Interactions among co-infecting parasite species: a mechanism maintaining genetic variation in parasites?

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Individuals of free-living organisms are often infected simultaneously by a community of parasites. If the co-infecting parasites interact, then this can add significantly to the diversity of host genotype × parasite genotype interactions. However, interactions between parasite species are usually not examined considering potential variation in interactions between different strain combinations of co-infecting parasites. Here, we examined the importance of interactions between strains of fish eye flukes Diplostomum spathaceum and Diplostomum gasterostei on their infectivity in naive fish hosts. We assessed the infection success of strains of both species in single-strain exposures and in co-exposures with a random strain of the other species. Parasite infection success did not consistently increase or decrease in the co-exposure treatment, but depended on the combinations of co-infecting parasite strains. This disrupted the relative infectivity of D. spathaceum strains observed in single-strain exposures. The infection success of D. gasterostei strains was independent of exposure type. These results suggest that interactions among parasite species may be strain specific and potentially promote maintenance of genetic polymorphism in parasite populations.

Keywords: concomitant infections; mixed infections; multiple infections; Diplostomum spathaceum; Diplostomum gasterostei; Trematoda

1. INTRODUCTION

Mechanisms that maintain genetic polymorphism of parasites are central for understanding epidemiology, virulence (disease severity) and evolution of host range. Often both host resistance and parasite infectivity are genetically polymorphic, and specific host genotype × parasite genotype interactions determine parasite infection success (e.g. Lively 1989; Carius et al. 2001) and virulence (Grech et al. 2006). However, the ‘host environment’ that the invading parasite needs to cope with may be affected not only by the host genotype, but also by the composition of the co-infecting parasite community (Cattadori et al. 2007). This is because individuals of free-living organisms are typically infected simultaneously by several parasite species (e.g. Holmes & Price 1986; Petney & Andrews 1998; Valtonen et al. 2001; Lello et al. 2004), which are frequently involved in interspecific interactions potentially affecting their fitness (reviewed by Christensen et al. 1987; Poulin 2001). For example, interacting parasites can directly compete for common host resources during invasion and establishment (e.g. Patrick 1991), and/or affect each other through cross-responsive host immune defences (e.g. Adams et al. 1989), thus reducing parasite success. On the other hand, parasite fitness may be higher in co-infections if increased heterogeneity of infection requires more host resources for immune system response, therefore leading to less effective host defences (Taylor et al. 1998; Jokela et al. 2000).

Most of the theory and empirical work on co-infections has focused on species specificity of interactions between parasites, while the possibility for strain-specific responses among co-infecting species has received less attention. To our knowledge, interspecific interactions between parasites have been studied at a genotype level only in diseases observed in co-infections with human immunodeficiency virus (HIV). For example, the genotype distribution of hepatitis C has been shown to differ between HIV+ and HIV− patients (Capa et al. 2007), but this does not apply to all other diseases (e.g. Perez-Ramirez et al. 1999). Examining strain-specific interactions between parasite species can be challenging, partly because ecologically different species are involved, and partly because empirical study systems rarely allow experiments addressing such questions. However, if the relative fitness of a parasite depends on its interactions with other parasites in a strain-specific manner, then this may affect parasite evolution and maintenance of genetic polymorphism in parasite traits. This is because each host individual is unlikely to carry a similar community of parasites, which could promote stochastic variation in the fitness of the parasite genotypes in the population. Such effects could lead to selection favouring different alleles in the parasite population depending on the composition of the parasite communities within individual hosts. Unfortunately, it is not known how common such interactions are, and how large effects they may have on parasite fitness. In this study, we asked whether parasitic eye flukes of fish, Diplostomum spathaceum and Diplostomum gasterostei, interact when co-infecting the same host individual. We specifically asked whether these interactions are specific to different combinations of interacting parasite strains, and...
whether parasite species differ in the importance of such effects on their fitness. The parasite species we chose for the experiment are common in several freshwater fishes, and co-infections are the rule in nature (Valtonen & Gibson 1997). To address the above questions, we experimentally exposed naïve rainbow trout (Oncorhyncus mykiss) to single-strain infections and to mixed-species infections using co-exposures of random combinations of single parasite strains. We measured the infection success of each strain in both exposure treatments to examine the effect of co-infection on parasites. We found that the infection success of D. spathaceum was affected by the co-exposure treatment, and that the interactions were specific to different combinations of interacting parasite strains. This disrupted the relative infectivity of D. spathaceum strains observed in single-strain exposures. The infection success of D. gasterostei strains was unaltered by co-exposure treatment.

