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Host infection history modifies co-infection success of multiple parasite genotypes

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Summary

1. Co-infections by multiple parasite genotypes are common and have important implications for host–parasite ecology and evolution through within-host interactions. Typically, these infections take place sequentially, and therefore, the outcome of co-infection may be shaped by host immune responses triggered by previous infections. For example, in vertebrates, specific immune responses play a central role in protection against disease over the course of life, but co-infection research has mostly focused on previously uninfected individuals.

2. Here, we investigated whether sequential exposure and activation of host resistance in rainbow trout *Oncorhynchus mykiss* affects infection success and interactions between co-infecting parasite genotypes of the trematode eye-fluke *Diplostomum pseudospathaceum*.

3. In accordance with earlier results, we show that a simultaneous attack of two parasite genotypes facilitates parasite establishment in previously uninfected hosts. However, we find for the first time that this facilitation in co-infection is lost in hosts with prior infection.

4. We conclude that vertebrate host infection history can affect the direction of within-host–parasite interactions. Our results may have significant implications for the evolution of co-infections and parasite transmission strategies.

Key-words: acquired immunity, co-infection, competition, *Diplostomum pseudospathaceum*, facilitation, genotype, within-host interaction

Introduction

Individual hosts are often simultaneously infected with a range of genotypes or strains of the same parasite species (Read & Taylor 2001; Balmer & Tanner 2011). Such co-infections may result in intraspecific interactions between the genotypes and have profound ecological and evolutionary consequences on parasite–host interactions (e.g. de Roode *et al.* 2005; Mideo 2009; Lopez-Villavicencio *et al.* 2011; Susi *et al.* 2015). For example, both theoretical and empirical studies have shown that co-infections can underlie altered infection risk and disease dynamics, shape the structure of parasite communities and drive the evolution of virulence (e.g. vanBaalen & Sabelis 1995; May & Nowak 1995; Frank 1996; Poulin 2001; Lello *et al.* 2004; Bell *et al.* 2006; Telfer *et al.* 2010; Karvonen *et al.* 2012). Co-infections typically have negative effects for parasites due to direct competition over host resources, direct interference competition via attack or exclusion of one genotype by the other and/or indirect immune-mediated apparent competition, where one genotype elicits an immune response that affects its competitors (reviewed in Read & Taylor 2001). However, as the genetic heterogeneity of infection increases, the challenge for the host immune system becomes also more complex. Thus, co-infection is expected to reduce the effectiveness of the host immune defence and increase the demand on resources (Jokela, Schmid-Hempel & Rigby 2000). If this leads to a higher *per capita* infection success among the genotypes, co-infection can also be beneficial for parasites. In some systems, this can be further shaped by kin selection, favouring cooperation between co-infecting genotypes based on the level of their relatedness (Griffin, West & Buckling 2004; Buckling & Brockhurst 2008). Empirical studies have indeed shown that mixed genotype infections can increase parasite success through increased infectivity (Taylor, Walliker & Read 1997; Ganz & Ebert 2010; Karvonen *et al.* 2012), virulence (Taylor, Mackinnon & Read 1998; Davies, Fairbrother & Webster 2002; Hodgson *et al.* 2004) and increased transmission success (Susi *et al.* 2015).

In the wild, most host individuals remain unexposed to parasites for only a short time relative to their lifespan and are subsequently repeatedly infected by multiple parasite species. Among vertebrate hosts, that typically acquire at least partial immunity after the first contact

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with a parasite, such sequential exposure has been shown to affect community dynamics of co-infecting parasite species. For example, competitive interactions between two parasite species may depend on the sequence of host invasion if infection by one species causes cross-reactive immunity to another, but not vice versa, as findings of a recent study on trematode parasites infecting amphibian hosts suggest (Hoverman, Hoye & Johnson 2013). Another study on co-infecting trematode parasites of fresh water fish showed that a prior infection with one of the species eroded positive associations between the species in a subsequent experimental exposure (Karvonen, Seppälä & Valtonen 2009). Thus, by altering interspecific parasite interactions, immune activation may strongly affect the pathology and infection success of co-infecting parasite species.

