	-	-	-	-	-

TGF-β1												
Treatment	0.539	2	0.764	0.022	2	0.989	0.893	2	0.640	0.245	2	0.885
Time point	10.235	2	**0.006**	-	-	-	-	-	-	-	-	-
Treatment x Time point	1.874	4	0.759	-	-	-	-	-	-	-	-	-
TLR2												
Treatment	0.671	2	0.715	2.194	2	0.334	0.022	2	0.989	0.559	2	0.756
Time point	0.752	2	0.687	-	-	-	-	-	-	-	-	-
Treatment x Time point	0.193	4	0.996	-	-	-	-	-	-	-	-	-

b) Infected & control, NO fish	Overall			5 weeks PE			7 weeks PE			9 weeks PE		
	F	D.F.	*P*-value	F	D.F.	*P*-value	F	D.F.	*P*-value	F	D.F.	*P*-value
IgM												
Treatment	0.918	2	0.632	1.820	2	0.402	0.609	2	0.737	0.300	2	0.860
Time point	0.104	2	0.949	-	-	-	-	-	-	-	-	-
Treatment x Time point	2.368	4	0.668	-	-	-	-	-	-	-	-	-
IL-1ß												
Treatment	7.580	2	**0.022**	6.744	2	**0.034**	1.940	2	0.380	1.636	2	0.441
Time point	1.631	4	0.442	-	-	-	-	-	-	-	-	-
Treatment x Time point	5.377	4	0.251	-	-	-	-	-	-	-	-	-
MHC-IIß												
Treatment	1.844	2	0.398	0.478	2	0.787	1.081	2	0.582	4.577	2	0.101
Time point	3.846	2	0.146	-	-	-	-	-	-	-	-	-
Treatment x Time point	6.820	4	0.146	-	-	-	-	-	-	-	-	-
MIF												
Treatment	3.199	2	0.202	6.486	2	**0.039**	1.712	2	0.423	5.160	2	0.076
Time point	2.313	2	0.315	-	-	-	-	-	-	-	-	-
Treatment x Time point	8.205	4	0.084	-	-	-	-	-	-	-	-	-
SOD2												
Treatment	0.503	2	0.777	0.301	2	0.860	10.500	2	**0.005**	2.380	2	0.304
Time point	2.636	2	0.268	-	-	-	-	-	-	-	-	-
Treatment x Time point	7.107	4	0.130	-	-	-	-	-	-	-	-	-
TGF-ß1												
Treatment	10.490	2	**0.005**	6.229	2	**0.044**	0.929	2	0.628	4.839	2	0.089
Time point	4.192	2	0.123	-	-	-	-	-	-	-	-	-
Treatment x Time point	3.012	4	0.556	-	-	-	-	-	-	-	-	-
TLR2												
Treatment	6.400	2	**0.041**	4.536	2	0.104	1.513	2	0.469	10.694	2	**0.005**
Time point	3.200	2	0.202	-	-	-	-	-	-	-	-	-
Treatment x Time point	3.153	4	0.533	-	-	-	-	-	-	-	-	-

Supplementary table S6

Linear model on the gene expression fold difference to control fish in the different experimental treatments overall and within time point. Tables show the results of pairwise comparison between experimental treatments (DE-(DE), DE-(NO), DE-ctrl or NO-(NO), NO-(DE), NO-ctrl), time points (5, 7 and 9 weeks PE, respectively noted here as T1, T2 and T3) and the interaction for **a)** DE infected and control fish (CF); **b)** NO infected and control fish; significant *P*-values (<0.05) in bold.

a) Infected and control, DE fish	Estimate	SE	D.F.	*Z*-value	*P*-value
IL-1ß (7 weeks PE)					
DE-(NO) - DE-(DE)	0.078	0.059	12	1.333	0.375
DE-(NO) - DE-ctrl	0.188	0.050	12	3.761	**<0.001**
DE-(DE) - DE-ctrl	0.109	0.062	12	1.756	0.183
IL-1ß (9 weeks PE)					
DE-(NO) - DE-(DE)	0.166	0.053	14	3.129	**0.005**
DE-(NO) - DE-ctrl	0.172	0.053	14	3.217	**0.004**
DE-(DE) - DE-ctrl	0.006	0.047	14	0.125	0.991
MIF (overall)					
5 weeks PE - 7 weeks PE	-0.035	0.026	49	-1.340	0.373
5 weeks PE - 9 weeks PE	0.053	0.027	49	1.970	0.119
7 weeks PE - 9 weeks PE	0.088	0.028	49	3.140	**0.005**
MIF (overall)					
DE-(DE).T1 - DE-(NO).T1	>0.001	>0.001	43	-1.234	0.947
DE-ctrl.T1 - DE-(NO).T1	>0.001	>0.001	43	-2.336	0.313
DE-(NO).T2 - DE-(NO).T1	>0.001	>0.001	43	-1.340	0.916
DE-(DE).T2 - DE-(NO).T1	>0.001	>0.001	43	0.233	1.000
DE-ctrl.T2 - DE-(NO).T1	>0.001	>0.001	43	-1.620	0.786
DE-(NO).T3 - DE-(NO).T1	>0.001	>0.001	43	1.970	0.556
DE-(DE).T3 - DE-(NO).T1	>0.001	>0.001	43	2.980	0.068
DE-ctrl.T3 - DE-(NO).T1	>0.001	>0.001	43	-1.685	0.748
DE-ctrl.T1 - DE-(DE).T1	>0.001	>0.001	43	-0.964	0.988
DE-(NO).T2 - DE-(DE).T1	>0.001	>0.001	43	0.112	1.000
DE-(DE).T2 - DE-(DE).T1	>0.001	>0.001	43	1.355	0.910
DE-ctrl.T2 - DE-(DE).T1	>0.001	>0.001	43	-0.368	1.000
DE-(NO).T3 - DE-(DE).T1	>0.001	>0.001	43	2.774	0.119
DE-(DE).T3 - DE-(DE).T1	>0.001	>0.001	43	4.381	**<0.010**
DE-ctrl.T3 - DE-(DE).T1	>0.001	>0.001	43	-0.380	1.000
DE-(NO).T2 - DE-ctrl.T1	>0.001	>0.001	43	1.120	0.970
DE-(DE).T2 - DE-ctrl.T1	>0.001	>0.001	43	1.998	0.535
DE-ctrl.T2 - DE-ctrl.T1	>0.001	>0.001	43	0.691	0.999
DE-(NO).T3 - DE-ctrl.T1	>0.001	>0.001	43	3.591	**<0.010**
DE-(DE).T3 - DE-ctrl.T1	>0.001	>0.001	43	4.593	**<0.010**
DE-ctrl.T3 - DE-ctrl.T1	>0.001	>0.001	43	0.714	0.999
DE-(DE).T2 - DE-(NO).T2	>0.001	>0.001	43	1.215	0.951

DE-ctrl.T2 - DE-(NO).T2	>0.001	>0.001	43	-0.512	1.000
DE-(NO).T3 - DE-(NO).T2	>0.001	>0.001	43	3.140	**0.043**
DE-(DE).T3 - DE-(NO).T2	>0.001	>0.001	43	4.136	**<0.010**
DE-ctrl.T3 - DE-(NO).T2	>0.001	>0.001	43	-0.533	1.000
DE-ctrl.T2 - DE-(DE).T2	>0.001	>0.001	43	-1.553	0.824
DE-(NO).T3 - DE-(DE).T2	>0.001	>0.001	43	1.211	0.952
DE-(DE).T3 - DE-(DE).T2	>0.001	>0.001	43	2.299	0.336
DE-ctrl.T3 - DE-(DE).T2	>0.001	>0.001	43	-1.595	0.801
DE-(NO).T3 - DE-ctrl.T2	>0.001	>0.001	43	3.134	**0.045**
DE-(DE).T3 - DE-ctrl.T2	>0.001	>0.001	43	4.231	**<0.010**
DE-ctrl.T3 - DE-ctrl.T2	>0.001	>0.001	43	0.000	1.000
DE-(DE).T3 - DE-(NO).T3	>0.001	>0.001	43	0.985	0.987
DE-ctrl.T3 - DE-(NO).T3	>0.001	>0.001	43	-3.244	**0.031**
DE-ctrl.T3 - DE-(DE).T3	>0.001	>0.001	43	-4.396	**<0.010**
MIF (9 weeks PE)					
DE-(NO) - DE-(DE)	0.030	0.022	15	1.365	0.359
DE-(NO) - DE-ctrl	-0.097	0.023	15	-4.115	**>0.001**
DE-(DE) - DE-ctrl	-0.127	0.021	15	-5.943	**>0.001**
SOD2 (overall)					
5 weeks PE - 7 weeks PE	0.083	0.028	49	2.982	**0.008**
5 weeks PE - 9 weeks PE	0.079	0.029	49	2.710	**0.018**
7 weeks PE - 9 weeks PE	-0.004	0.030	49	-0.141	0.989
TGF-β1 (overall)					
5 weeks PE - 7 weeks PE	0.118	0.037	49	3.199	**0.004**
5 weeks PE - 9 weeks PE	0.052	0.037	49	1.429	0.326
7 weeks PE - 9 weeks PE	-0.065	0.039	49	-1.679	0.213

b) Infected and control, NO fish	Estimate	SE	D.F.	*Z*-value	*P*-value
IL-1ß (overall)					
NO-(NO) - NO-(DE)	0.201	0.073	59	2.740	**0.017**
NO-(NO) - NO-ctrl	0.047	0.063	59	0.746	0.735
NO-(DE) - NO-ctrl	-0.154	0.077	59	-1.984	0.115
IL-1ß (5 weeks PE)					
NO-(NO) - NO-(DE)	0.196	0.077	21	2.554	**0.028**
NO-(NO) - NO-ctrl	0.035	0.069	21	0.517	0.863
NO-(DE) - NO-ctrl	-0.161	0.082	21	-1.966	0.120
MIF (5 weeks PE)					
NO-(NO) - NO-(DE)	-0.051	0.036	21	-1.434	0.322
NO-(NO) - NO-ctrl	-0.086	0.035	21	-2.487	**0.034**
NO-(DE) - NO-ctrl	-0.035	0.039	21	-0.883	0.650
SOD2 (7 weeks PE)					
NO-(NO) - NO-(DE)	-0.017	0.024	13	-0.697	0.764
NO-(NO) - NO-ctrl	-0.065	0.020	13	-3.220	**0.004**
NO-(DE) - NO-ctrl	-0.049	0.026	13	-1.868	0.146
TGF-ß1 (overall)					
NO-(NO) - NO-(DE)	0.109	0.040	49	2.707	**0.018**
NO-(NO) - NO-ctrl	-0.022	0.038	49	-0.592	0.824
NO-(DE) - NO-ctrl	-0.132	0.043	49	-3.042	**0.007**
TGF-ß1 (5 weeks PE)					
NO-(NO) - NO-(DE)	0.120	0.051	21	2.372	**0.046**
NO-(NO) - NO-ctrl	0.006	0.044	21	0.137	0.990
NO-(DE) - NO-ctrl	-0.114	0.053	21	-2.133	0.082
TLR2 (overall)					
NO-(NO) - NO-(DE)	-0.005	0.089	53	-0.055	0.998
NO-(NO) - NO-ctrl	0.212	0.092	53	2.305	0.055
NO-(DE) - NO-ctrl	0.216	0.100	53	2.172	0.076
TLR2 (9 weeks PE)					
NO-(NO) - NO-(DE)	0.174	0.061	19	2.846	**0.012**
NO-(NO) - NO-ctrl	0.194	0.071	19	2.716	**0.018**
NO-(DE) - NO-ctrl	0.020	0.070	19	0.280	0.958

Supplementary table S7

Summary table of sample size used in the gene expression fold differences analysis.

Treatment	Time	IgM	IL-1ß	MHC-IIß	MIF	SOD2	TGF-ß1	TLR2
DE-(DE)	5	5	5	5	5	5	5	5
	7	3	3	3	3	3	3	3
	9	7	7	6	7	7	7	6
DE-(NO)	5	8	8	6	8	8	8	7
	7	7	7	7	7	7	7	7
	9	5	4	5	5	5	5	5
DE-ctrl	5	6	6	4	6	6	6	3
	7	5	5	4	5	5	5	5
	9	6	6	5	6	6	6	6
DE fish	5	19	19	15	19	19	19	15
	7	15	15	14	15	15	15	15
	9	18	17	16	18	18	18	17
	Overall	**52**	**51**	**45**	**52**	**52**	**52**	**47**
NO-(NO)	5	11	11	9	11	11	11	9
	7	8	8	7	8	8	8	5
	9	8	8	8	8	8	8	8
NO-(DE)	5	5	5	4	5	5	5	5
	7	3	3	3	3	3	3	3
	9	9	9	9	9	9	9	9
NO-ctrl	5	8	8	8	8	8	8	8
	7	5	5	5	5	5	5	4
	9	5	5	5	5	5	5	5
NO fish	5	24	24	21	24	24	24	22
	7	16	16	15	16	16	16	12
	9	22	22	22	22	22	22	22
	Overall	**62**	**62**	**58**	**62**	**62**	**62**	**56**

Supplementary table S8

PERMANOVA on the gene expression profile of different comparison overall and within time point. Tables show the results for different data subsets of PERMANOVA considering the effect of experimental fish origin (DE, NO), worm origin (DE, NO), infection status (infected, control) and/or time point (5, 7 and 9 weeks PE) and their interaction on the gene expression profile of **a)** all immune genes; **b)** antigen recognition genes; **c)** Th2 response genes; significant *P*-values (<0.05) in bold.

a) All immune genes	**Overall**			**5 weeks PE**			**7 weeks PE**			**9 weeks PE**		
	F	**D.F.**	***P*-value**	**F**	**D.F.**	***P*-value**	**F**	**D.F.**	***P*-value**	**F**	**D.F.**	***P*-value**
Controls												
Fish origin	6.531	1	0.129	1.902	1	1.000	6.255	1	1.000	3.351	1	1.000
Time point	0.950	2	0.799	-	-	-	-	-	-	-	-	-
Fish origin x Time point	2.715	2	**0.032**	-	-	-	-	-	-	-	-	-
DE control vs. DE-(DE)												
Infectious status	1.998	1	0.168	1.041	1	1.000	0.820	1	0.455	1.662	1	0.090
Time point	2.350	2	0.080	-	-	-	-	-	-	-	-	-
Infectious status x Time point	0.850	2	0.825	-	-	-	-	-	-	-	-	-
DE control vs. DE-(NO)												
Infectious status	3.870	1	**0.014**	0.572	1	0.794	2.498	1	0.091	3.379	1	**0.020**
Time point	3.469	2	0.126	-	-	-	-	-	-	-	-	-
Infectious status x Time point	0.702	2	0.843	-	-	-	-	-	-	-	-	-
NO control vs. NO-(NO)												
Infectious status	6.765	1	**0.002**	2.956	1	0.108	2.560	1	0.083	2.268	1	0.076
Time point	3.614	2	**0.006**	-	-	-	-	-	-	-	-	-
Infectious status x Time point	0.466	2	0.917	-	-	-	-	-	-	-	-	-
NO control vs. NO-(DE)												
Infectious status	3.353	1	**0.021**	2.450	1	0.067	1.113	1	0.388	1.930	1	0.074
Time point	2.983	2	**0.005**	-	-	-	-	-	-	-	-	-
Infectious status x Time point	1.251	2	0.261	-	-	-	-	-	-	-	-	-

DE infected fish												
Worm origin	1.208	1	0.315	0.393	1	0.814	0.641	1	0.595	1.429	1	0.125
Time point	2.204	2	0.053	-	-	-	-	-	-	-	-	-
Worm origin x Time point	0.944	2	0.450	-	-	-	-	-	-	-	-	-
NO infected fish												
Worm origin	3.322	1	**0.020**	1.917	1	0.103	1.406	1	0.196	3.241	1	**0.006**
Time point	4.109	2	**0.001**	-	-	-	-	-	-	-	-	-
Worm origin x Time point	1.505	2	0.158	-	-	-	-	-	-	-	-	-
DE worm												
Fish origin	1.386	1	**0.035**	1.480	1	0.253	3.650	1	0.200	1.524	1	0.100
Time point	1.853	2	0.065	-	-	-	-	-	-	-	-	-
Fish origin x Time point	1.839	2	0.073	-	-	-	-	-	-	-	-	-
NO worm												
Fish origin	0.945	1	**0.030**	2.555	1	1.000	5.408	1	1.000	1.651	1	1.000
Time point	0.915	2	0.624	-	-	-	-	-	-	-	-	-
Fish origin x Time point	3.473	2	**0.004**	-	-	-	-	-	-	-	-	-
	Overall			**5 vs. 7 weeks PE**			**5 vs.9 weeks PE**			**7 vs. 9 weeks PE**		
	F	**D.F.**	***P*-value**	**F**	**D.F.**	***P*-value**	**F**	**D.F.**	***P*-value**	**F**	**D.F.**	***P*-value**
DE-ctrl												
Time point	2.619	2	0.119	3.167	1	1.000	3.078	1	1.000	1.989	1	0.141
NO-ctrl												
Time point	1.311	2	0.225	1.966	1	0.056	0.437	1	0.903	2.200	1	0.131
DE-(DE)												
Time point	1.194	2	0.295	0.734	1	0.507	1.582	1	0.175	0.928	1	0.410
DE-(NO)												
Time point	2.018	2	0.129	3.324	1	0.138	0.739	1	0.434	1.522	1	0.195
NO-(NO)												
Time point	2.340	2	**0.033**	3.769	1	**0.024**	1.057	1	0.377	3.465	1	**0.031**
NO-(DE)												
Time point	2.563	2	**0.024**	2.851	1	0.134	1.965	1	0.113	3.049	1	**0.007**

b) Antigen recognition genes	Overall			5 weeks PE			7 weeks PE			9 weeks PE		
	F	D.F.	*P*-value	F	D.F.	*P*-value	F	D.F.	*P*-value	F	D.F.	*P*-value
Controls												
Fish origin	5.715	1	0.093	1.709	1	1.000	7.306	1	1.000	2.483	1	1.000
Time point	0.350	2	0.868	-	-	-	-	-	-	-	-	-
Fish origin x Time point	4.462	2	**0.028**	-	-	-	-	-	-	-	-	-
DE control vs. DE-(DE)												
Infectious status	1.657	1	0.279	0.421	1	1.000	0.145	1	0.872	1.369	1	0.217
Time point	2.315	2	0.140	-	-	-	-	-	-	-	-	-
Infectious status x Time point	0.489	2	0.916	-	-	-	-	-	-	-	-	-
DE control vs. DE-(NO)												
Infectious status	0.715	1	0.503	0.755	1	0.753	0.585	1	0.445	0.569	1	0.622
Time point	4.847	2	0.083	-	-	-	-	-	-	-	-	-
Infectious status x Time point	0.503	2	0.847	-	-	-	-	-	-	-	-	-
NO control vs. NO-(NO)												
Infectious status	8.473	1	**0.002**	3.751	1	0.105	1.917	1	0.134	3.522	1	0.051
Time point	4.682	2	**0.006**	-	-	-	-	-	-	-	-	-
Infectious status x Time point	0.462	2	0.775	-	-	-	-	-	-	-	-	-
NO control vs. NO-(DE)												
Infectious status	2.028	1	0.127	2.438	1	0.108	0.639	1	0.589	0.343	1	0.762
Time point	4.021	2	**0.008**	-	-	-	-	-	-	-	-	-
Infectious status x Time point	1.168	2	0.350	-	-	-	-	-	-	-	-	-
DE infected fish												
Worm origin	0.729	1	0.514	0.319	1	0.777	0.379	1	0.600	0.738	1	0.598
Time point	2.520	2	0.052	-	-	-	-	-	-	-	-	-
Worm origin x Time point	0.614	2	0.680	-	-	-	-	-	-	-	-	-
NO infected fish												
Worm origin	1.831	1	0.150	0.201	1	0.867	1.574	1	0.180	4.203	1	**0.025**
Time point	5.185	2	**0.003**	-	-	-	-	-	-	-	-	-
Worm origin x Time point	1.236	2	0.300	-	-	-	-	-	-	-	-	-