2. MATERIAL AND METHODS
(a) Study organisms
Both D. spathaceum and D. gasterostei have three-host life cycles (see Williams 1966; Chappell et al. 1994). Worms mature in the intestine of fish-eating birds, and their eggs are released with birds’ faeces. When reaching water, eggs hatch into free-swimming miracidia larvae. Miracidia of D. spathaceum infect mainly Lymnaea stagnalis snails (Chappell et al. 1994) whereas D. gasterostei infects mainly Radix balthica and Myxas glutinosa snails (Karvonen et al. 2006). Miracidia penetrate into snail gonads where they develop into sporocysts, which multiply asexually and produce thousands of free-swimming cercariae larvae. Cercariae leave the snail and seek the fish host. Because multiplication of Diplostomum parasites in the snail host is asexual, all cercariae originating from a single miracidia infection are genetically identical.

Cercariae of both parasite species infect fish by penetrating the gills and skin, and migrate to the eyes where they develop into metacercariae. Metacercariae of D. spathaceum locate themselves in the lenses (Chappell et al. 1994) whereas D. gasterostei locates itself in the vitreous body (Williams 1966; Karvonen et al. 2006). For successful transmission back to the avian definitive host, an infected fish has to be eaten by a piscivorous bird. Both parasite species are commonly observed in several freshwater fish species, and individual fish can carry tens, or even hundreds of Diplostomum metacercariae (Valtonen & Gibson 1997; Mar cogliese et al. 2001). Furthermore, multiple-species and multiple-genotype infections are common in nature (Valtonen & Gibson 1997; Rauch et al. 2005).

To investigate the infection success of parasite strains in single-species exposures and in mixed-species co-exposures, we collected infected L. stagnalis and M. glutinosa snails from Lake Konnevesi (62°37’ N, 26°21’ E) in Central Finland. Parasite species produced by different snail hosts were identified according to the behaviour and morphology of the cercariae (see Karvonen et al. 2006). In the experiment and the analyses, we considered that each L. stagnalis snail produced a strain of cercariae of D. spathaceum, and each M. glutinosa snail produced a strain of cercariae of D. gasterostei. Thus, cercariae expelled from different snail individuals were considered to represent genetically distinct random parasite strains, which is likely because the miracidia that infect snails are produced sexually. It is possible, however, that some snails harboured more than one parasite genotype that was shedding cercaria. In our system, the probability of multiple-genotype infection within a snail host is linearly related to the abundance of parasite transmission stages in the environment, a crude proxy of which is the prevalence of infection in the population (Louhi et al. 2008, personal observations). In other words, in populations where prevalence of infection is low, the frequency of multiple-genotype infections is also low. In the present work, the prevalence of infection in L. stagnalis was 11.3 per cent and in M. glutinosa 14.0 per cent (prevalence of infection can go up to 50–70% in natural populations). Thus, based on these prevalence estimates, the probability of multiple-genotype infections was relatively low, one-fifth to one-quarter of the snails being likely to carry more than one parasite genotype (Louhi et al. 2008, personal observations). Furthermore, in the cases of multiple-genotype infections, the majority of cercariae are typically produced by a single parasite genotype (Rauch et al. 2005). Therefore, we believe that the possible ‘noise’ in the data caused by multiple-genotype infections is likely to be small and it would lead to a conservative error. If multiple infections were a rule in our experiment, then the chances of finding differences among the cercariae shed from different snail individuals would be reduced because among-strain variance would be confounded with within-strain variance.

We used juvenile (0 + year old) rainbow trout (O. mykiss) as a fish host in the experiment. We chose to use immature fish to reduce potential effects of host sex on the susceptibility of fish to infections. We obtained the fish from a commercial fish farm where they had been reared in indoor tanks supplied with ground water. Thus, the fish had no previous experience of eye flukes or other helminth parasites, and they were thus able to use only innate immunity to resist infections (see Manning 1994; Magnadottir 2006). In other words, we examined the interactions between parasite strains in the absence of recent coevolutionary background between the host and the parasite strains.