Similar immune-mediated effects could also be expected in sequentially infecting genotypes of one parasite species, particularly due to an increased likelihood of cross-reactive immunity. However, genotype interactions in parasites infecting vertebrates have so far mainly been studied in previously unexposed individuals (Rauch, Kalbe & Reusch 2008; Balmer *et al*. 2009; Vardo-Zalik & Schall 2009; Karvonen *et al*. 2012). A single study on competitively interacting malaria clones found no alteration of clone interactions due to immunization of the host (Grech *et al*. 2008). Here, we study the infection success of single and co-infecting parasite genotypes in naïve and previously infected hosts using the trematode eye-fluke *Diplostomum pseudospathaceum* and one of its intermediate hosts, the rainbow trout *Oncorhynchus mykiss*. *Diplostomum pseudospathaceum* is a widespread freshwater parasite that has a complex, three-host life cycle (reviewed in Chappell, Hardie & Secombes 1994). Its definitive host is a piscivorous bird that releases sexually produced parasite eggs (each carrying one unique genotype) via faeces to aquatic systems. There, eggs hatch into free-living miracidia and infect their first intermediate host, the freshwater snail *Lymnaea stagnalis*. A single snail can be infected with one or several parasite genotypes (Rauch, Kalbe & Reusch 2005; Louhi *et al*. 2013b) and co-infecting genotypes face competition for resources within the snail (Karvonen *et al*. 2012). Asexual reproduction within the snail results in the release of large numbers of clonal free-living cercariae that infect the second intermediate host, a freshwater fish. As cercarial shedding takes place during the whole summer, fish become repeatedly exposed during the season (Karvonen, Seppälä & Valtonen 2004b, 2009; Karvonen, Halonen & Seppälä 2010). After penetrating the fish, cercariae migrate to its eye lenses. The lens, which lacks blood circulation, is an immunologically privileged site, and thus, the parasite is only vulnerable to the host immune defence during migration, which occurs within 24 h from exposure (Chappell, Hardie & Secombes 1994). Earlier work has shown that fish hosts acquire partial immunity to *D. pseudospathaceum* after the first exposure: they produce specific antibodies against the parasite (Whyte *et al*. 1987) and infection success at re-exposure is significantly reduced (Karvonen *et al*. 2005; Karvonen, Seppälä & Valtonen 2009; Karvonen, Halonen & Seppälä 2010). This acquired immune response is cross-reactive among *D. pseudospathaceum* genotypes, that is it provides protection at re-exposure with other genotypes (Rellstab *et al*. 2013). Once established in the lens, the parasites develop to metacercariae that are long-lived and induce cataracts. Upon predation of an infected fish, the definitive host completes the life cycle.

Recent research on this particular trematode–fish system (*D. pseudospathaceum* and *O. mykiss*) demonstrated an increased infection success when two genotypes attack simultaneously compared to single-genotype attacks in immunologically naïve hosts (Karvonen *et al*. 2012). This was evident both in trials using transmission stages originating from naturally double-infected snails (including effects of within-snail competition between the genotypes) as well as in those using experimental mixtures of two genotypes originating from single-infected snails (excluding effects of within-snail competition). Interestingly, among artificial mixtures of two parasite genotypes in different proportions, infection success in the previously unexposed fish increased as the proportions became more even (Karvonen *et al*. 2012). This suggests a decrease in the efficiency of host innate immune defence as the genetic diversity of the infection increases. Here, we were interested in exploring how a prior infection and activation of the host specific immune system shapes the infection success of co-infecting parasite genotypes. We followed the approach of Karvonen *et al*. (2012) and exposed naïve and previously infected, immune activated fish to (i) single parasite genotypes, (ii) two genotypes originating from double-infected snails or (iii) artificial mixtures of two genotypes originating from two single-infected snails. We predicted an increased infection success of co-infecting genotypes compared to single genotypes in previously unexposed fish as in Karvonen *et al*. (2012), but significant alterations of this interaction in fish previously exposed to the same parasite. We observed that a prior infection of the fish host eroded the benefit of a synchronous attack by co-infecting parasite genotypes. This may have significant implications for the outcomes of parasite co-infections and the evolution of transmission strategies.