DE worm												
Fish origin	1.427	1	0.099	1.120	1	0.314	3.905	1	0.200	1.872	1	1.000
Time point	1.538	2	0.191	-	-	-	-	-	-	-	-	-
Fish origin x Time point	1.972	2	0.107	-	-	-	-	-	-	-	-	-
NO worm												
Fish origin	1.397	1	**0.015**	3.911	1	1.000	6.516	1	1.000	2.051	1	1.000
Time point	0.427	2	0.818	-	-	-	-	-	-	-	-	-
Fish origin x Time point	4.782	2	**0.001**	-	-	-	-	-	-	-	-	-
	Overall			**5 vs. 7 weeks PE**			**5 vs.9 weeks PE**			**7 vs. 9 weeks PE**		
	F	**D.F.**	***P*-value**	**F**	**D.F.**	***P*-value**	**F**	**D.F.**	***P*-value**	**F**	**D.F.**	***P*-value**
DE-ctrl												
Time point	3.122	2	0.171	3.762	1	1.000	6.631	1	1.000	1.896	1	0.166
NO-ctrl												
Time point	1.929	2	0.126	3.254	1	0.053	0.109	1	1.000	3.455	1	0.051
DE-(DE)												
Time point	0.967	2	0.443	0.754	1	0.488	1.330	1	0.259	0.618	1	0.453
DE-(NO)												
Time point	2.608	2	0.092	4.649	1	0.107	0.755	1	0.369	1.793	1	0.205
NO-(NO)												
Time point	2.645	2	**0.037**	4.738	1	**0.016**	0.462	1	0.694	4.302	1	**0.028**
NO-(DE)												
Time point	2.976	2	**0.042**	2.913	1	0.143	2.204	1	0.135	4.100	1	**0.021**

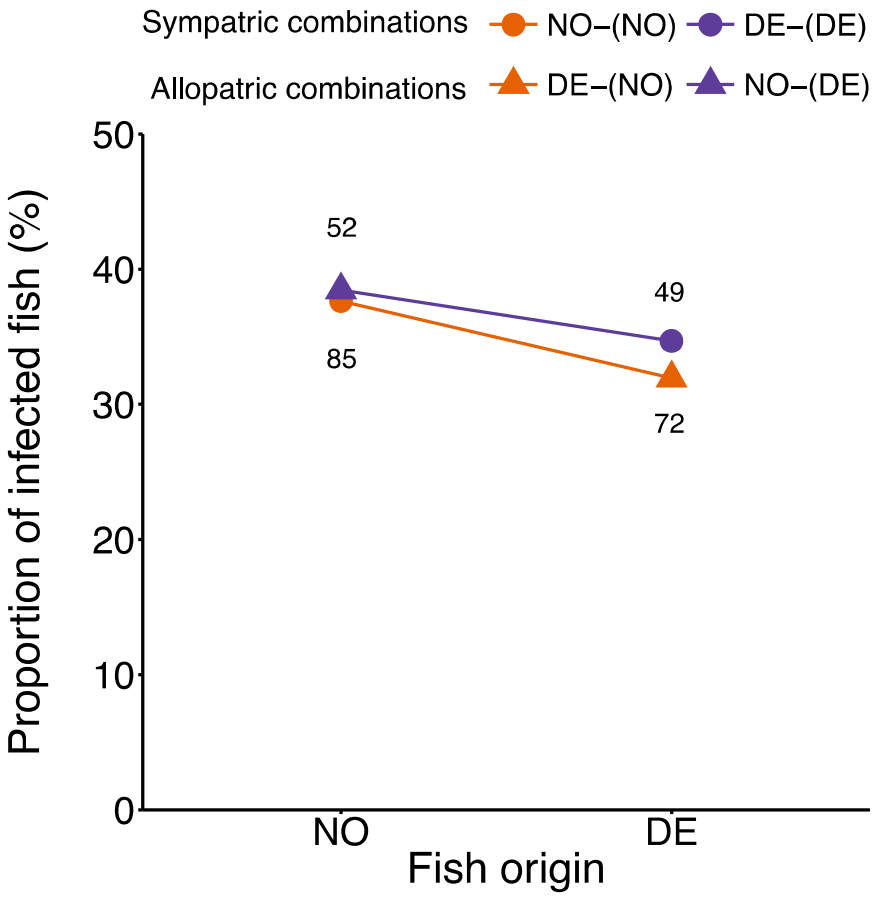
c) Th2 response genes	Overall			5 weeks PE			7 weeks PE			9 weeks PE		
	F	D.F.	*P*-value	F	D.F.	*P*-value	F	D.F.	*P*-value	F	D.F.	*P*-value
Controls												
Fish origin	8.106	1	0.331	1.631	1	1.000	6.129	1	1.000	3.126	1	1.000
Time point	0.801	2	0.857	-	-	-	-	-	-	-	-	-
Fish origin x Time point	1.908	2	0.096	-	-	-	-	-	-	-	-	-
DE control vs. DE-(DE)												
Infectious status	0.843	1	0.536	1.268	1	1.000	0.770	1	0.417	0.232	1	0.889
Time point	2.446	2	0.095	-	-	-	-	-	-	-	-	-
Infectious status x Time point	0.796	2	0.766	-	-	-	-	-	-	-	-	-
DE control vs. DE-(NO)												
Infectious status	3.622	1	**0.034**	0.187	1	1.000	2.901	1	0.080	3.071	1	**0.016**
Time point	2.848	2	0.301	-	-	-	-	-	-	-	-	-
Infectious status x Time point	0.567	2	0.875	-	-	-	-	-	-	-	-	-
NO control vs. NO-(NO)												
Infectious status	8.209	1	**0.001**	3.527	1	0.123	2.389	1	0.103	2.895	1	0.072
Time point	4.386	2	**0.020**	-	-	-	-	-	-	-	-	-
Infectious status x Time point	0.367	2	0.244	-	-	-	-	-	-	-	-	-
NO control vs. NO-(DE)												
Infectious status	3.500	1	**0.019**	3.045	1	**0.045**	0.797	1	0.477	1.873	1	0.132
Time point	2.754	2	**0.020**	-	-	-	-	-	-	-	-	-
Infectious status x Time point	1.389	2	0.244	-	-	-	-	-	-	-	-	-
DE infected fish												
Worm origin	1.518	1	0.244	0.213	1	0.903	0.656	1	0.513	2.674	1	**0.043**
Time point	2.554	2	0.071	-	-	-	-	-	-	-	-	-
Worm origin x Time point	0.876	2	0.501	-	-	-	-	-	-	-	-	-
NO infected fish												
Worm origin	4.474	1	**0.007**	2.247	1	0.089	0.468	1	0.618	4.503	1	**0.004**
Time point	4.977	2	**0.003**	-	-	-	-	-	-	-	-	-
Worm origin x Time point	1.511	2	0.165	-	-	-	-	-	-	-	-	-

DE worm												
Fish origin	0.614	1	0.020	1.925	1	0.170	3.292	1	0.200	0.575	1	1.000
Time point	1.814	2	0.074	-	-	-	-	-	-	-	-	-
Fish origin x Time point	2.570	2	0.025	-	-	-	-	-	-	-	-	-
NO worm												
Fish origin	0.495	1	**0.034**	2.821	1	1.000	7.718	1	1.000	1.779	1	1.000
Time point	0.684	2	0.756	-	-	-	-	-	-	-	-	-
Fish origin x Time point	4.227	2	**0.004**	-	-	-	-	-	-	-	-	-
	Overall			**5 vs. 7 weeks PE**			**5 vs.9 weeks PE**			**7 vs. 9 weeks PE**		
	F	**D.F.**	***P*-value**	**F**	**D.F.**	***P*-value**	**F**	**D.F.**	***P*-value**	**F**	**D.F.**	***P*-value**
DE-ctrl												
Time point	1.605	2	0.324	2.766	1	1.000	1.173	1	1.000	1.021	1	0.324
NO-ctrl												
Time point	1.127	2	0.295	1.514	1	**0.036**	0.450	1	0.641	1.983	1	0.160
DE-(DE)												
Time point	1.582	2	0.140	1.017	1	0.358	1.558	1	0.179	2.081	1	**0.041**
DE-(NO)												
Time point	1.924	2	0.146	3.053	1	0.191	0.595	1	0.483	1.845	1	0.162
NO-(NO)												
Time point	3.041	2	**0.019**	5.207	1	**0.015**	0.838	1	0.517	5.895	1	**0.004**
NO-(DE)												
Time point	2.569	2	**0.039**	2.962	1	0.104	2.413	1	0.077	2.193	1	0.081

Supplementary table S9

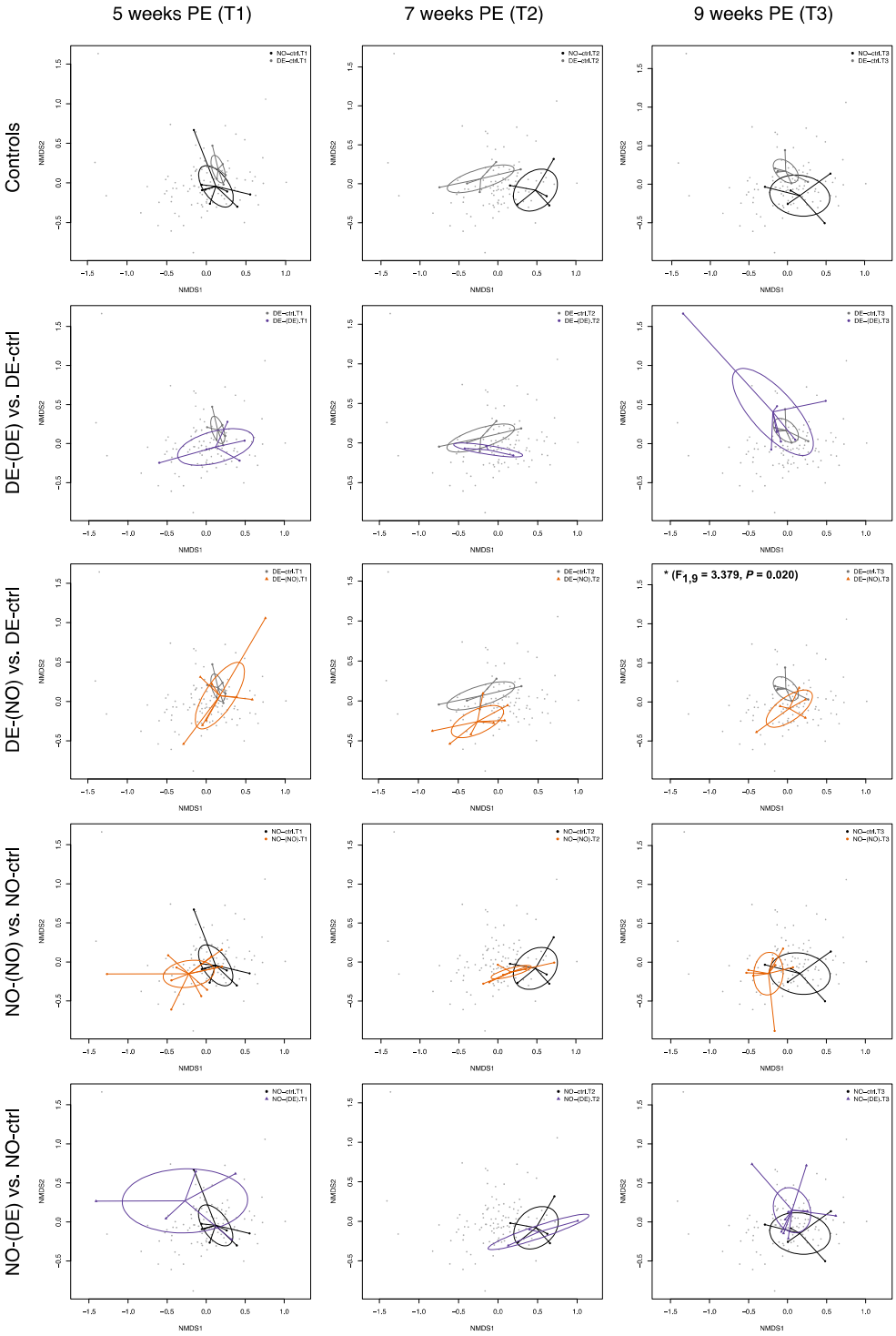
Summary of sample size used in the gene expression profiles analysis.

Subset	5 weeks PE	7 weeks PE	9 weeks PE	Overall
Controls	14	10	11	35
DE control vs. DE-(DE)	11	8	13	32
DE control vs. DE-(NO)	14	12	11	37
NO control vs. NO-(NO)	19	13	13	45
NO control vs. NO-(DE)	13	8	14	35
Infected fish	29	21	29	79
DE infected fish	13	10	12	35
NO infected fish	16	11	17	44
DE worm	10	6	16	32
NO worm	19	15	13	47
Subset	**5 vs. 7 weeks PE**	**5 vs. 9 weeks PE**	**7 vs. 9 weeks PE**	**Overall**
DE controls	11	12	11	17
NO controls	13	13	10	18
DE-(DE)	8	12	10	15
DE-(NO)	15	13	12	20
NO-(NO)	19	19	16	27
NO-(DE)	8	14	12	17

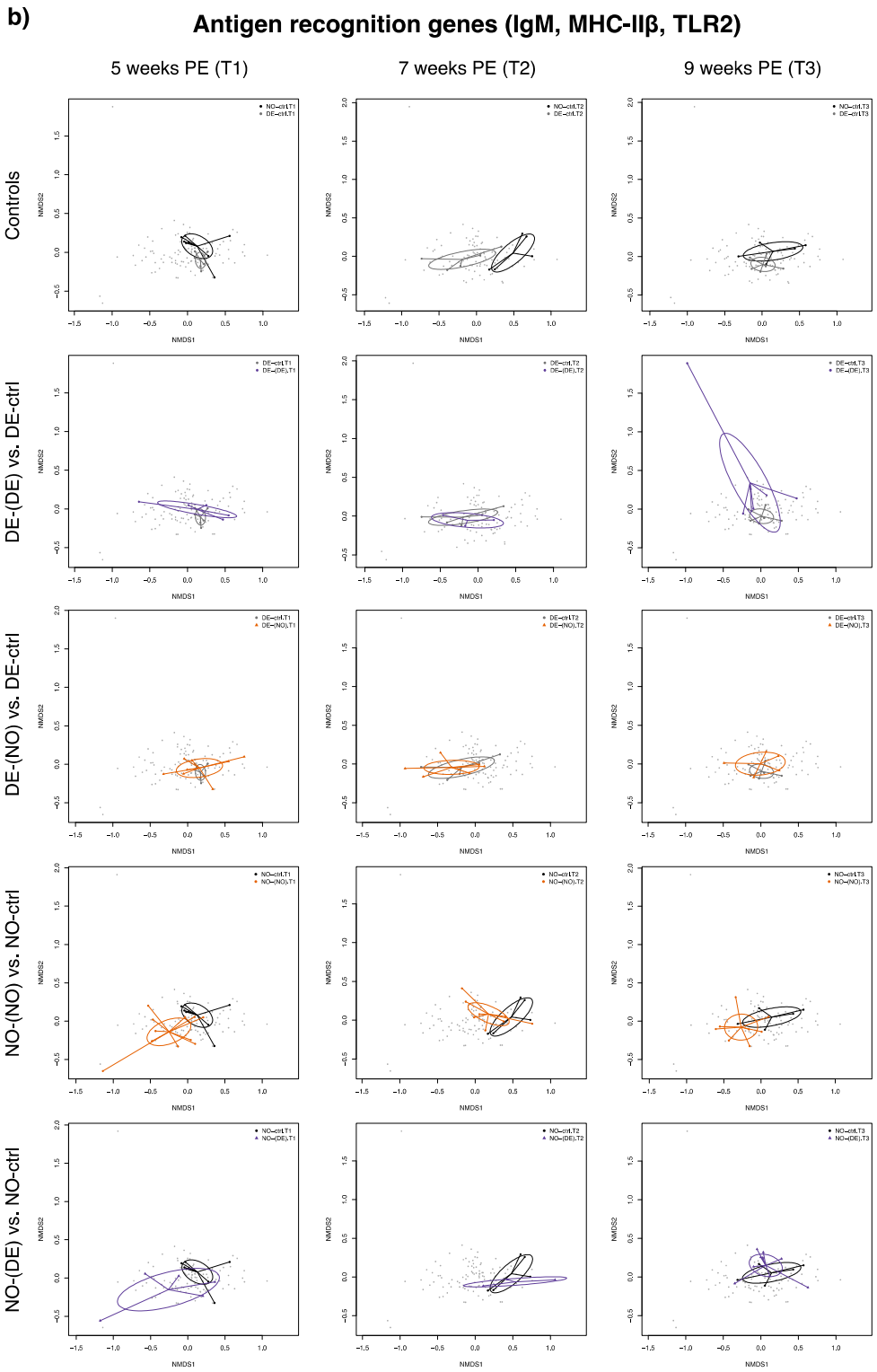


Supplementary figure S1
Proportion of infected fish (in %) in the different infection combinations (sympatric NO-(NO), sympatric DE-(DE), allopatric NO-(DE), allopatric DE-(NO)) across all time points (group sample sizes are indicated).