(b) Experimental design
We randomly divided infected snails into seven pairs, each with one L. stagnalis producing cercaria of D. spathaceum and one M. glutinosa producing cercariae of D. gasterostei. We placed the snails individually in glass jars containing 2 dl of water and allowed them to produce cercariae for 12 hours. We estimated the number of produced cercariae from ten 1 ml samples from each jar. We exposed randomly selected fish to cercariae by placing them individually into containers with 0.5 l of water and 100 cercariae for 20 min at 14.4°C. Using the cercaria produced by snails in each pair, we exposed each of 10 fish to 100 cercariae of D. spathaceum, each of 10 fish to 100 cercariae of D. gasterostei and each of 10 fish to 50 cercariae of both parasite species. After the exposure, we transferred the fish to 901 tanks for 12 days. We used a short maintenance period because the experiment was designed to examine interactions between parasites during invasion, and eye flukes successfully established in the eyes are known not to compete intensively (Rauch et al. 2006). We killed the fish with an overdose of 0.01 per cent MS 222 (Sigma Chemical Co., St Louis, MO, USA), measured the length (± 1 mm) and mass (± 0.1 g) of each fish, and counted the number of D. spathaceum and D. gasterostei parasites in fish eyes by dissecting the lenses and vitreous body separately. The average (± s.e.) body length and mass of the fish.
were $93 \pm 0.7$ mm and $7.5 \pm 0.2$ g, respectively. Of a total of 210 fish, 14 died during the maintenance period and were excluded from the data.

With this experiment, our goal was to record the relative infection success of strains of both parasite species in single-strain exposures and when interacting with a random strain of the other species. The design mimics the most likely situation of co-exposure under natural conditions as a certain parasite strain invades a fish either separately or simultaneously with one strain of the other species. Thus, our design reveals not only the possible interaction between *D. spathaceum* and *D. gasterostei*, but also shows if these interactions are specific to different combinations of interacting parasite strains. In other words, our experiment tests whether co-exposure disrupts the relative infectivity of parasite strains. Note that our goal was not to find how genetic identity of the co-infecting parasites affects the infection success of a particular parasite strain by competing each strain against several random strains of the other species. However, such an experiment would be interesting as it would examine the potential for genotype x genotype interactions between parasites (see Carius et al. 2001; Grech et al. 2006, e.g. about $G \times G$ interactions between host and parasite genotypes). We also did not include a treatment where several strains of a single species were co-infecting the same host individuals (see Rauch et al. 2008).

(c) Statistical analyses
We analysed the variation in parasite infection success using mixed-model analysis of variance (ANOVA). We recorded the infection success of each parasite strain as a proportion of parasite cercariae successfully infecting the fish (each fish was exposed either to 100 or 50 cercariae of a specific strain depending upon exposure treatment). First, we analysed the variation in infection success between parasite species and strains in single-strain exposures. In the analysis, we used parasite species (*D. spathaceum* and *D. gasterostei*) as a fixed, and parasite strain nested within species (seven strains per species) as a random factor. This analysis reveals general differences in infectivity of different parasite species and strains when not interacting with co-infecting parasites. In the second ANOVA, we examined the effect of co-exposure on parasite infection success separately for *D. spathaceum* and *D. gasterostei*. In the analyses, we used exposure type (single-strain exposure and mixed-species co-exposure) as a fixed, and parasite strain (seven strains) as a random factor. These analyses reveal both the general effect of heterogeneity of exposure on parasite infection success as well as strain specificity of interactions for both parasite species.

To examine whether the infectivity of parasite strains in single-species exposures significantly predicted their infection success in co-exposures with another species, we analysed the relationship between the infection success of the parasite strains in different exposure treatments. In these models, we used the mean infectivity (proportion of parasites successfully infecting the fish) of each strain in the mixed-species co-exposure as a dependent variable and the mean infectivity of the same strains in their respective single species exposures as an independent variable. Furthermore, we investigated whether the difference in infection success of parasite strains between exposure types was determined by the infectivity of co-infecting parasite strains using linear regressions. As above, we did the analyses separately for *D. spathaceum* and *D. gasterostei*. In these analyses, we used the difference in mean infection success between exposure treatments for each strain as a dependent variable and the mean infectivity of the co-infecting strains in single-strain exposures as an independent variable. In other words, we took the infectivity of parasite strains in single-species exposures to indicate their performance and asked whether that can explain their effect on infectivity of another species.

**Table 1.** Nested mixed-model ANOVA for the infection success of *Diplodostomum* eye flukes by parasite species (*D. spathaceum* and *D. gasterostei*) and parasite strain nested within species (seven strains per species).