Materials and methods

PARASITE COLLECTION

Lymnaea stagnalis snails were collected in June 2014 from Lake Vuojärvi (Central Finland, 62°N, 25°E) and transferred to the laboratory. Infection with *D. pseudospathaceum* was verified by placing snails individually in small containers with lake water and following potential cercarial production for 12 h. To determine the number of parasite genotypes infecting each snail, 15 cercariae were haphazardly collected from each container and

genotyped individually with four highly polymorphic microsatellite markers (Diplo06, Diplo09, Diplo23 and Diplo29; Reusch, Rauch & Kalbe 2004). The protocol for genotyping and separating single from double-genotype-infected snails has been earlier described in detail (Karvonen *et al.* 2012; Louhi *et al.* 2013a,b). We found that one-third of the infected snails harboured two genotypes and among those, the rarer genotype represented on average 24·4% of the cercarial output (range 6·7–37·5%). It is important to note that each genotype is unique and can only infect a single snail. Further, this parasite shows no detectable population genetic structure in the snail hosts due to high levels of gene flow, which is facilitated by infected avian definitive hosts moving over a large geographical scale (Louhi *et al.* 2010). After sampling, infected snails were kept at 4 °C in individual containers with 1 L of lake water and lettuce *ad libitum* for 5 weeks until the challenge infections. The snails were brought to room temperature once a week for 4–6 h to sustain cercarial production.

FIRST INFECTION (ACTIVATION OF HOST RESISTANCE)

Six snails were placed individually in 200 mL of lake water after collection and were allowed to produce cercariae for 4 h, after which the suspensions from the snails were combined. Cercariae were not genotyped for this phase, and therefore, the mixture contained a minimum of six unique genotypes of *D. pseudospathaceum*. Cercarial density was estimated from ten 1 mL samples of the mixture by counting the number of cercariae in each sample using a microscope. Juvenile rainbow trout (average weight 3·5 g) were obtained from a fish farm using ground water for maintenance, which ensured that they had not been previously exposed to *D. pseudospathaceum* or any other parasite. The fish were randomly distributed among 6 tanks, each holding 200 fish in 72 L of ground water (17 °C). The fish in three randomly chosen tanks were exposed to an estimated number of 2000 cercariae each (10 cercariae per fish). The fish in the other three tanks were sham exposed with ground water (see Fig. S1, Supporting information). The exposure lasted 30 min, after which the water volume was brought to 500 L. The fish were kept in these conditions for 5 weeks, which is sufficient time for the development of induced host responses (Rellstab *et al.* 2013). During the maintenance, fish were fed daily with commercial fish pellets.

All exposed fish became infected and harboured an average of 10·4 ± 0·2 (SE) metacercariae (sum for right and left eye lens 5 weeks after exposure; see below). Host infection history had no effect on fish growth, as length of the infected and naïve fish did not differ 5 weeks after exposure (GLM, $F_{1,832} = 0{\cdot}42$, $P = 0{\cdot}517$). Additionally, the condition of the fish (residuals from regression of length and mass) did not differ between the groups ($F_{1,832} = 1{\cdot}15$, $P = 0{\cdot}284$).

SECOND INFECTION (CHALLENGE)

After 5 weeks, 10 single-genotype-infected and seven double-genotype-infected snails were transferred to room temperature, placed individually in 200 mL of lake water and allowed to produce cercariae for 5 h. Ten 1 mL samples were then taken from each container to estimate cercarial density. Experimental exposure of fish followed the protocol in Karvonen *et al.* (2012). From the three replicate tanks of previously infected and naïve fish, a total of 420 fish were each haphazardly taken and individually exposed to either (i) parasites originating from single-infected snails (single-genotype exposure, 10 parasite genotypes from 10 snails, 10 replicate fish each), (ii) parasites originating from double-infected snails (exposure to two genotypes inhabiting the same snail, 7 snails, 10 replicate fish each) or (iii) artificial mixtures of parasites originating from two single-infected snails (exposure to two genotypes inhabiting two different snails, 10 genotypes from 10 snails in 5 randomly assigned pairs). See Fig. S1 (Supporting information) for a schematic draft of the experimental design. The exposure dose was 100 cercariae per fish. Artificial mixtures were prepared by combining the two genotypes within a pair at five different proportions: 10:90, 25:75, 50:50, 75:25 and 90:10 and 10 replicate fish were used for each proportion of a pair. The same 10 parasite genotypes were used for single-genotype infections (i) and for the 5 genotype pairs in artificial combinations (iii). Exposure took place in containers with 500 mL of ground water (17 °C) for 30 min. Afterwards, each group of 10 replicate fish were kept in mesh cages (35 × 35 × 35 cm) randomly distributed among eight 500-L tanks (17 °C) for 48 h to allow parasite establishment in the eye lenses. Subsequently, all fish were euthanized with an overdose of MS-222 anaesthetic, weighed (mean ± SE = 5·7 ± 0·1 g), their length measured (84·3 ± 0·2 mm) and dissected to count the number of parasites established using a microscope. Metacercariae established during the first exposure and the re-exposure were differentiated by their clear size difference (Sweeting 1974).