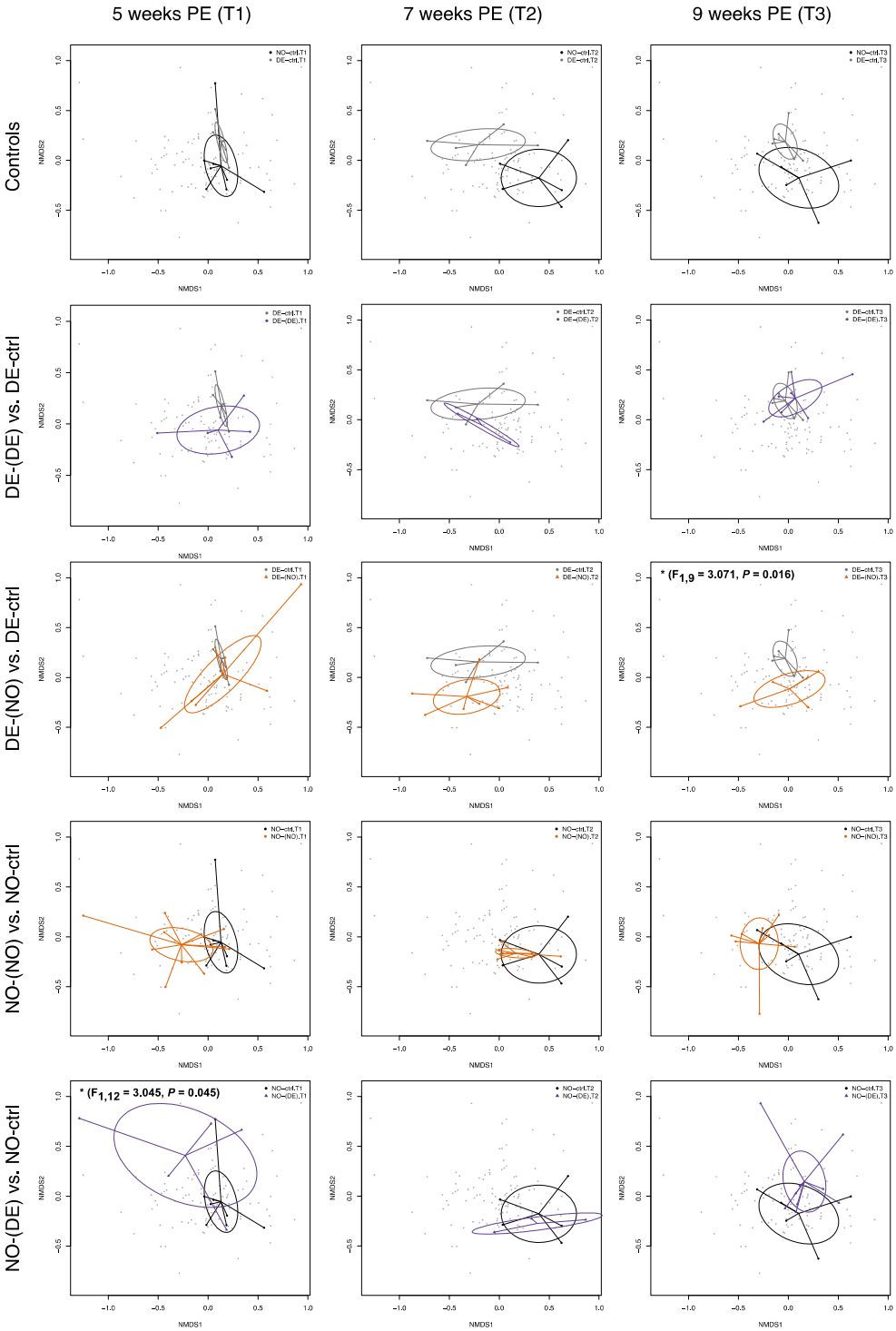
a) **Immune genes (IgM, IL-1β, MHC-IIβ, MIF, SOD2, TGF-β1, TLR2)**



b)


Antigen recognition genes (IgM, MHC-IIβ, TLR2)
5 weeks PE (T1)
7 weeks PE (T2)
9 weeks PE (T3)
Controls
DE-(DE) vs. DE-ctrl
DE-(NO) vs. DE-ctrl
NO-(NO) vs. NO-ctrl
NO-(DE) vs. NO-ctrl
NMDS1
NMDS2

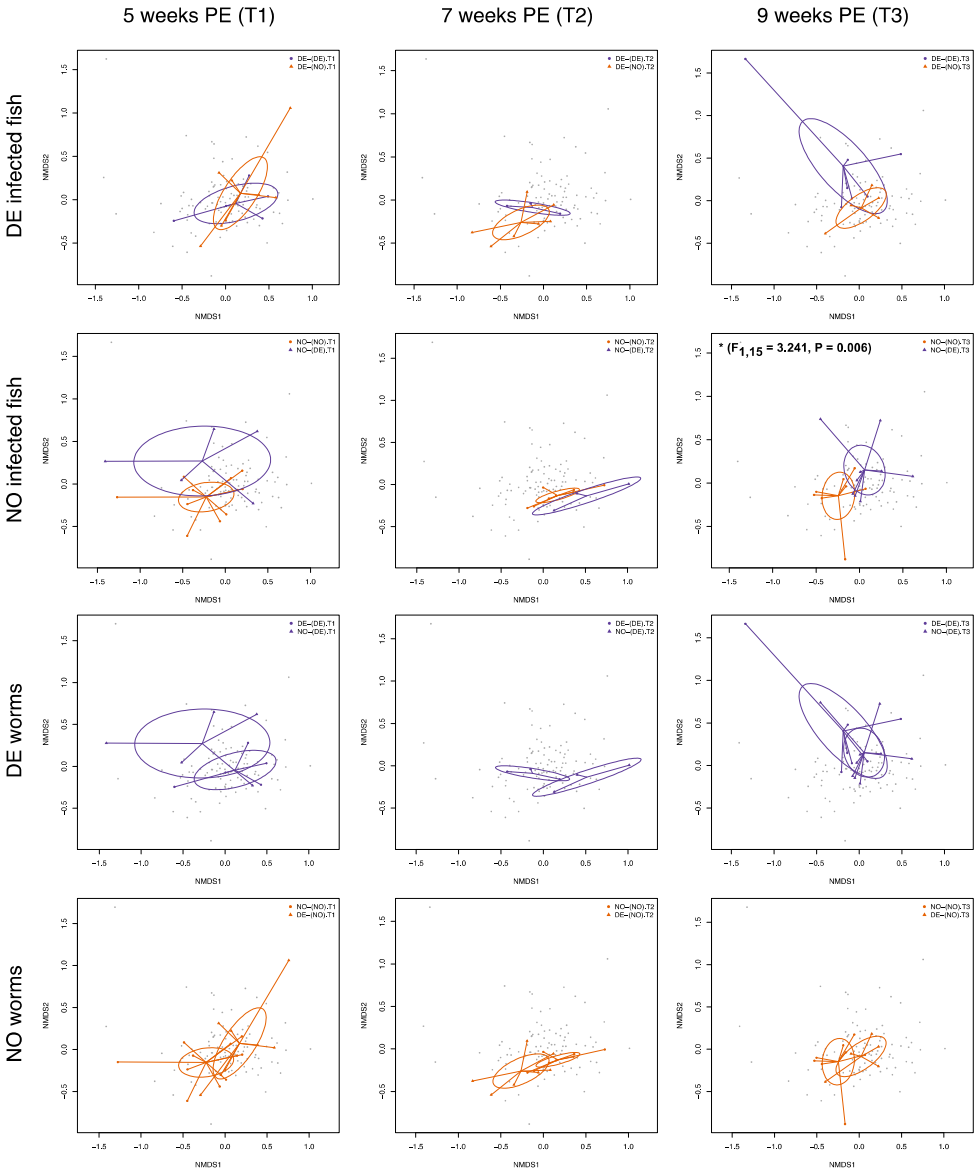
c) **Th2 response genes (IL-1β, SOD2, TGF-β1, TLR2)**



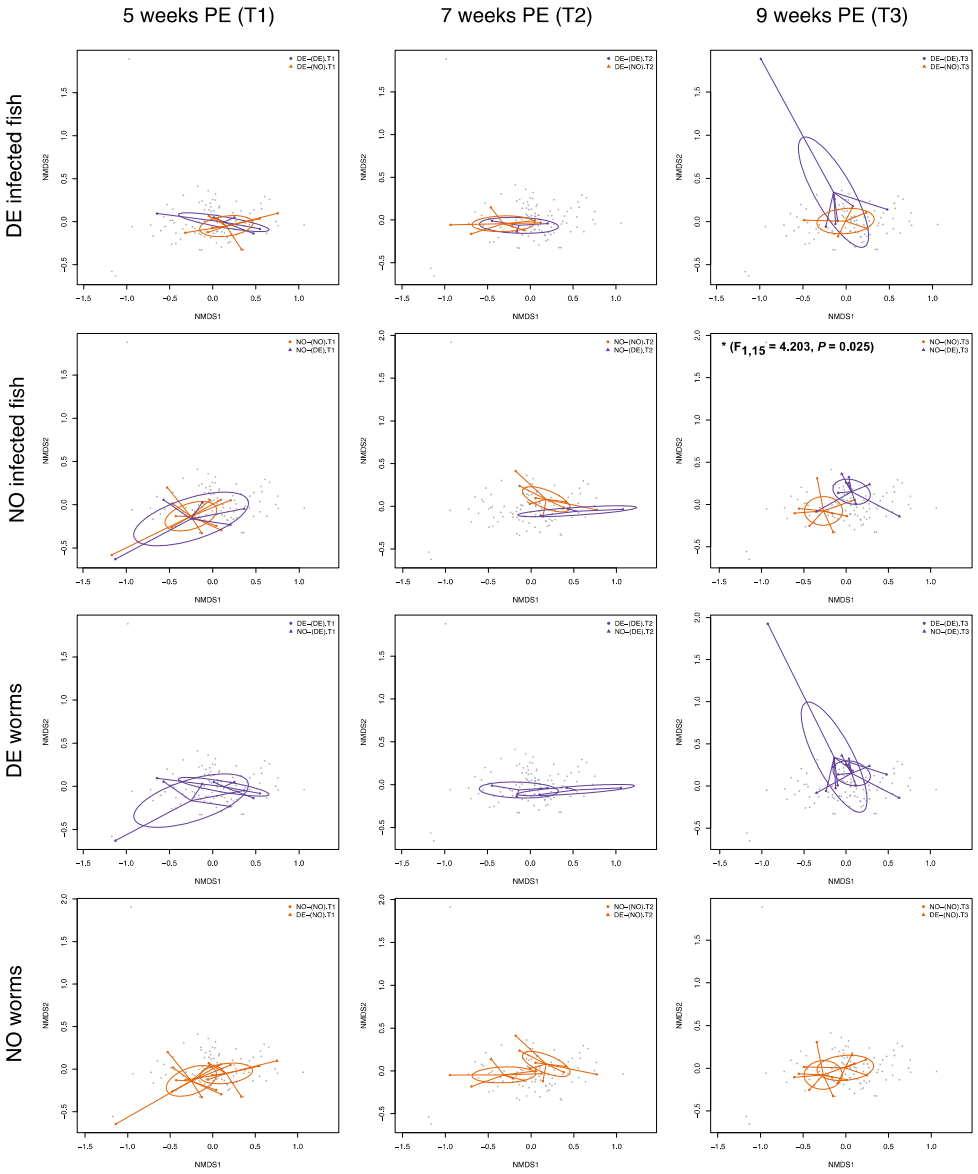
Supplementary figure S2

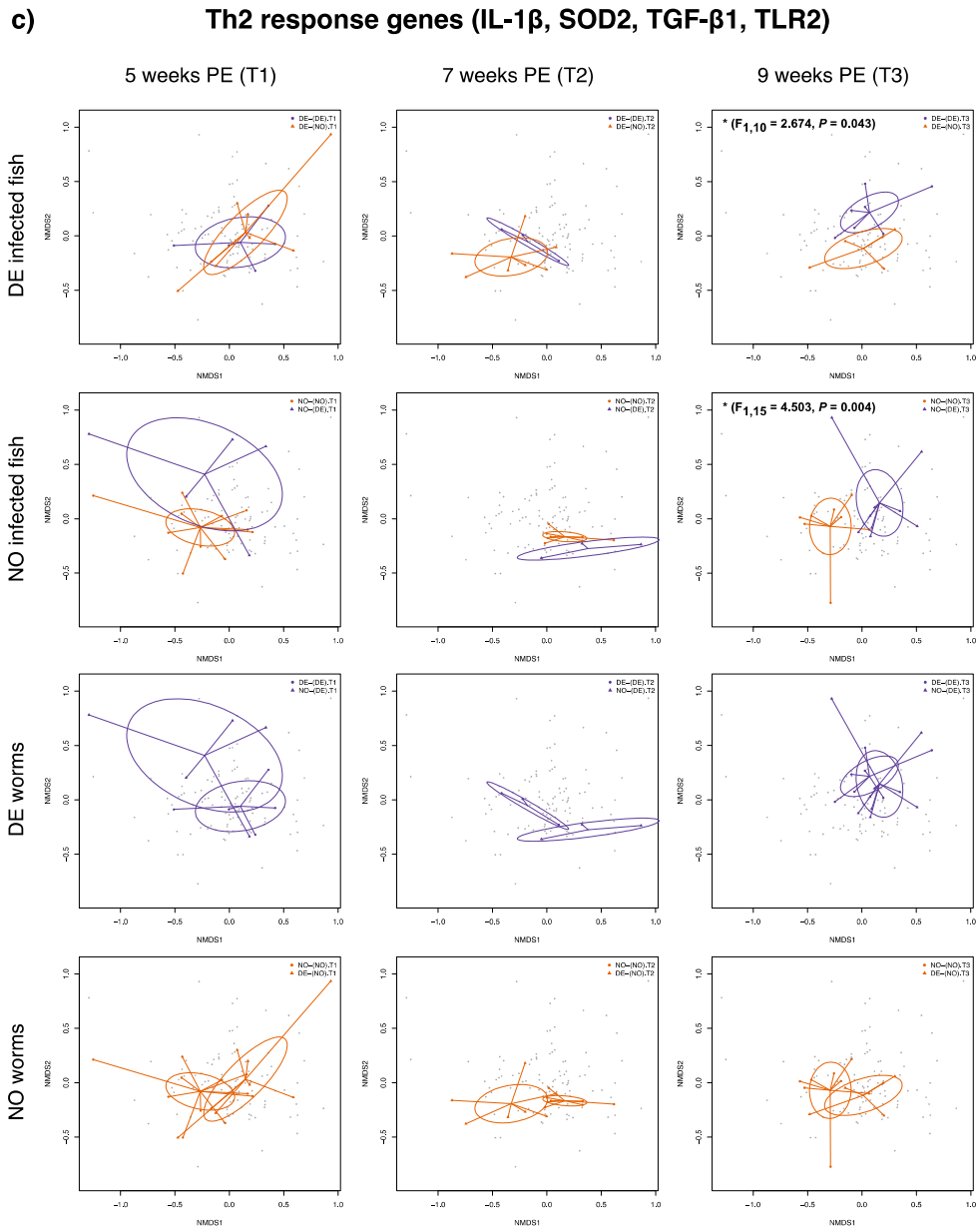
Non-metric multidimensional scaling (NMDS) ordination plots comparing infected fish to their respective controls. Euclidian dissimilarities based on CNRQs from **a)** seven immune genes (IgM, IL-1β, MHC-IIβ, MIF, SOD2, TGF-β1, TLR2) **b)** three antigen recognition genes (IgM, MHC-IIβ, TLR2) **c)** four Th2 response genes (IL-1β, SOD2, TGF-β1, TLR2).

a) Immune genes (IgM, IL-1β, MHC-IIβ, MIF, SOD2, TGF-β1, TLR2)



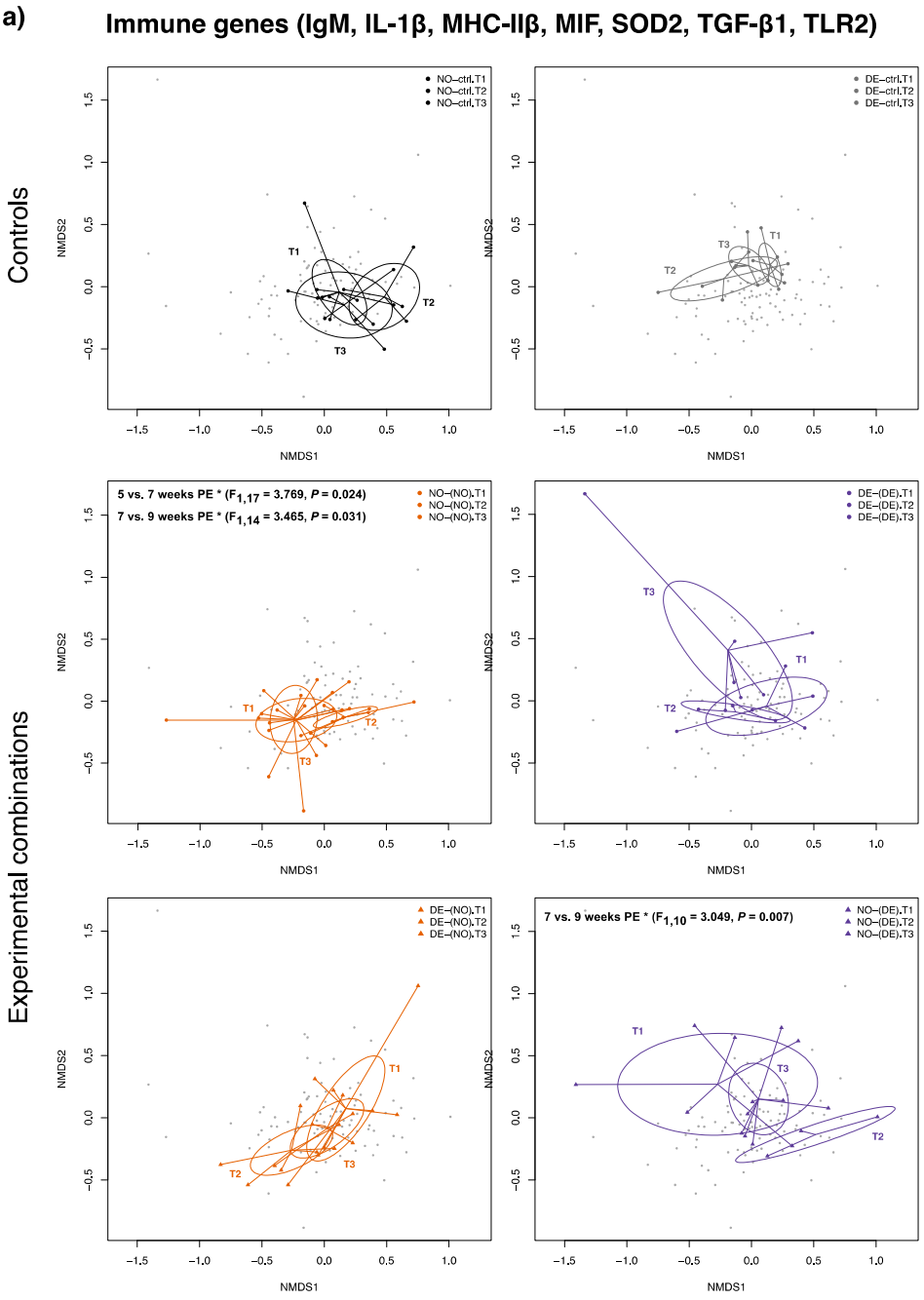
b) **Antigen recognition genes (IgM, MHC-IIβ, TLR2)**