STATISTICS

All statistical tests were conducted using SAS v. 9.3 (SAS Institute, Cary, NC, USA). Ten fish (three naïve and seven previously infected) died during the experiment and were excluded from the analysis. Infection success (sum of parasites established in the left and right lens) was analysed using generalized linear mixed models (GLMMs) with negative binomial error structure and log link. *P*-values in pairwise comparisons of least-square means were adjusted using the *Bonferroni* correction. First, infection success was compared between single-genotype exposures (i) and exposures to two genotypes from double-infected snails (ii) among fish with different infection history, that is previously infected or naïve fish.

Fish length was added as covariate and snail ID ($N = 17$) was included as random factor. Secondly, infection success was compared among the different proportions of artificial mixtures of two genotypes originating from single-infected snails (iii), including infection success of these genotypes at single-genotype exposures (i), in both previously infected and naïve fish. For this, all combinations containing equal proportions (e.g. 10:90 and 90:10) were folded up, such that we had four proportions in the analysis (100:0, 90:10, 75:25 and 50:50). To account for this, we included a random factor that labelled all 10 replicate fish exposed to the same genotype pair at the same proportion (10:90, 25:75, 50:50, 75:25 or 90:10) with an individual ID ($N = 34$). This ID was nested within genotype pair ($N = 5$). Fish length was added as covariate.

Results

SINGLE- VS. DOUBLE-GENOTYPE INFECTIONS

Infection success of cercariae originating from both single- and double-genotype-infected snails was significantly affected by host infection history ($F_{1,305} = 4{\cdot}15$, $P = 0{\cdot}042$) as fewer parasites established in previously infected fish compared to naïve fish (Fig. 1). Infection success also tended to be higher for parasites originating from snails with double-genotype infections compared to single-genotype infections (Fig. 1), but this difference was not significant at the five per cent level ($F_{1,15} = 3{\cdot}73$, $P = 0{\cdot}073$). Considering fish with different infection history separately, co-infecting parasites increased their infection success by 43·6% compared to single genotypes when attacking naïve hosts ($t_{17{\cdot}7} = 2{\cdot}33$, $P = 0{\cdot}021$), but only by 20·1% when attacking previously infected hosts ($t_{18{\cdot}1} = 1{\cdot}35$, $P = 0{\cdot}179$). However, the interaction between the number of genotypes and host infection history was not significant in the full model ($F_{1,305} = 2{\cdot}45$, $P = 0{\cdot}119$). Fish length had no effect on infection success ($F_{1,319} = 0{\cdot}02$, $P = 0{\cdot}896$).

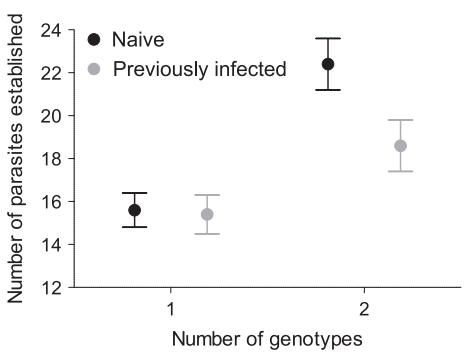

Fig. 1. Mean number of parasites established (± SE, left and right eye combined) in naïve and previously infected fish in single-genotype infections (10 genotypes from 10 naturally single-infected snail hosts) and double-genotype infections (14 genotypes from 7 naturally double-infected snail hosts). Infection success was significantly reduced in previously infected fish compared to naïve fish.

GENOTYPE PROPORTIONS

Infection success of cercariae originating from single-genotype-infected snails that were combined in artificial mixtures was significantly affected by the interaction of host infection history and genotype proportion (Table 1). *Post hoc* pairwise comparisons showed that while infection success increased with the evenness in the proportion of genotypes infecting naïve fish (100:0 vs. 50:50; $t_{40} = -2{\cdot}00$, $P = 0{\cdot}046$; Fig. 2), there were no significant differences between genotype proportions within previously infected fish (all $P > 0{\cdot}405$). Importantly, infection success at genotype proportion 50:50 was highest among naïve fish and lowest within the previously infected fish ($t_{640} = 3{\cdot}79$, $P < 0{\cdot}001$).