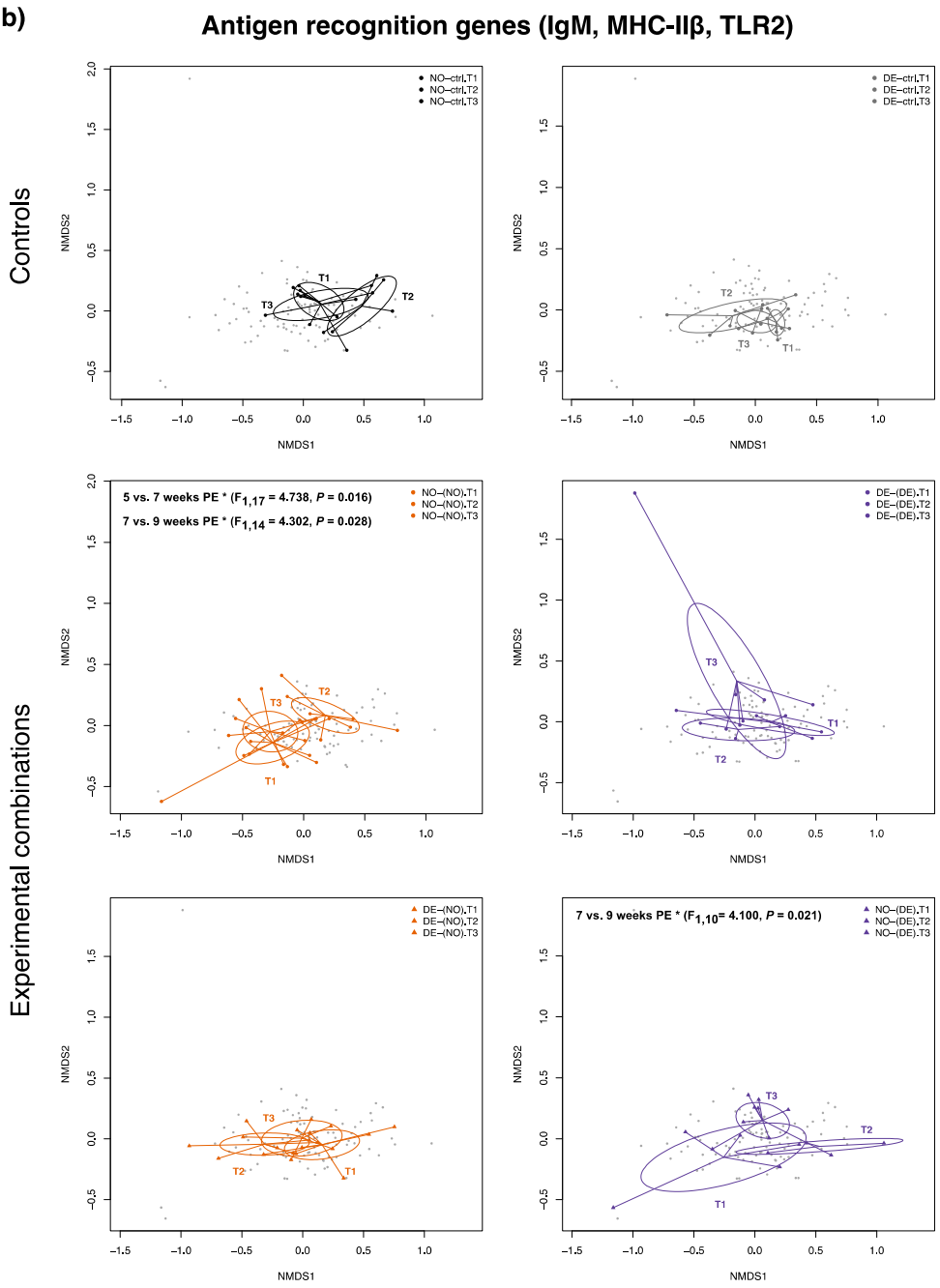


Supplementary figure S3
Non-metric multidimensional scaling (NMDS) ordination plots comparing the different experimental combinations. Euclidian dissimilarities based on CNRQs from **a)** seven immune genes (IgM, IL-1ß, MHC-IIß, MIF, SOD2, TGF-ß1, TLR2) **b)** three antigen recognition genes (IgM, MHC-IIß, TLR2) **c)** four Th2 response genes (IL-1ß, SOD2, TGF-ß1, TLR2).

a)


Immune genes (IgM, IL-1β, MHC-IIβ, MIF, SOD2, TGF-β1, TLR2)
Controls
Experimental combinations
NO–ctrl.T1
NO–ctrl.T2
NO–ctrl.T3
DE–ctrl.T1
DE–ctrl.T2
DE–ctrl.T3
5 vs. 7 weeks PE * ($F_{1,17}$ = 3.769, P = 0.024)
7 vs. 9 weeks PE * ($F_{1,14}$ = 3.465, P = 0.031)
NO–(NO).T1
NO–(NO).T2
NO–(NO).T3
DE–(DE).T1
DE–(DE).T2
DE–(DE).T3
DE–(NO).T1
DE–(NO).T2
DE–(NO).T3
7 vs. 9 weeks PE * ($F_{1,10}$ = 3.049, P = 0.007)
NO–(DE).T1
NO–(DE).T2
NO–(DE).T3
NMDS1
NMDS2

b)


Antigen recognition genes (IgM, MHC-IIβ, TLR2)
Controls
Experimental combinations
NO–ctrl.T1
NO–ctrl.T2
NO–ctrl.T3
DE–ctrl.T1
DE–ctrl.T2
DE–ctrl.T3
5 vs. 7 weeks PE * ($F_{1,17}$ = 4.738, P = 0.016)
7 vs. 9 weeks PE * ($F_{1,14}$ = 4.302, P = 0.028)
NO–(NO).T1
NO–(NO).T2
NO–(NO).T3
DE–(DE).T1
DE–(DE).T2
DE–(DE).T3
DE–(NO).T1
DE–(NO).T2
DE–(NO).T3
7 vs. 9 weeks PE * ($F_{1,10}$= 4.100, P = 0.021)
NO–(DE).T1
NO–(DE).T2
NO–(DE).T3
NMDS1
NMDS2

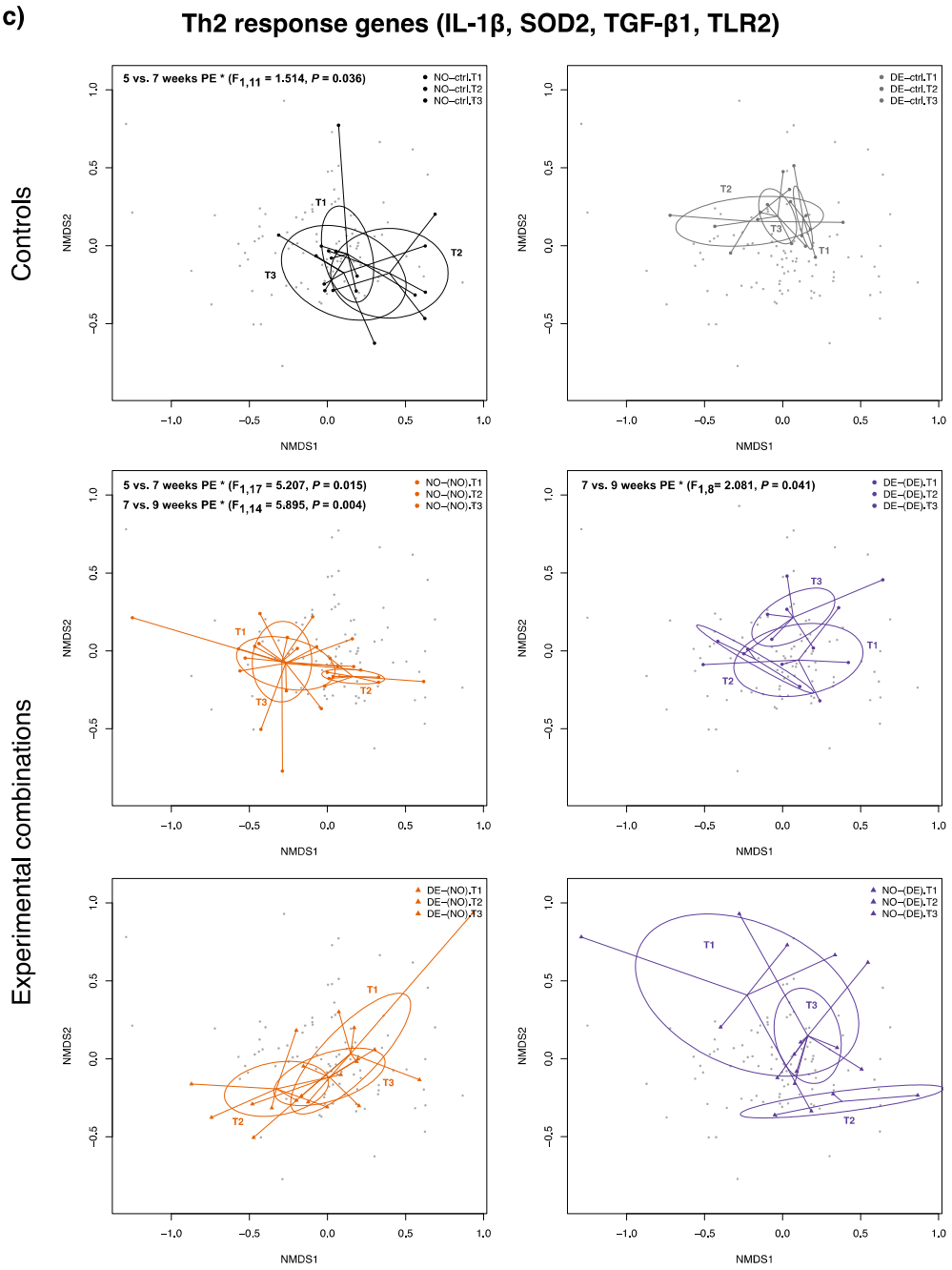


Figure S4

Non-metric multidimensional scaling (NMDS) ordination plots comparing time points for each experimental treatment. Euclidian dissimilarities based on CNRQs from **a)** seven immune genes (IgM, IL-1β, MHC-IIβ, MIF, SOD2, TGF-β1, TLR2) **b)** three antigen recognition genes (IgM, MHC-IIβ, TLR2) **c)** four Th2 response genes (IL-1β, SOD2, TGF-β1, TLR2).

Supplementary Analysis SA1: Reference gene selection using geNorm

To select the most stable reference gene to be use in our RT-qPCR assay, we tested the stability of expression of the four most stable housekeeping genes from Hibbeler *et al*. (2008): ubiquitin (UBC), L13A ribosomal binding protein (RPL13a), glyceraldehyd-3-phosphate dehydrogenase (GAPD) and hypoxanthine phosphoribosyltransferase 1 (HPRT1). These different housekeeping genes are involved in different cellular pathways, reducing the likelihood of gene co-regulation (Vandesompele *et al*. 2002).

We used a representative subset of our experimental samples by selecting, for each organ (HK and SP), two individuals for each sympatric and allopatric combinations and controls in the time points 5 and 9 weeks PE (table SA1.1). We also restricted the samples to only one fish family and only one worm origin, for each population respectively (NO and DE).

The cDNA samples were diluted 1:10 fold and measured using a Nanodrop-1000 spectrophotometer (Thermo Scientific). We then adjusted the concentration of each sample to 7.5 ng/µl of cDNA. RT-qPCR reactions were run following the protocol described in the Material & Method section.

We used the geNorm algorithm (Vandesompele *et al*. 2002) integrated into qBase^{+} (Biogazelle, Zwijnaarde, Belgium) to measure gene expression stability and rank the four housekeeping gene accordingly.

In head-kidney, spleen or both tissues combined, the most stable gene was identified to be UBC, followed by RPL13, HPRT1 and GADPH (fig. SA1.1 and table SA1.2). Therefore, we used UBC as reference gene in our RT-qPCR assay.

Supplementary table SA1.1

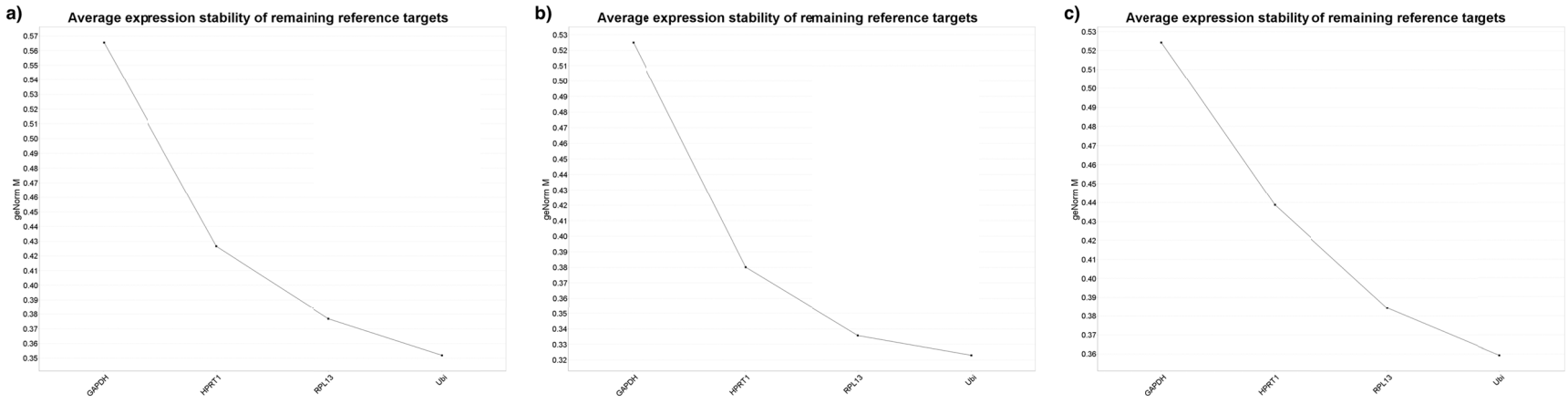
Samples used to select the most stable housekeeping gene for each organ (spleen or head-kidney). Individuals were from one fish family exposed to one worm family for each origin, respectively.

Combination	5 weeks +PE		9 weeks +PE	
	uninfected	infected	uninfected	infected
Sympatric				
DE-(DE)	2	2	2	2
NO-(NO)	2	2	2	2
Allopatric				
DE-(NO)	2	2	2	2
NO-(DE)	2	2	2	2
Control	control		control	
DE	2		2	
NO	-		2	

Supplementary table SA1.2

Evaluation and ranking of gene expression stability of four housekeeping genes (UBC, RPL13, HPRT1 and GADPH) in all samples, head-kidneys or spleen samples (M: stability values; CV: standard error).

	All samples (N = 75)			Head-kidney (N = 38)			Spleen (N = 37)		
Housekeeping gene	**Ranking**	**M**	**CV**	**Ranking**	**M**	**CV**	**Ranking**	**M**	**CV**
UBC	1	0.469	0.165	1	0.444	0.161	1	0.435	0.155
RPL13	2	0.537	0.243	2	0.480	0.197	2	0.500	0.223
HPRT1	3	0.551	0.222	3	0.507	0.195	3	0.552	0.251
GAPDH	4	0.704	0.348	4	0.670	0.326	4	0.610	0.266
Average		0.565	0.245		0.525	0.220		0.524	0.224



Supplementary figure SA1.1

Average expression stability of four housekeeping genes (UBC, RPL13, HPRT1 and GADPH) in **a)** all samples, **b)** head-kidneys samples **c)** spleen samples.

Supplementary Analysis SA2: Standard curves analysis

According to Pfaffl, "different tissues exhibit different PCR efficiencies, caused by RT inhibitors, PCR inhibitors and by variations in the total RNA fraction pattern extracted" (Pfaffl 2004). We wanted to determine the PCR efficiencies and the dynamic range (Bustin *et al*. 2009) of our different genes in the two tissue types (HK and SP).

We pooled 1 µl of each experimental sample to create four different cDNA pools, one for each tissue type and fish origin: HK-NO, HK-DE, SP-NO, SP-DE. We made nine serial dilutions of each pool (1x, 2x, 5x, 10x, 50x, 250x, 1250x, 6250x, 31250x). All dilutions were run in triplicates for all the eight target genes and the reference gene following the protocol described in the Material & Method section.

We used qBase^{+} (Biogazelle, Zwijnaarde, Belgium) to visualise and calculate different parameters of the amplification efficiency curves, and to determine the linear dynamic ranges (see table SA2.1 and fig. SA2.1).

Overall, amplification efficiency was worst in the spleen than in the head-kidney pools. We exclude IL-1ß, MIF and TLR2 from further analysis in the spleen samples because their linear dynamic range was extremely limited. For the other genes, we used the exclusion criteria for "outlier" replicates of the general assay (as described in Supplementary Analysis SA3) and calculated the calibrated normalized relative quantities using the estimated amplification efficiencies (E) of each gene. However, the proportion of successful individual measurement in the spleen samples was again worse than in the head-kidney samples. This often decreased by more than a quarter the already small sample size available per experimental groups (see table SA2.2), compromising statistical testing.

This demonstrates further that in this assay, spleen samples had low qPCR efficiencies for our immune candidate genes, likely due to the presence of inhibitors or to lower gene expression levels in this tissue (limit of detection LOD, Bustin *et al*. 2009). All the spleen samples results were therefore excluded from further analysis.

Supplementary table SA2.1

Parameters of the RT-qPCR amplification efficiency curves for each immune genes (IgM, IL-1ß, MHC-IIß, MIF, SOD2, TGF-ß1, TLR2, TNFα) and the reference gene (UBC): amplification efficiency (E), standard error (SE), R-square (R^2), slope, y-intercept, number of dilutions forming the linear dynamic range and linear dynamic range (LDR, in Ct values).

Gene	E	SE	R^2	Slope	Y-intercept	Dilution points	LDR
				Head-kidneys			
IgM	2.036	0.009	0.998	-3.238 ±0.020	18.12 ±0.053	8	19-32
IL-1ß	2.158	0.078	0.941	-2.994 ±0.141	25.893 ±0.202	5	27-33
MHC-IIß	2.056	0.017	0.994	-3.194 ±0.037	17.961 ±0.098	8	19-32
MIF	2.029	0.026	0.987	-3.254 ±0.059	20.078 ±0.130	7	21-32
SOD2	2.078	0.021	0.993	-3.149 ±0.043	21.513 ±0.094	7	22-34
TGF-ß1	2.078	0.019	0.993	-3.149 ±0.040	21.014 ±0.089	7	22-33
TLR2	2.333	0.048	0.981	-2.717 ±0.065	24.217 ±0.115	6	25-33
TNFα	2.189	0.066	0.964	-2.939 ±0.113	26.838 ±0.144	5	28-33
UBC	2.001	0.011	0.997	-3.319 ±0.025	14.723 ±0.076	9	16-32
				Spleen			
IgM	1.811	0.030	0.979	-3.876 ±0.108	18.006 ±0.248	6	21-33
IL-1ß	2.616	0.470	0.657	-2.395 ±0.447	27.575 ±0.421	4	-
MHC-IIß	1.846	0.061	0.915	-3.756 ±0.203	17.75 ±0.477	6	20-33
MIF	69.541	133.096	0.235	-0.543 ±0.245	31.111 ±0.604	3	-
SOD2	1.918	1.121	0.849	-3.536 ±0.342	23.093 ±0.536	4	25-33
TGF-ß1	1.873	0.051	0.954	-3.669 ±0.159	20.979 ±0.304	5	23-33
TLR2	2.226	0.204	0.761	-2.877 ±0.329	25.685 ±0.480	5	-
TNFα	2.271	0.227	0.828	-2.807 ±0.342	26.626 ±0.457	4	28-33
UBC	1.833	0.028	0.978	-3.799 ±0.097	16.381 ±0.258	7	19-33

Supplementary table SA2.2

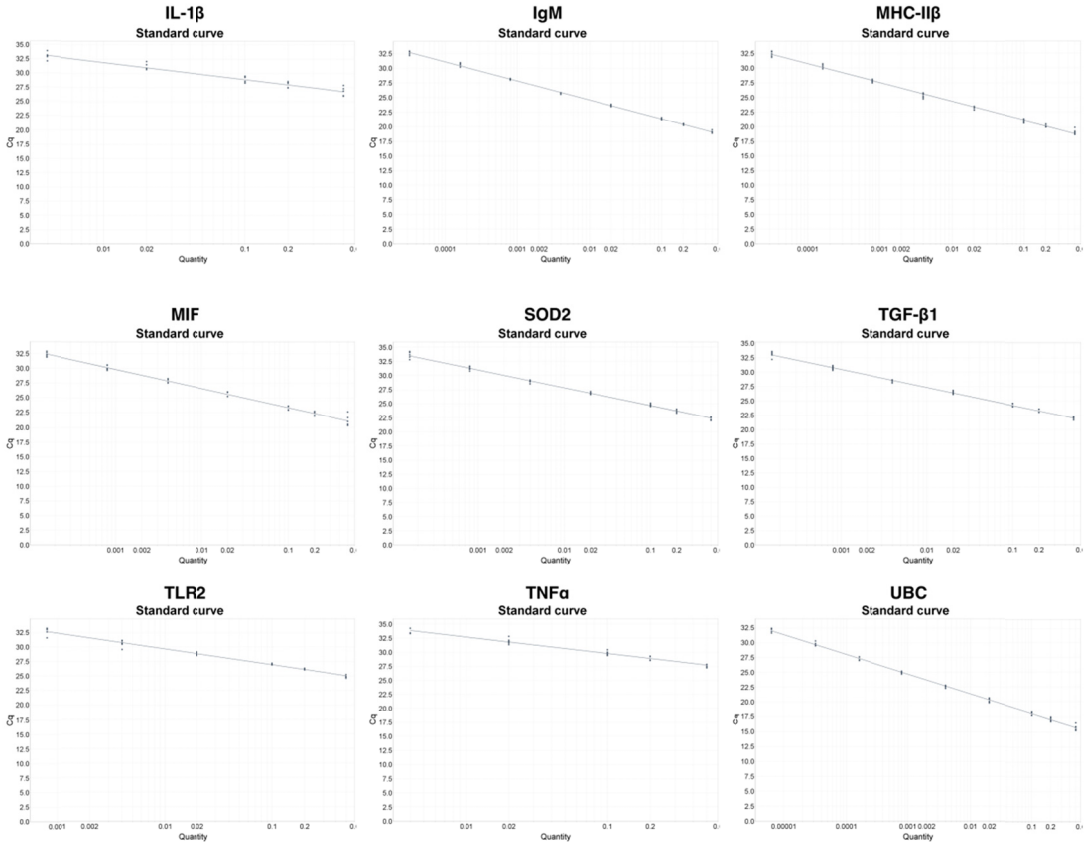
Success rate of gene expression measurements. Proportion of samples successfully measured (in %) for gene expression for each of the eight candidate genes (IgM, IL-1ß, MHC-IIß, MIF, SOD2, TGF-ß1, TLR2, TNFα) in the different treatment groups, time points (in weeks PE) and infectious status for **a)** head-kidney samples and **b)** spleen samples (N: sample size; in blue: 100%; light blue: 80-100%; yellow: 50-80% ;red:<50% samples successfully measured).

a) Time point	Fish origin	Infectious status	N	IgM	IL-1ß	MHC-IIß	MIF	SOD2	TGF- ß1	TLR2	TNFα	Total
5	NO-ctrl	control	8	100.0	100.0	100.0	100.0	100.0	100.0	62.5	25,0	**85.9**
5	DE-ctrl	control	6	100.0	100.0	66.7	100.0	100.0	100.0	50.0	50,0	**83.3**
7	NO-ctrl	control	6	83.3	83.3	100.0	83.3	83.3	83.3	83.3	16,7	**77.1**
7	DE-ctrl	control	5	100.0	100.0	80.0	100.0	100.0	100.0	100.0	100,0	**97.5**
9	NO-ctrl	control	9	55.6	55.6	100.0	55.6	55.6	55.6	100.0	100,0	**72.2**
9	DE-ctrl	control	8	75.0	75.0	87.5	75.0	75.0	87.5	87.5	100,0	**82.8**
5	NO-(NO)	infected	12	91.7	91.7	75.0	91.7	91.7	91.7	83.3	58,3	**84.4**
5	NO-(DE)	infected	5	100.0	100.0	80.0	100.0	100.0	100.0	100.0	40,0	**90.0**
5	DE-(NO)	infected	10	80.0	80.0	80.0	80.0	80.0	80.0	90.0	40,0	**76.3**
5	DE-(DE)	infected	6	83.3	100.0	100.0	83.3	83.3	83.3	100.0	100,0	**91.7**
7	NO-(NO)	infected	8	100.0	100.0	87.5	100.0	100.0	100.0	62.5	62,5	**89.1**
7	NO-(DE)	infected	3	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100,0	**100.0**
7	DE-(NO)	infected	7	100.0	100.0	100.0	100.0	100.0	100.0	100.0	71,4	**96.4**
7	DE-(DE)	infected	3	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100,0	**100.0**
9	NO-(NO)	infected	8	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100,0	**100.0**
9	NO-(DE)	infected	9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	77,8	**97.2**
9	DE-(NO)	infected	6	83.3	66.7	100.0	83.3	83.3	83.3	83.3	83,3	**83.3**
9	DE-(DE)	infected	8	87.5	87.5	87.5	87.5	87.5	87.5	87.5	100,0	**89.1**
5	NO-(NO)	uninfected	7	57.1	57.1	100.0	57.1	57.1	57.1	85.7	71,4	**67.9**
5	NO-(DE)	uninfected	7	100.0	100.0	100.0	100.0	100.0	100.0	100.0	71,4	**96.4**
5	DE-(NO)	uninfected	9	66.7	77.8	100.0	66.7	66.7	66.7	88.9	77,8	**76.4**

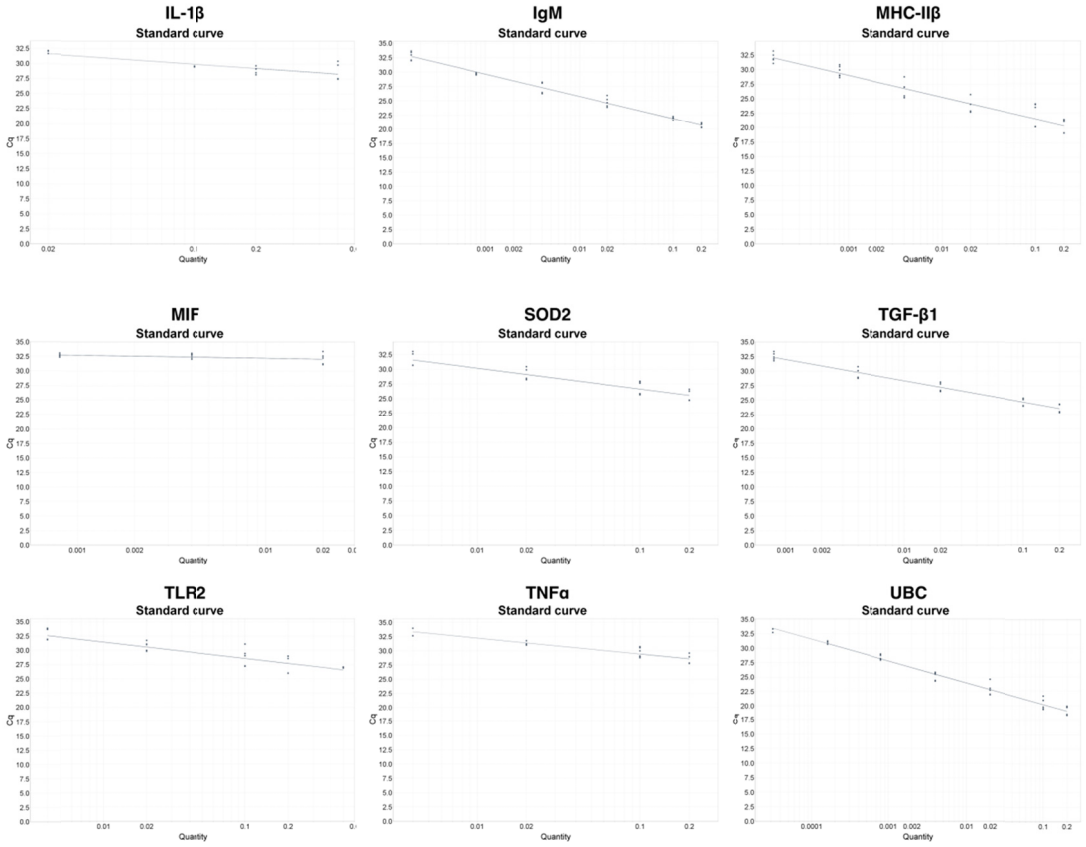
5	DE-(DE)	uninfected	11	90.9	90.9	90.9	90.9	90.9	90.9	90.9	100,0	**92.0**
7	NO-(NO)	uninfected	14	85.7	78.6	92.9	85.7	85.7	78.6	92.9	57,1	**82.1**
7	NO-(DE)	uninfected	5	80.0	80.0	100.0	100.0	100.0	100.0	100.0	100,0	**95.0**
7	DE-(NO)	uninfected	10	80.0	80.0	50.0	80.0	80.0	80.0	70.0	60,0	**72.5**
7	DE-(DE)	uninfected	5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100,0	**100.0**
9	NO-(NO)	uninfected	16	93.8	93.8	87.5	93.8	93.8	93.8	87.5	62,5	**88.3**
9	NO-(DE)	uninfected	9	88.9	88.9	66.7	88.9	88.9	88.9	55.6	77,8	**80.6**
9	DE-(NO)	uninfected	15	73.3	86.7	93.3	73.3	73.3	73.3	86.7	80,0	**80.0**
9	DE-(DE)	uninfected	4	100.0	100.0	75.0	100.0	100.0	100.0	100.0	75,0	**93.8**
Total			**239**	**88.5**	**90.0**	**89.2**	**89.1**	**89.2**	**89.4**	**88.3**	**75.3**	**87.4**

b) Time point	Fish origin	Infectious status	N	IgM	IL-1β	MHC-IIβ	MIF	SOD2	TGF-β1	TLR2	TNFα	Total
5	NO-ctrl	control	8	62.5	-	87.5	-	75.0	100.0	-	62.5	**77.5**
5	DE-ctrl	control	6	66.7	-	83.3	-	100.0	100.0	-	50.0	**80.0**
7	NO-ctrl	control	6	100.0	-	50.0	-	50.0	83.3	-	50.0	**66.7**
7	DE-ctrl	control	5	100.0	-	100.0	-	100.0	100.0	-	100.0	**100.0**
9	NO-ctrl	control	9	33.3	-	66.7	-	66.7	66.7	-	55.6	**57.8**
9	DE-ctrl	control	9	33.3	-	66.7	-	66.7	66.7	-	66.7	**60.0**
5	NO-(NO)	infected	11	90.9	-	27.3	-	81.8	90.9	-	81.8	**74.5**
5	NO-(DE)	infected	8	75.0	-	12.5	-	100.0	100.0	-	87.5	**75.0**
5	DE-(NO)	infected	10	90.0	-	80.0	-	90.0	90.0	-	70.0	**84.0**
5	DE-(DE)	infected	6	100.0	-	100.0	-	100.0	100.0	-	83.3	**96.7**
7	NO-(NO)	infected	8	100.0	-	37.5	-	100.0	100.0	-	37.5	**75.0**
7	NO-(DE)	infected	3	100.0	-	100.0	-	100.0	100.0	-	100.0	**100.0**
7	DE-(NO)	infected	7	100.0	-	14.3	-	85.7	85.7	-	28.6	**62.9**
7	DE-(DE)	infected	3	100.0	-	100.0	-	100.0	100.0	-	100.0	**100.0**
9	NO-(NO)	infected	8	75.0	-	25.0	-	75.0	100.0	-	100.0	**75.0**
9	NO-(DE)	infected	9	100.0	-	33.3	-	88.9	88.9	-	11.1	**64.4**

9	DE-(NO)	infected	5	40.0	-	100.0	-	100.0	60.0	-	100.0	**80.0**
9	DE-(DE)	infected	8	12.5	-	87.5	-	12.5	100.0	-	100.0	**62.5**
5	NO-(NO)	uninfected	9	100.0	-	22.2	-	66.7	66.7	-	44.4	**60.0**
5	NO-(DE)	uninfected	8	87.5	-	50.0	-	100.0	100.0	-	62.5	**80.0**
5	DE-(NO)	uninfected	10	100.0	-	60.0	-	50.0	60.0	-	40.0	**62.0**
5	DE-(DE)	uninfected	11	45.5	-	54.5	-	90.9	90.9	-	90.9	**74.5**
7	NO-(NO)	uninfected	14	78.6	-	64.3	-	78.6	100.0	-	50.0	**74.3**
7	NO-(DE)	uninfected	5	100.0	-	100.0	-	100.0	100.0	-	100.0	**100.0**
7	DE-(NO)	uninfected	10	100.0	-	40.0	-	70.0	90.0	-	40.0	**68.0**
7	DE-(DE)	uninfected	5	100.0	-	100.0	-	80.0	80.0	-	100.0	**92.0**
9	NO-(NO)	uninfected	16	75.0	-	56.3	-	62.5	87.5	-	12.5	**58.8**
9	NO-(DE)	uninfected	9	88.9	-	66.7	-	88.9	100.0	-	11.1	**71.1**
9	DE-(NO)	uninfected	15	40.0	-	46.7	-	6.7	66.7	-	6.7	**33.3**
9	DE-(DE)	uninfected	4	25.0	-	75.0	-	25.0	100.0	-	100.0	**65.0**
Total			**245**	**77.3**	**-**	**63.6**	**-**	**77.0**	**89.1**	**-**	**64.8**	**74.4**



Supplementary figure SA2.1
Standard amplification curves (head-kidney). Linear dynamic range of qPCR amplification in head-kidney for each of the eight candidate genes (IgM, IL-1β, MHC-IIβ, MIF, SOD2, TGF-β1, TLR2, TNFα).



Supplementary figure SA2.2
Standard amplification curves (spleen). Linear dynamic range of qPCR amplification in spleen for each of the eight candidate genes (IgM, IL-1ß, MHC-IIß, MIF, SOD2, TGF-ß1, TLR2, TNFα).

Supplementary Analysis SA3: Quality control of qPCR reactions and triplicates

Two qPCR plates had positive NTC and had to be repeated. For all other plates, NTC were negative with Ct>32 and no amplification of the target gene revealed by the dissociation analysis.

All samples were checked using the following quality control criteria: i) replicates showing sign of cross-contamination from the dissociation analysis; ii) replicates with Ct value superior or equal to 35; iii) replicates with Ct value out of the linear dynamic range; iv) triplicates with a standard error (SE) >0.2; v) triplicates with a coefficient of variation (CV, a measure of inter-assay variation and reproducibility) >4.0. The qPCR reactions that met criteria i) and ii) were excluded from the analysis. For all qPCR reactions that met criteria iii) to v), samples were further checked by calculating pairwise difference in the Ct value of the triplicates to measurement reproducibility. For samples with one replicate exceeding a pairwise difference of Ct0.5, the deviating replicate was removed. Samples where at least two replicates exceeded the Ct0.5 pairwise difference were excluded from the analysis.

Supplementary table SA3.1

Summary of the number of qPCR reactions or replicates that failed the different quality controls and that were excluded from analysis for the different tissues (HK: head-kidneys; SP: spleen) and samples (ctrl: control; inf.: infected; uninf.: uninfected; IRC). All numbers are counts, except percentage that are indicated in parenthesis and italic.