Discussion

Mixed genotype infections are widespread in nature and may lead to within-host interactions between co-infecting genotypes. Although a number of theoretical and empirical studies have examined such interactions, most research has focused on competitive relationships (reviewed in Mideo 2009). However, the degree of competition may depend on within-host resource availability and need. For example, some intermediate hosts in complex parasite life cycles may not be intensively exploited for reproduction, but primarily used as transmission vehicles, increasing the opportunity for parasite facilitation. Further, relatedness between competing genotypes is expected to reduce competition (Buckling & Brockhurst 2008). Finally, fitness costs paid by competition can be reduced or even negated by impairment of the host immune system (Jokela, Schmid-Hempel & Rigby 2000). Here, we show that a simultaneous attack of two genotypes facilitates parasite establishment in previously uninfected hosts, which is in accordance with earlier results (Karvonen *et al.* 2012). This is unlikely to be explained by genotype relatedness as it is generally low in this parasite (Louhi *et al.* 2010) and because relatedness has been shown not to correlate with parasite co-infection success in this system (Karvonen

Table 1. General liner mixed model (GLMM) analyses of infection success of *D. pseudospathaceum* genotypes explained by host infection history (I, naïve vs. previously infected), genotype proportion (P, 100:0, 90:10, 75:25 and 50:50), their interaction and fish length. A factor accounting for folding up the same proportions within parasite genotype pairs (see Methods for details) nested within genotype pair is included in the model as random factor

Factors	d.f. denominator	d.f. numerator	*F*	*P*
I	1	648	14·30	0·002
P	3	30	0·64	0·597
I*P	3	650	2·95	0·032
Fish length	1	667	0·95	0·331

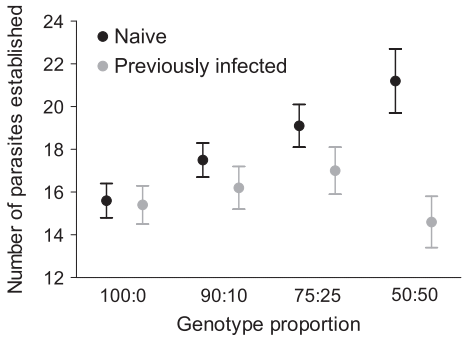

Fig. 2. Mean number of parasites established (± SE, left and right eye combined) in naïve and previously infected fish in single-genotype infections (100:0, 10 genotypes) and in artificial co-infection mixtures of the single genotypes (5 randomly assigned pairs). Infection success increased significantly with evenness of proportion within naïve fish and differed significantly between naïve and previously infected fish at parasite genotype proportion 50:50. Note that the data in the genotype proportion 100:0 are the same as the data in single-genotype infections in Fig. 1.

et al. 2012). Instead, parasite infection success increased with evenness in proportion of two genotypes, re-enforcing the hypothesis that host immune efficiency is reduced as the diversity of an attack increases.

However, hosts are not uniform stable entities, and therefore, the interactions between co-infecting parasites may be context dependent. Earlier research has shown that the outcome and direction of within-host interactions can depend on host ecology (Hodgson *et al*. 2004) and host genotype (de Roode *et al*. 2004; Susi *et al*. 2015). Here, we demonstrate that within-host interactions between parasite genotypes co-infecting a vertebrate host can depend on infection history: when the fish host had been previously infected by the same parasite species, the advantageous effect of co-attack was lost and infection success did not differ between single- and double-genotype infections. In general, the immune system of vertebrates can respond in two ways against a parasitic infection depending on previous exposure history. When a pathogen invades a host for the first time, an innate immune response is immediately elicited, which is typically non-specific (Murphy 2011). Acquired immune responses are, on the other hand, activated more slowly, but often create immunological memory, which allows a fast and efficient response at recurring infections of the same parasite (Murphy 2011). In the present case, the exact mechanism is not clear, but it appears that previously infected rainbow trout, which typically activate an acquired immune response against this parasite (Whyte *et al*. 1987), are not only more efficient in tackling a re-infection (Karvonen *et al*. 2005), but can also handle a genetically heterogeneous attack as well as a homogeneous attack. Our results are in line with previous work on plants, which showed that host responses to prior infections affect the direction of within-host interactions between fungal pathogen strains (Laine 2011). Other immune-mediated effects on within-host interactions have been shown for the rodent malaria parasite *Plasmodium chabaudi*, where mice host immune responses cause competitive suppression of avirulent genotypes by virulent genotypes (immune-mediated apparent competition, Råberg *et al*. 2006).