Tissue	**HK**					**SP***				
Sample types	**ctrl**	**inf.**	**uninf.**	**IRC**	**Total**	**ctrl**	**inf.**	**uninf.**	**IRC**	**Total**
individuals	42	86	115	1	241	43	87	117	1	248
Samples	121	271	342	24	755	122	233	312	23	690
qPCR reactions	1308	2919	3644	289	8160	981	1848	2517	192	5538
Quality control criteria										
i) Cross-contamination	0 (*0.0*)	0 (*0.0*)	0 (*0.0*)	0 (*0.0*)	0 (*0.0*)	5 (*0.5*)	1 (*0.1*)	3 (*0.1*)	0 (*0.0*)	9 (*0.2*)
ii) Ct ≥35	17 (*1.3*)	10 (*0.3*)	25 (*0.7*)	0 (*0.0*)	52 (*0.6*)	47 (*4.8*)	30 (*1.6*)	97 (*3.9*)	4 (*2.1*)	178 (*3.2*)
iii) Out of dynamic range	53 (*4.1*)	93 (*3.2*)	149 (*4.1*)	45 (*15.6*)	340 (*4.2*)	71 (*7.2*)	114 (*6.2*)	151 (*6.0*)	65 (*33.9*)	401 (*7.2*)
iv) $SE_{triplicates}$ >0.2	147 (*11.2*)	277 (*9.5*)	334 (*9.2*)	27 (*9.3*)	785 (*9.6*)	100 (*10.2*)	152 (*8.2*)	238 (*9.5*)	16 (*8.3*)	506 (*9.1*)
v) $CV_{triplicates}$ >4.0	3 (0.2)	14 (*0.5*)	13 (*0.4*)	2 (*0.7*)	32 (*0.4*)	2 (*0.2*)	5 (*0.3*)	3 (*0.1*)	0 (*0.0*)	10 (*0.2*)
Excluded replicates										
1 out of 3 triplicates	87 (*6.7*)	160 (*5.5*)	195 (*5.4*)	13 (*4.5*)	455 (*5.6*)	64 (*6.5*)	80 (*4.3*)	147 (*5.8*)	4 (*2.1*)	295 (*5.3*)
3 out of 3 triplicates	57 (*4.4*)	93 (*3.2*)	138 (*3.8*)	24 (*8.3*)	312 (*3.8*)	43 (*4.4*)	75 (*4.1*)	96 (*3.8*)	29 (*15.1*)	243 (*4.4*)
Total excluded replicates	**144 (*11.0*)**	**253 (*8.7*)**	**333 (*9.1*)**	**37 (*12.8*)**	**767 (*9.4*)**	**107 (*10.9*)**	**155 (*8.4*)**	**243 (*9.7*)**	**33 (*17.2*)**	**538 (*9.7*)**

***** IL-1 ß, TLR2 and MIF reactions are excluded from the count

Appendix – Chapter 4

Supplementary table S1

Summary table of the experimental exposure in the different treatment groups. For each experimental exposure a realm of outcomes was possible: no infection (uninfected fish), a single infection (single Hv or Lv infected fish) or a double infection (double homologous or double heterologous infected fish). Here, we summarize the sample size of the different exposure outcomes for each experimental treatment. The sample sizes of the groups of interest (experimental groups) used in the results analysis are indicated in bold.

Experimental treatment	Exposed	Dead	Unexposed control	Exposure outcomes				
				Uninfected	Single Hv	Single Lv	Double homologous	Double heterologous
Control	-	12	***Control:*** **72**	-	-	-	-	-
Hv	249	4	-	165	***Single Hv:*** **80**	-	-	-
Lv	308	3	-	192	-	***Single Lv:*** **113**	-	-
Hv+Hv	253	9	-	118	93	-	***Double Hv+Hv:*** **33**	-
Lv+Lv	342	4	-	174	-	111	***Double Lv+Lv:*** **53**	-
Hv+Lv	356	6	-	166	61	76	-	***Double Hv+Lv:*** **47**

Supplementary Analysis SA1: Experimental trial effect

Supplementary table SA1.1

Linear mixed effect model on the total parasite index (tPI) for the two independent experimental trials. Table shows the results of pairwise comparison between infection types (Lv single homologous, Hv single homologous, Lv+Lv double homologous, Hv+Hv double homologous or Hv+Lv double heterologous), significant *P*-values (<0.05) in bold.

tPI	Experimental trial 1					Experimental trial 2				
Infection type	Estimate	S.E.	D.F.	*T*-value	*P*-value	Estimate	S.E.	D.F.	*T*-value	*P*-value
Hv - Hv+Hv	-16.493	2.203	145.26	-7.486	**<0.001**	-12.703	1.198	141.91	-10.603	**<0.001**
Hv - Hv+Lv	-5.456	1.895	148.97	-2.879	**0.036**	-3.913	1.043	164.03	-3.752	**0.002**
Hv - Lv	20.407	1.282	148.86	15.911	**<0.001**	15.207	0.945	135.16	16.083	**<0.001**
Hv - Lv+Lv	17.610	1.598	145.92	11.022	**<0.001**	17.311	1.129	73.00	15.335	**<0.001**
Hv+Hv - Hv+Lv	11.037	2.488	148.20	4.435	**<0.001**	8.789	1.237	161.09	7.106	**<0.001**

Supplementary table SA1.2

Linear mixed effect model on the discrete parasite index (dPI) for the two independent experimental trials. Table shows the results of pairwise comparison between the interaction of infection types and parasite strains (Lv single, Hv single, Lv double homologous, Hv double homologous, Hv double heterologous or Lv double heterologous), significant *P*-values (<0.05) in bold; standard error (S.E.).

dPI	Experimental trial 1					Experimental trial 2				
Infection type x Parasite strain	Estimate	S.E.	D.F.	*T*-value	*P*-value	Estimate	S.E.	D.F.	*T*-value	*P*-value
Hv heterologous – Lv heterologous	2.088	1.701	193.88	1.227	0.823	3.893	0.885	235.68	4.397	**<0.001**
Hv heterologous – Hv homologous	-4.158	1.673	199.79	-2.485	0.133	-2.455	0.837	229.60	-2.934	**0.042**
Hv heterologous – Lv homologous	12.719	1.409	198.03	9.027	**<0.001**	12.559	0.821	142.26	15.295	**<0.001**
Hv heterologous – Hv single	-12.382	1.509	199.73	-8.204	**<0.001**	-9.125	0.830	243.95	-10.995	**<0.001**
Hv heterologous – Lv single	8.220	1.357	197.91	6.058	**<0.001**	6.097	0.833	218.92	7.322	**<0.001**
Lv heterologous – Hv homologous	-6.246	1.673	199.79	-3.733	**0.003**	-6.349	0.837	229.60	-7.586	**<0.001**
Lv heterologous – Lv homologous	10.631	1.409	198.03	7.545	**<0.001**	8.665	0.821	142.26	10.554	**<0.001**
Lv heterologous – Hv single	-14.470	1.509	199.73	-9.588	**<0.001**	-13.019	0.830	243.95	-15.686	**<0.001**
Lv heterologous – Lv single	6.131	1.357	197.91	4.519	**<0.001**	2.204	0.833	218.92	2.647	0.091
Hv homologous – Lv homologous	16.877	1.344	195.64	12.556	**<0.001**	15.014	0.815	26.01	18.413	**<0.001**
Hv homologous – Hv single	-8.224	1.394	188.86	-5.898	**<0.001**	-6.670	0.789	182.33	-8.449	**<0.001**
Hv homologous – Lv single	12.378	1.287	191.42	9.614	**<0.001**	8.553	0.810	63.99	10.553	**<0.001**
Lv homologous – Hv single	-25.102	1.092	190.27	-22.983	**<0.001**	-21.684	0.747	80.69	-29.041	**<0.001**
Lv homologous – Lv single	-4.500	0.928	198.74	-4.846	**<0.001**	-6.461	0.716	221.11	-9.025	**<0.001**
Hv single – Lv single	20.602	1.024	199.70	20.112	**<0.001**	15.223	0.750	212.28	20.304	**<0.001**

Supplementary table SA1.3

Linear mixed effect model on the condition factor (CF) for the two independent experimental trials. Table shows the results of pairwise comparison between experimental treatments (unexposed control, Lv single homologous, Hv single homologous, Lv+Lv double homologous, Hv+Hv double homologous or Hv+Lv double heterologous), significant *P*-values (<0.05) in bold.

CF	Experimental trial 1					Experimental trial 2				
Treatment	Estimate	S.E.	D.F.	*T*-value	*P*-value	Estimate	S.E.	D.F.	*T*-value	*P*-value
Hv - Hv+Hv	0.097	0.027	179.70	3.537	**0.007**	0.033	0.020	196.70	1.671	0.552
Hv - Hv+Lv	-0.019	0.024	184.22	-0.808	0.966	-0.007	0.018	192.27	-0.385	0.999
Hv - Lv	-0.030	0.016	183.73	-1.922	0.392	-0.028	0.017	196.31	-1.670	0.553
Hv - Lv+Lv	-0.054	0.020	181.77	-2.742	0.072	-0.029	0.020	193.16	-1.450	0.696
Hv - control	-0.047	0.018	183.43	-2.689	0.082	-0.058	0.017	194.38	-3.338	**0.013**
Hv+Hv - Hv+Lv	-0.116	0.031	182.49	-3.720	**0.004**	-0.040	0.021	194.44	-1.918	0.394
Hv+Hv - Lv	-0.127	0.026	181.54	-4.880	**<0.001**	-0.061	0.022	186.95	-2.823	0.058
Hv+Hv - Lv+Lv	-0.151	0.029	183.77	-5.275	**<0.001**	-0.063	0.024	184.00	-2.553	0.114
Hv+Hv - control	-0.144	0.027	180.25	-5.267	**<0.001**	-0.092	0.021	195.88	-4.262	**<0.001**
Hv+Lv - Lv	-0.011	0.022	182.76	-0.517	0.995	-0.021	0.018	196.18	-1.156	0.857
Hv+Lv - Lv+Lv	-0.035	0.024	182.51	-1.425	0.712	-0.022	0.021	195.54	-1.070	0.893
Hv+Lv - control	-0.028	0.023	183.70	-1.217	0.828	-0.052	0.019	192.46	-2.770	0.067
Lv - Lv+Lv	-0.024	0.018	183.66	-1.347	0.758	-0.001	0.018	192.65	-0.074	1.000
Lv - control	-0.017	0.015	182.90	-1.120	0.873	-0.031	0.017	193.65	-1.791	0.474
Lv+Lv - control	0.007	0.019	183.47	0.343	0.999	-0.029	0.020	196.05	-1.463	0.688

Supplementary table SA1.4

Linear mixed effect model on the hepatosomatic index (HSI) for the two independent experimental trials. Table shows the results of pairwise comparison between experimental treatments (unexposed control, Lv single homologous, Hv single homologous, Lv+Lv double homologous, Hv+Hv double homologous or Hv+Lv double heterologous), significant *P*-values (<0.05) in bold.

HSI	Experimental trial 1					Experimental trial 2				
Treatment	Estimate	S.E.	D.F.	*T*-value	*P*-value	Estimate	S.E.	D.F.	*T*-value	*P*-value
Hv - Hv+Hv	-0.117	0.080	183.57	-1.451	0.696	-0.026	0.063	196.62	-0.419	0.998
Hv - Hv+Lv	0.005	0.069	184.91	0.080	1.000	-0.129	0.055	193.93	-2.355	0.177
Hv - Lv	-0.031	0.047	183.97	-0.663	0.986	-0.227	0.051	194.60	-4.423	**<0.001**
Hv - Lv+Lv	-0.118	0.059	184.93	-2.011	0.340	-0.156	0.062	185.70	-2.519	0.124
Hv - control	-0.249	0.052	182.17	-4.793	**<0.001**	-0.501	0.055	193.20	-9.110	**<0.001**
Hv+Hv - Hv+Lv	0.122	0.091	182.60	1.347	0.758	-0.103	0.065	195.79	-1.579	0.613
Hv+Hv - Lv	0.085	0.076	183.57	1.118	0.873	-0.201	0.067	172.38	-2.997	**0.036**
Hv+Hv - Lv+Lv	-0.002	0.084	184.19	-0.020	1.000	-0.130	0.076	166.33	-1.716	0.523
Hv+Hv - control	-0.132	0.080	183.37	-1.651	0.566	-0.474	0.067	193.01	-7.119	**<0.001**
Hv+Lv - Lv	-0.036	0.062	181.73	-0.591	0.992	-0.098	0.056	195.36	-1.745	0.504
Hv+Lv - Lv+Lv	-0.124	0.071	182.33	-1.741	0.507	-0.027	0.065	192.73	-0.418	0.998
Hv+Lv - control	-0.254	0.067	183.51	-3.793	**0.003**	-0.372	0.058	193.70	-6.396	**<0.001**
Lv - Lv+Lv	-0.087	0.052	183.32	-1.665	0.557	0.071	0.057	193.96	1.251	0.811
Lv - control	-0.218	0.045	182.21	-4.830	**<0.001**	-0.273	0.053	194.23	-5.095	**<0.001**
Lv+Lv - control	-0.131	0.057	184.71	-2.276	0.209	-0.344	0.062	196.60	-5.508	**<0.001**

Supplementary table SA1.5

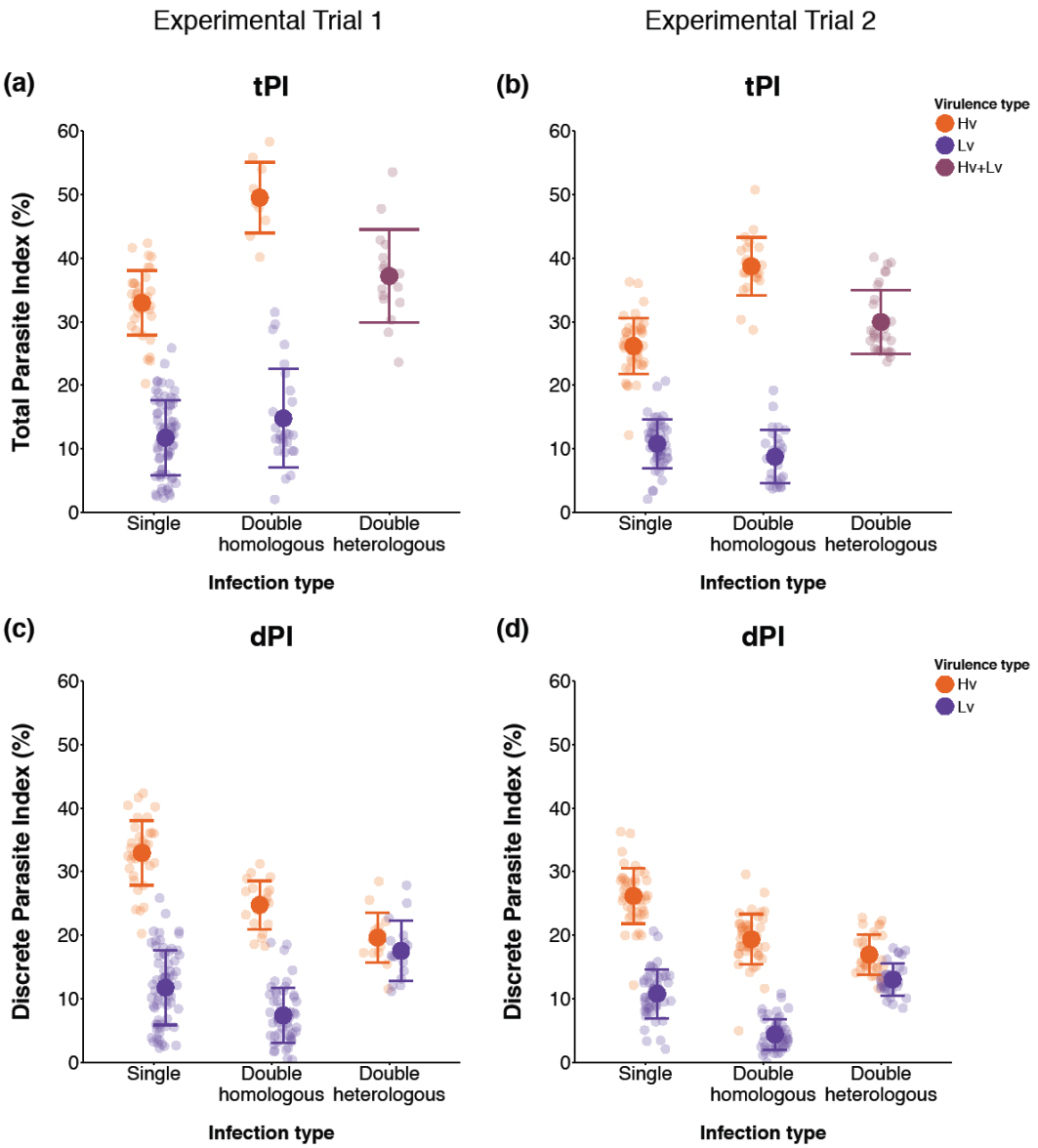
Linear mixed effect model on the splenosomatic index (SSI) for the two independent experimental trials. Table shows the results of pairwise comparison between experimental treatments (unexposed control, Lv single homologous, Hv single homologous, Lv+Lv double homologous, Hv+Hv double homologous or Hv+Lv double heterologous), significant *P*-values (<0.05) in bold.

SSI	Experimental trial 1					Experimental trial 2				
Treatment	Estimate	S.E.	D.F.	*T*-value	*P*-value	Estimate	S.E.	D.F.	*T*-value	*P*-value
Hv - Hv+Hv	0.067	0.025	184.54	2.642	0.092	0.016	0.016	194.87	0.987	0.923
Hv - Hv+Lv	0.036	0.021	185.56	1.677	0.549	-0.001	0.014	195.65	-0.069	1.000
Hv - Lv	0.035	0.014	184.64	2.385	0.167	0.019	0.013	188.85	1.456	0.693
Hv - Lv+Lv	0.027	0.018	184.91	1.501	0.664	0.020	0.016	165.09	1.266	0.803
Hv - control	0.071	0.016	184.05	4.334	**0.001**	0.039	0.014	194.87	2.710	0.078
Hv+Hv - Hv+Lv	-0.030	0.028	183.12	-1.075	0.891	-0.017	0.017	196.34	-1.009	0.915
Hv+Hv - Lv	-0.031	0.024	184.40	-1.314	0.777	0.003	0.017	144.75	0.187	1.000
Hv+Hv - Lv+Lv	-0.040	0.026	184.97	-1.472	0.683	0.004	0.020	132.17	0.211	0.100
Hv+Hv - control	0.004	0.025	184.37	0.162	1.000	0.023	0.017	184.77	1.316	0.776
Hv+Lv - Lv	-0.001	0.019	182.61	-0.053	1.000	0.020	0.015	191.14	1.394	0.730
Hv+Lv - Lv+Lv	-0.009	0.022	181.96	-0.383	0.999	0.021	0.017	183.16	1.267	0.803
Hv+Lv - control	0.034	0.021	184.27	1.646	0.569	0.040	0.015	194.99	2.628	0.095
Lv - Lv+Lv	-0.007	0.017	170.57	-0.453	0.998	0.001	0.015	195.37	0.059	1.000
Lv - control	0.035	0.014	183.22	2.516	0.125	0.020	0.014	194.90	1.389	0.733
Lv+Lv - control	0.043	0.018	182.13	2.382	0.168	0.019	0.016	194.01	1.140	0.864

Supplementary table SA1.6

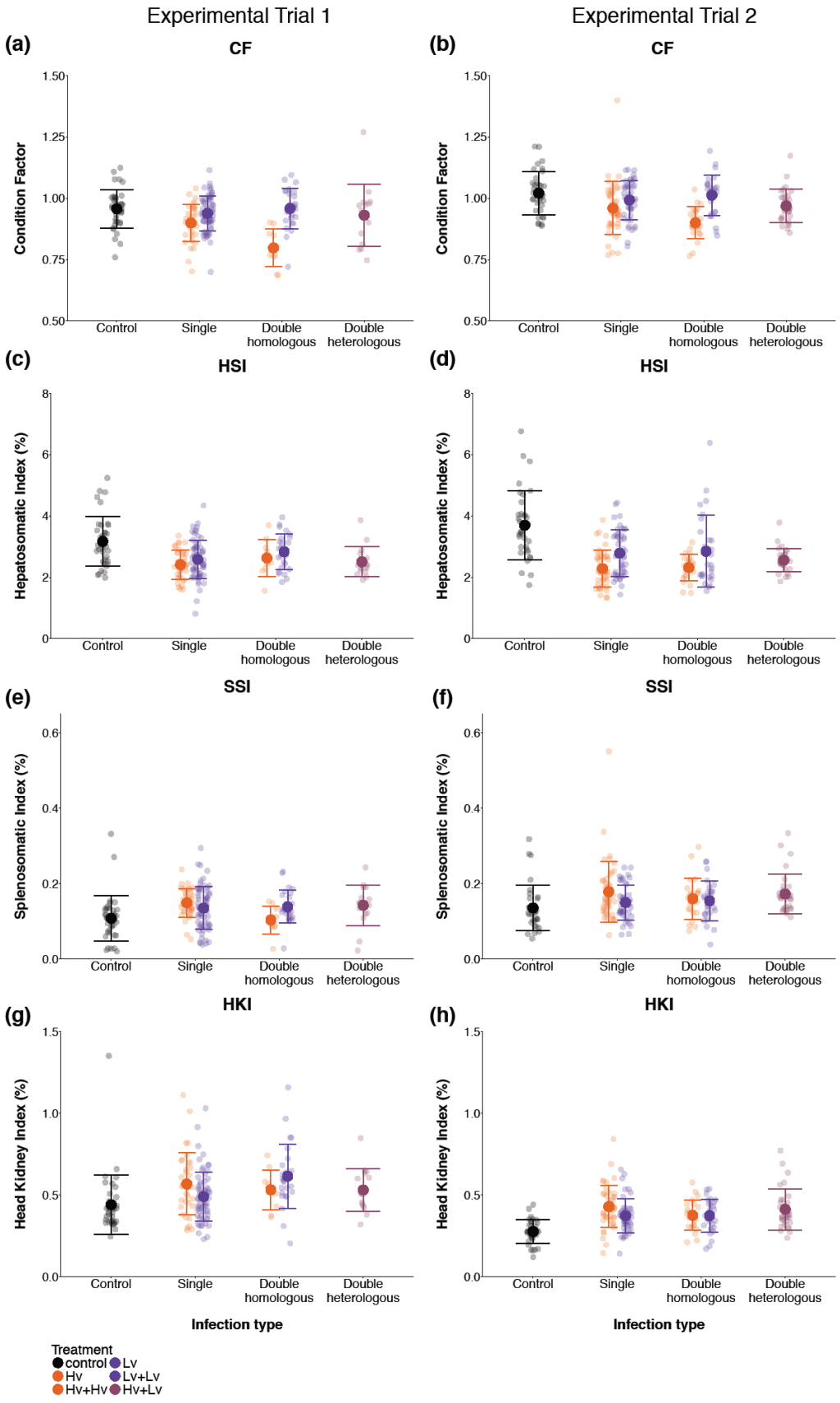
Linear mixed effect model on the head-kidney index (HKI) for the two independent experimental trials. Table shows the results of pairwise comparison between experimental treatments (unexposed control, Lv single homologous, Hv single homologous, Lv+Lv double homologous, Hv+Hv double homologous or Hv+Lv double heterologous), significant *P*-values (<0.05) in bold.

HKI	Experimental trial 1					Experimental trial 2				
Treatment	Estimate	S.E.	D.F.	*T*-value	*P*-value	Estimate	S.E.	D.F.	*T*-value	*P*-value
Hv - Hv+Hv	0.008	0.021	184.27	0.381	0.999	0.014	0.014	196.31	0.980	0.924
Hv - Hv+Lv	0.023	0.018	184.82	1.262	0.805	0.001	0.012	193.73	0.064	1.000
Hv - Lv	0.034	0.012	184.22	2.811	0.060	0.029	0.011	194.78	2.485	0.134
Hv - Lv+Lv	-0.014	0.015	184.94	-0.889	0.949	0.031	0.014	186.15	2.235	0.227
Hv - control	0.050	0.014	182.77	3.669	**0.004**	0.077	0.012	193.18	6.188	**<0.001**
Hv+Hv - Hv+Lv	0.015	0.024	182.40	0.621	0.989	-0.013	0.015	196.10	-0.887	0.949
Hv+Hv - Lv	0.026	0.020	183.96	1.321	0.773	0.015	0.015	173.46	0.988	0.921
Hv+Hv - Lv+Lv	-0.022	0.022	184.66	-0.980	0.924	0.017	0.017	167.54	1.019	0.911
Hv+Hv - control	0.042	0.021	183.87	1.997	0.348	0.063	0.015	193.77	4.177	**<0.001**
Hv+Lv - Lv	0.012	0.016	181.92	0.726	0.978	0.028	0.013	195.81	2.206	0.240
Hv+Lv - Lv+Lv	-0.036	0.019	182.69	-1.947	0.377	0.030	0.014	193.18	2.087	0.298
Hv+Lv - control	0.027	0.017	183.53	1.551	0.632	0.076	0.013	194.12	5.791	**<0.001**
Lv - Lv+Lv	-0.048	0.014	181.40	-3.481	**0.008**	0.002	0.013	194.23	0.191	1.000
Lv - control	0.015	0.012	182.50	1.310	0.779	0.048	0.012	194.56	3.956	**0.001**
Lv+Lv - control	0.064	0.015	184.51	4.224	**<0.001**	0.045	0.014	196.97	3.219	**0.019**



Supplementary figure SA1.1

a-b) Mean total parasite index (tPI ±SD) and **c-d)** mean discrete parasite index (dPI ±SD) in the different treatment groups (Lv single homologous, Hv single homologous, Lv+Lv double homologous, Hv+Hv double homologous or Hv+Lv double heterologous) for the two independent experimental trials 1 and 2.


Experimental Trial 1
Experimental Trial 2
(a)
CF
(b)
CF
Condition Factor
1.50
1.25
1.00
0.75
0.50
Control
Single
Double homologous
Double heterologous
(c)
HSI
(d)
HSI
Hepatosomatic Index (%)
8
6
4
2
0
(e)
SSI
(f)
SSI
Splenosomatic Index (%)
0.6
0.4
0.2
0.0
(g)
HKI
(h)
HKI
Head Kidney Index (%)
1.5
1.0
0.5
0.0
Infection type
Treatment
control
Lv
Hv
Lv+Lv
Hv+Hv
Hv+Lv

Supplementary figure SA1.2

a-b) Mean condition factor (CF ±SD), **c-d)** mean hepatosomatic index (HSI ±SD), **e-f)** mean splenosomatic index (SSI ±SD), and **g-h)** mean head-kidney index (HKI ±SD) in the different treatment groups (unexposed control, Lv single homologous, Hv single homologous, Lv+Lv double homologous, Hv+Hv double homologous or Hv+Lv double heterologous) for the two independent experimental trials 1 and 2.

Supplementary Analysis SA2: Fish family effects

The sample sizes of the different treatment groups for each of the six fish families are summarized in Table S1.

We used a linear model (lm function, stats library) and a type-III ANOVA (Anova function, car library) to compare the effect of the treatment (unexposed control, Lv single homologous, Hv single homologous, Lv+Lv double homologous, Hv+Hv double homologous or Hv+Lv double heterologous) and of the fish family within each experimental trial on the total parasite index (tPI), the discrete parasite index (dPI) of each individual parasite, the condition factor (CF), hepatosomatic index (HSI), splenosomatic index (SSI) and head kidney index (HKI). The response variables were transformed similarly as described in the main analysis.

The fish family had a significant effect on HKI in trial 1, and on CF and HSI in trial 2. However we found a significant effect of the interaction between treatment and fish family only in CF in trial 1 and HSI in trial 2 (see table S2.2). Tukey's tests (lsmeans function, lsmeans library) showed that in the case of CF, there was no significant difference between fish families within the same treatment; while for HSI, all the significant differences within treatment groups involved the fish family number 6, which presents the highest mean values for trial 2 (figure S2.4).

This indicates that while there are differences among fish families, overall, similar patterns are observed in the different fish families for the effect of the treatment (see figure S2.1 to S2.6).

Supplementary table SA2.1

Sample sizes in the different fish families for the different treatment groups. *One fish out of 80 could not be assigned to a fish family.

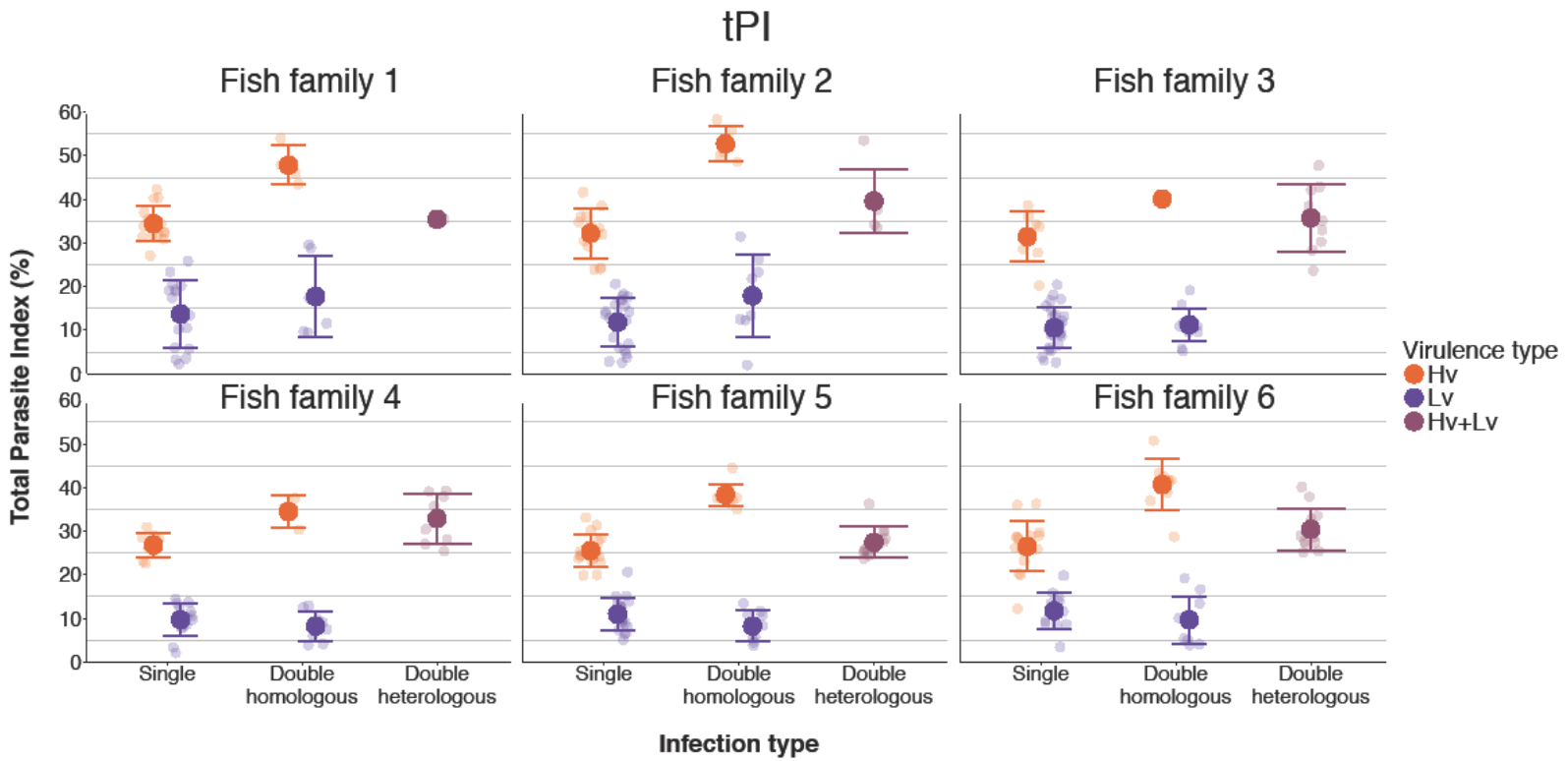
Experimental treatment	Experimental trial 1			Experimental trial 2			Total
	Fish family 1	Fish family 2	Fish family 3	Fish family 4	Fish family 5	Fish family 6	
Control	13	10	15	13	12	9	**72**
Hv	15	13	8	9	17	17	**79***
Lv	16	23	28	14	19	13	**113**
Hv+Hv	4	5	1	3	11	9	**33**
Lv+Lv	6	8	12	8	9	10	**53**
Hv+Lv	1	6	9	8	11	12	**47**
Total	**55**	**65**	**73**	**55**	**79**	**70**	**397**

Supplementary table SA2.2

Results from linear mixed effect models for the two independent experimental trials 1 and 2 on **A:** the total parasite index (tPI), **B:** discrete parasite index (dPI), **C:** condition factor (CF), **D:** hepatosomatic index (HSI), **E:** splenosomatic index (SSI), **F:** head-kidney index (HKI), significant *P*-values (<0.05) in bold.

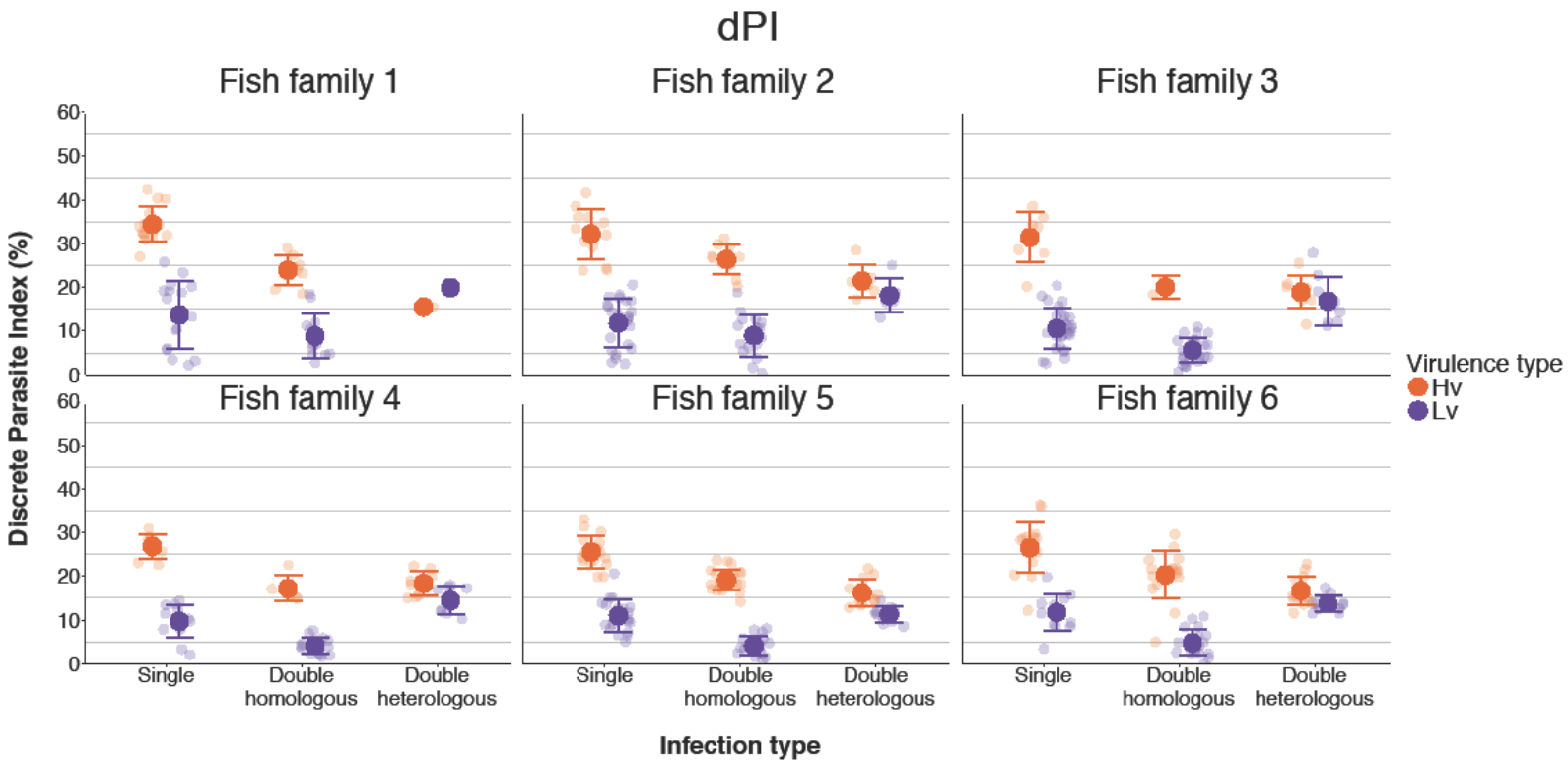
	Experimental trial 1			Experimental trial 2		
A: tPI	Chi-square	D.F.	*P*-value	Chi-square	D.F.	*P*-value
(Intercept)	414.010	1	**< 0.001**	352.448	1	**< 0.001**
Infection types	161.288	4	**< 0.001**	269.035	4	**< 0.001**
Fish family	1.429	2	0.489	2.709	2	0.439
Infection types x Fish family	6.482	8	0.593	11.675	8	0.166
B: dPI	Chi-square	D.F.	*P*-value	Chi-square	D.F.	*P*-value
(Intercept)	10.297	1	**< 0.001**	224.752	1	**< 0.001**
Infection types x Parasite virulence	221.183	5	**< 0.001**	290.335	5	**< 0.001**
Fish family	1.437	2	0.487	1.995	2	0.369
Infection types x Parasite virulence x Fish family	5.212	10	0.876	10.594	10	0.390
C: CF	Chi-square	D.F.	*P*-value	Chi-square	D.F.	*P*-value
(Intercept)	1351.213	1	**< 0.001**	1331.222	5	**< 0.001**
Infection types	33.184	5	**< 0.001**	9.603	5	0.087
Fish family	2.141	2	0.343	12.863	25	**0.002**
Infection types x Fish family	21.221	10	**0.020**	13.107	186	0.218
D: HSI	Chi-square	D.F.	*P*-value	Chi-square	D.F.	*P*-value
(Intercept)	133.032	1	**< 0.001**	61.671	1	**< 0.001**
Infection types	15.293	5	**0.009**	29.167	5	**< 0.001**
Fish family	3.573	2	0.167	25.512	2	**< 0.001**
Infection types x Fish family	7.668	10	0.661	27.173	10	**< 0.001**
E: SSI	Chi-square	D.F.	*P*-value	Chi-square	D.F.	*P*-value
(Intercept)	117.178	1	**< 0.001**	75.566	1	**< 0.001**