The reduction in the facilitation effect observed here can be interpreted as host-mediated, but also as a result of within-host apparent competition. The two simultaneously attacking *D. pseudospathaceum* parasites do not only interact with each other, but also indirectly with those that infected the fish host during the primary infection. Concomitant immunity, a form of apparent competition, suggests that the presence of a parasite prevents or reduces the establishment of newly invading parasites of the same species through the induction of an immune response that is not harming itself (Smithers & Terry 1967). Transplantation studies with adult schistosomes support the hypothesis of concomitant immunity, demonstrating a change in the immunological environment of the new host which provides resistance to subsequent infection by larval stages that have a different antigenic surface than adult worms (Smithers & Terry 1967). Similar mechanisms are possible also in our system. On entering the fish host, *D. pseudospathaceum* elicits an immune response effective at reinfections with other genotypes several weeks later (Rellstab *et al*. 2013), but escapes the immune system after settling in the eye lens, where it cannot be cleared (Chappell, Hardie & Secombes 1994). However, several aspects of the transmission biology of *D. pseudospathaceum* actually do not support concomitant immunity as parasite strategy. First, intraspecific competition within the fish eye lens is likely to be negligible, as the magnitude of host exploitation is relatively low compared to the previous snail host, and there is sufficient space for hundreds of metacercariae (Karvonen *et al*. 2012). Secondly, parasites may actually benefit from accumulating in host eye lenses as the intensity of the deleterious effects of the infection, eye cataracts, increases with parasite burden (Karvonen, Seppälä & Valtonen 2004a). High cataract intensity increases the susceptibility of the fish host to avian predators (Seppälä, Karvonen & Valtonen 2005) and therefore, the probability of transmission to the final host. Thirdly, co-infection in the fish host reduces the risk of parasite inbreeding during sexual reproduction in the final host if multiple parasite genotypes are transmitted as packages at the same time from one fish (Rauch, Kalbe & Reusch 2005). Thus, we suggest that the reduction in facilitation in previously infected hosts is more likely to be a host adaptive than a parasite-adaptive strategy.

Independent of the exact mechanisms, our results may have significant implications for parasite transmission strategies. Karvonen *et al*. (2012) suggested that co-infection of *D. pseudospathaceum* should be avoided in the first intermediate host (snail) due to within-host resource competition, but preferred in the second intermediate host (fish) due to facilitation of establishment success. Such an optimal infection strategy is possible, because snails typically occur locally concentrated and release cercariae

synchronously to the water column, where they can simultaneously attack their mobile fish hosts. The present results indicate that parasites would gain the highest benefit from simultaneous attack on immunologically naïve hosts. It is not known whether *D. pseudospathaceum* possesses recognition mechanisms for active host selection that would allow them to assess the infection history of their fish host. However, as recently hatched fish juveniles typically are unexposed, the parasite may benefit from focusing its effort in clonal production to the beginning of the summer, when cercarial release commences and hosts have not yet developed acquired immunity.

In summary, we show that prior infection of the host significantly alters the interaction between simultaneously co-infecting genotypes. The suggested immune-induced change of within-host interactions may affect parasite transmission strategies and select, for example for changes in seasonal efforts. Further, within-host interactions are expected to affect selective pressures on parasite traits (Read & Taylor 2001; Alizon, de Roode & Michalakis 2013), and thus, a change in the direction of the interaction may have important evolutionary consequences. For example, if there was a positive relationship between parasite competitive ability and virulence, within-host competition should generally select for higher levels of virulence (vanBaalen & Sabelis 1995; Frank 1996; Mosquera & Adler 1998). A recent epidemiological model on virulence evolution under co-infection confirms this prediction, but also shows that facilitation as a result of immune system impairment can select for decreased virulence at the parasite population level (Choisy & de Roode 2010). By modifying host responses and parasite–parasite interactions, host infection history could therefore potentially change these outcomes. To date, most theoretical models of within-host interactions have not considered effects of host immune responses (but see Alizon & van Baalen 2008; Choisy & de Roode 2010) and empirical studies on parasite co-infection have mainly focused on immunologically naïve hosts (but see Grech *et al*. 2008; Laine 2011). As immunological naïvety is a rare status of hosts in any system, our results suggest that sequential activation of host responses should be considered in theoretical and empirical studies on within-host–parasite interactions.

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Data accessibility

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.1264j (Klemme, Louhi & Karvonen 2015).

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. Schematic overview of experimental setup.