Infection types	13.926	5	**0.016**	7.261	5	0.202
Fish family	2.883	2	0.236	5.351	2	0.069
Infection types x Fish family	9.385	10	0.496	7.073	10	0.718
F: (HKI)^1/4	Chi-square	D.F.	*P*-value	Chi-square	D.F.	*P*-value
(Intercept)	2322.768	1	**< 0.001**	1810.6610	1	**< 0.001**
Infection types	3.919	5	0.561	20.1560	5	**0.001**
Fish family	12.857	2	**0.002**	3.8356	2	0.147
Infection types x Fish family	11.462	10	0.323	3.2167	10	0.976



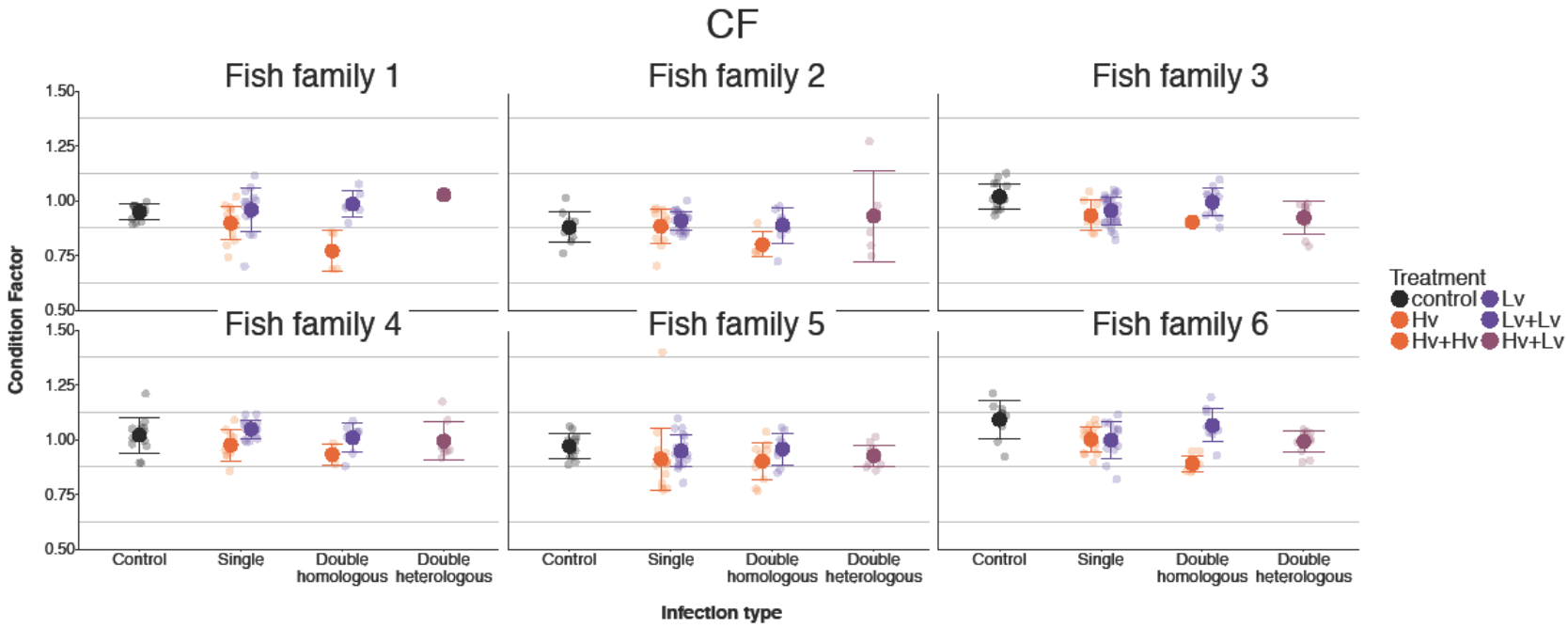
Supplementary figure SA2.1

Mean total parasite index (tPI ±SD) in the different treatment groups and fish families.



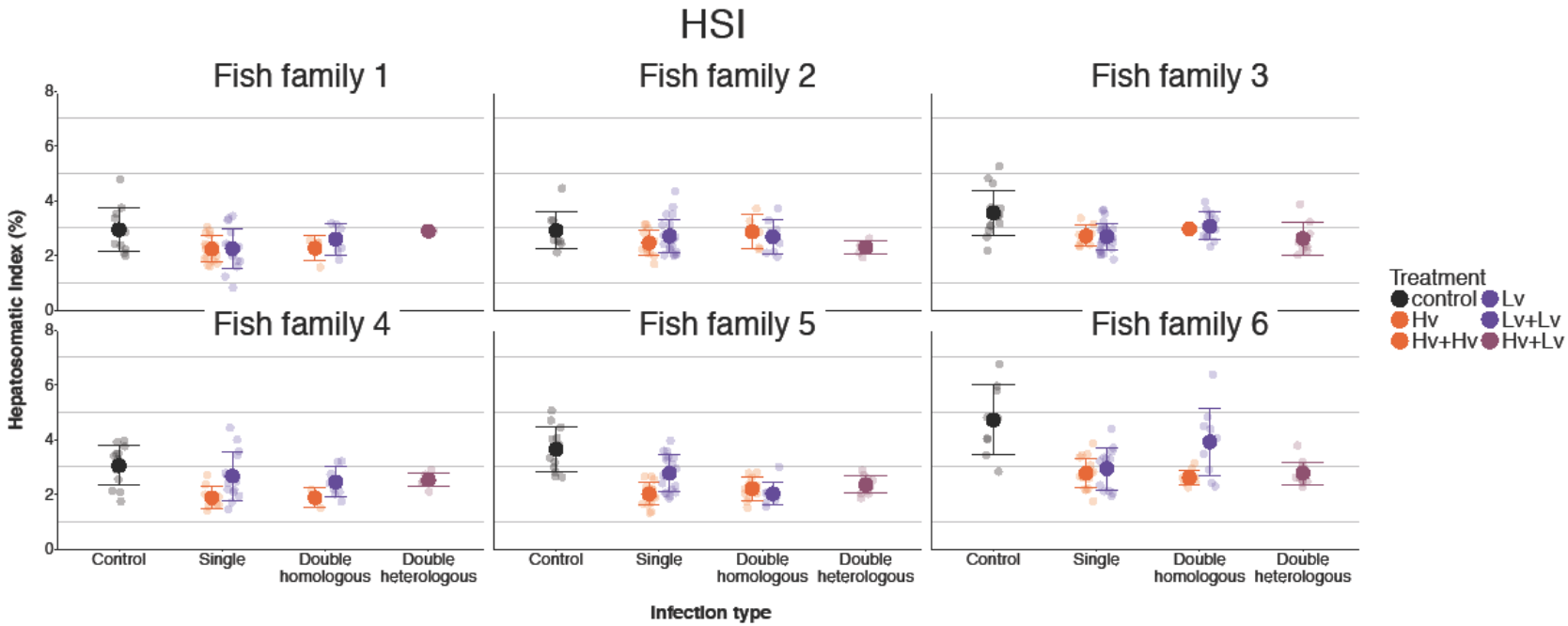
Supplementary figure SA2.2

Virulence of individual parasites as mean discrete parasite index (dPI ±SD) in the different treatments and fish families.



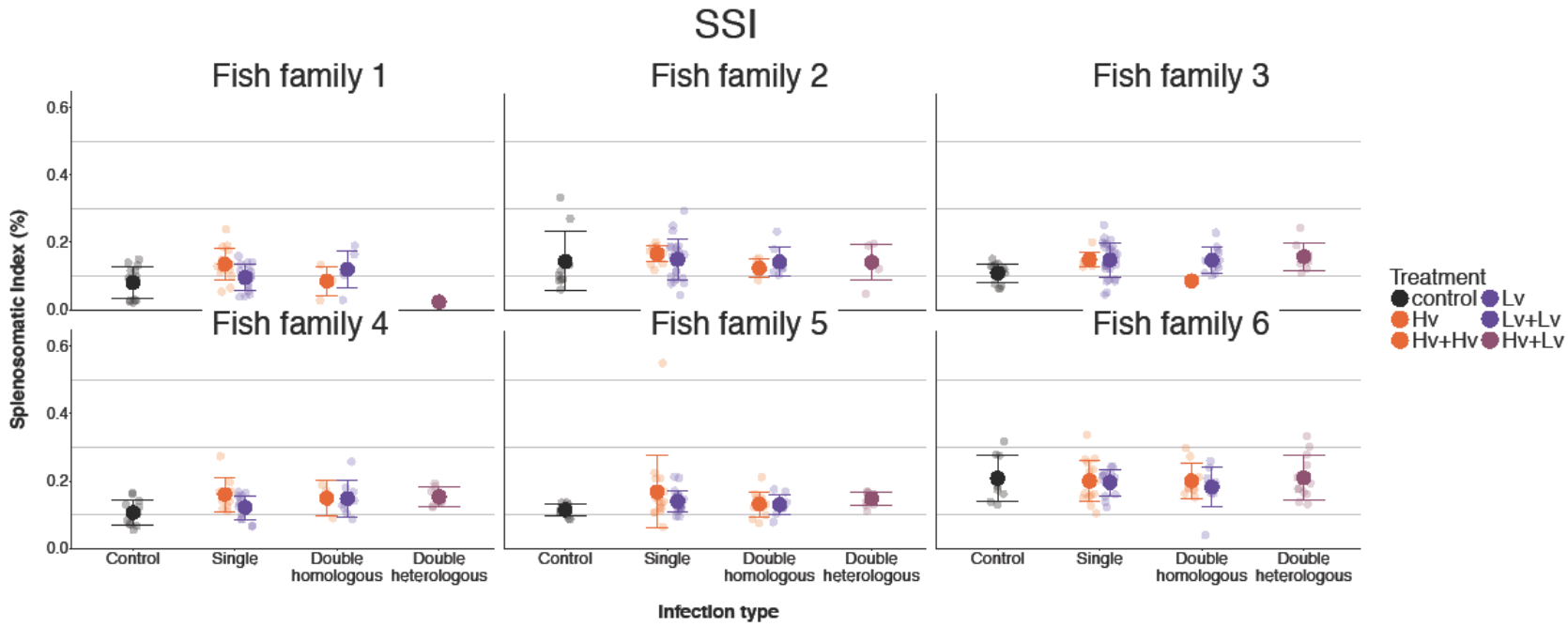
Supplementary figure SA2.3

The means condition factor (CF ±SD) in the different treatment groups and the six different fish families.



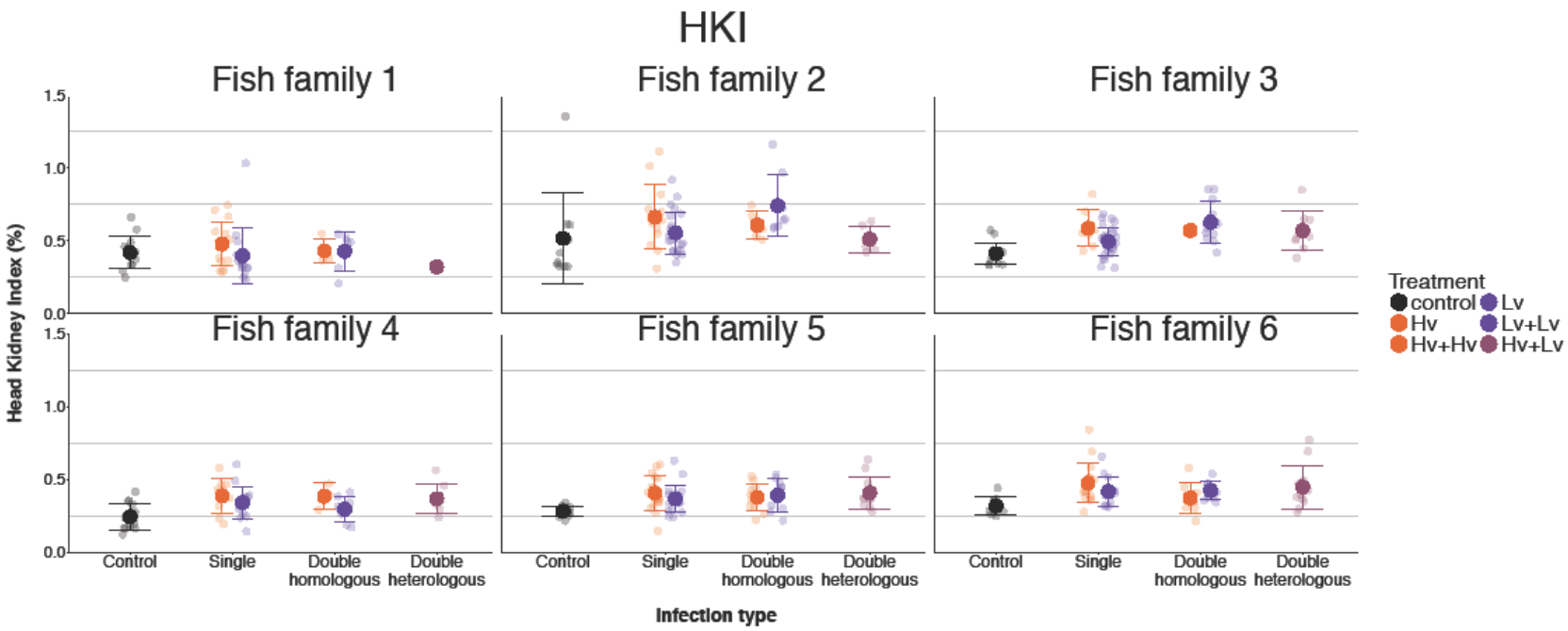
Supplementary figure SA2.4

The means hepatosomatic index (HSI ±SD) in the different treatment groups and the six different fish families.



Supplementary figure SA2.5

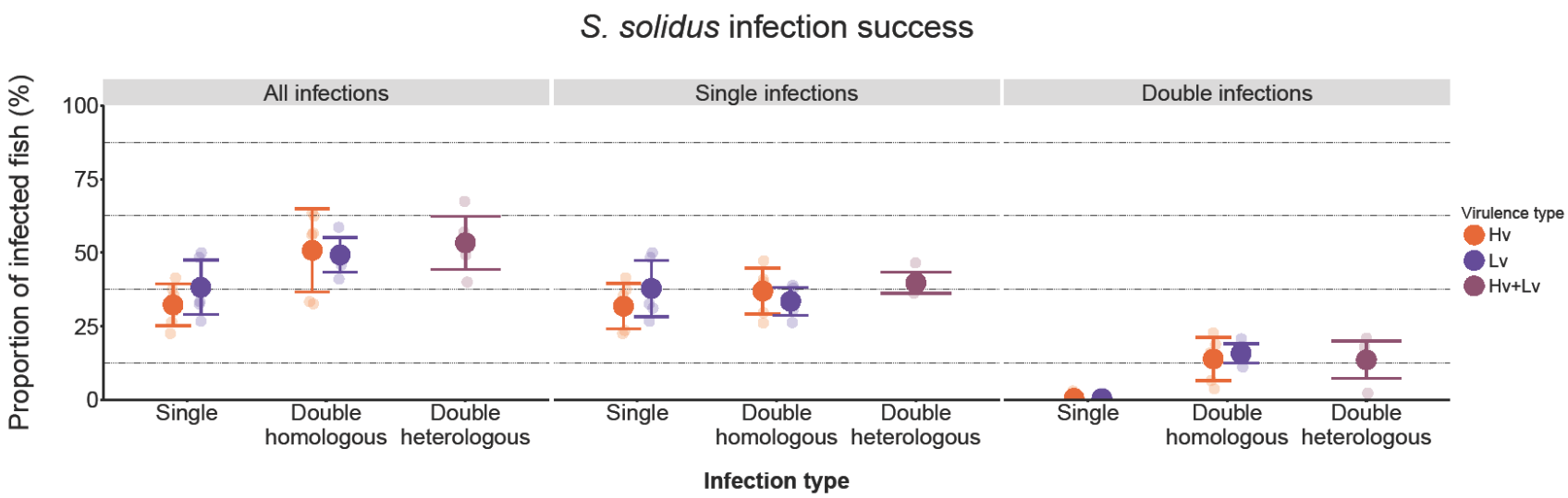
Mean splenosomatic index (SSI ±SD) in the different treatment groups and the six different fish families.



Supplementary figure SA2.6

Mean head kidney index (HKI ±SD) in the different treatment groups and the six different fish families.

Supplementary Analysis SA3: *Schistocephalus solidus* infection success



Supplementary figure SA3.1

Proportion of fish infected with *S. solidus* per fish family in the different treatment groups.

Supplementary table SA4.1

Sample sizes in the different treatment groups for the different worm family combinations

Experimental	*Trial*	*Worm family*	*Hv*	*Lv*	*Hv+Hv*	*Lv+Lv*	*Hv+Lv*
Hv	1	Hv1	18				
		Hv2	18				
		Hv3	-				
	2	Hv4	16				
		Hv5	13				
		Hv6	15				
Lv	1	Lv1		21			
		Lv2		15			
		Lv3		31			
	2	Lv4		22			
		Lv5		13			
		Lv6		11			
Hv+Hv	1	Hv1+Hv2			2		
		Hv2+Hv3			2		
		Hv3+Hv1			6		
	2	Hv4+Hv6			10		
		Hv5+Hv4			5		
		Hv6+Hv5			8		
Lv+Lv	1	Lv1+Lv2				4	
		Lv2+Lv3				10	
		Lv3+Lv1				12	
	2	Lv4+Lv6				9	
		Lv5+Lv4				12	
		Lv6+Lv5				6	
Hv+Lv	1	Hv1+Lv3					7
		Hv2+Lv1					-
		Hv3+Lv2					9
	2	Hv4+Lv4					11
		Hv5+Lv6					9
		Hv6+Lv5					11
	Total		***80***	***113***	***33***	***53***	***47***

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Declaration

Hereby I declare that

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

ii) this thesis has not already been submitted either partially or wholly as part of a doctoral degree to another examination body, and no other materials are published or submitted for publication than indicated in the thesis;

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

Kiel, 20 March 2017

Noémie I. Erin