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>parasite species (P)</td>
<td>1</td>
<td>3.922</td>
<td>13.851</td>
<td>0.003</td>
</tr>
<tr>
<td>strain [S(P)]</td>
<td>12</td>
<td>0.291</td>
<td>10.141</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>error</td>
<td>116</td>
<td>0.029</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a S(P) as the error term.*
Table 2. Mixed-model ANOVA for the infection success of *D. spathaceum* and *D. gasterostei* by exposure type (single-strain exposure and mixed-species co-exposure) and parasite strain (seven strains per species). ($\eta^2$ shows the proportion of total variance explained by each factor.)

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>MS</th>
<th>F</th>
<th>p</th>
<th>$\eta^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. spathaceum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>exposure type (T)</td>
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<td>0.023</td>
<td>0.090</td>
<td>0.774</td>
<td>0.002</td>
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<tr>
<td>strain (S)</td>
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<td>0.637</td>
<td>2.478</td>
<td>0.617</td>
<td>0.335</td>
</tr>
<tr>
<td>T×S</td>
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<td>0.257</td>
<td>5.009</td>
<td>&lt;0.001</td>
<td>0.135</td>
</tr>
<tr>
<td>error</td>
<td>117</td>
<td>0.051</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>D. gasterostei</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>exposure type (T)</td>
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<td>0.01</td>
<td>1.099</td>
<td>0.311</td>
<td>0.002</td>
</tr>
<tr>
<td>strain (S)</td>
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<td>56.407</td>
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<td>0.528</td>
</tr>
<tr>
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<td>0.009</td>
<td>0.396</td>
<td>0.88</td>
<td>0.009</td>
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<tr>
<td>error</td>
<td>117</td>
<td>0.023</td>
<td></td>
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</tbody>
</table>

*T×S as the error term.

3. RESULTS

Parasite infectivity in single-strain exposures varied between parasite species and parasite strains (figure 1; table 1), strains of *D. spathaceum* being on average more infective than strains of *D. gasterostei*. The mean infectivity of *D. spathaceum* and *D. gasterostei* strains did not differ significantly between exposure types (figure 1; table 2), indicating that the increased diversity of infection in co-exposures did not have a general effect on parasite infection success. Infectivity of *D. gasterostei* was strongly strain specific, strain identity explaining 52.8 per cent of the total variance in infection success (table 2). In *D. spathaceum*, strain identity explained 33.5 per cent of the total variance in infection success, and the strains also expressed a strong strain-specific interaction with the exposure type (table 2). In total, 13.5 per cent of the variance in infection success of *D. spathaceum* was explained by the interaction term between parasite strain and the type of exposure, while in *D. gasterostei*, only 0.9 per cent of the variance was explained by the same interaction (table 2). Thus, the interactions between parasites were specific to different combinations of interacting parasite strains and asymmetric between parasite species.

Infectivity of *D. spathaceum* strains in single-strain exposures did not explain their infection success in mixed-species co-exposures (figure 1a; linear regression: $R^2=0.200, F_{1,5}=1.253, p=0.314$), while in *D. gasterostei*, infectivity of parasite strains in single-strain exposures was a significant predictor of their infection success in mixed-species exposures (figure 1b; linear regression: $R^2=0.932, F_{1,5}=68.456, p<0.001$). This suggests that co-infection affects *D. spathaceum* strains more than *D. gasterostei* strains, and that the presence of competing parasite strains in the host may significantly alter the fitness rank of *D. spathaceum* strains.

The difference in infection success of parasite strains between exposure treatments was not affected by the infectivity of their interacting partner neither in *D. spathaceum* (linear regression: $R^2=0.015, F_{1,5}=0.074, p=0.796$) nor in *D. gasterostei* (linear regression: $R^2=0.001, F_{1,5}=0.068, p=0.804$), as could have been predicted if more virulent strains of one species were better in inhibiting the success of the other species.

4. DISCUSSION

Contrary to some earlier studies where co-infections with multiple parasite species were applied (e.g. Christensen et al. 1987; Adams et al. 1989; Patrick 1991; Poulin 2001; Lello et al. 2004), we did not find a general positive or negative effect of the species interaction on the infection success of either *D. spathaceum* or *D. gasterostei*. Instead, we found that the effects of co-exposure were specific to the interacting pairs of parasite strains. Moreover, interactions between parasites were asymmetric, co-exposures affecting the infection success of *D. spathaceum*, but not of *D. gasterostei* strains. Interestingly, some *D. spathaceum* strains gained higher infection success in co-exposures, while others performed more poorly. Thus, for *D. spathaceum*, a significant component of strain’s fitness may be determined by the presence of another parasite species. This type of variation in fitness-related parasite traits is usually not measured experimentally.

The potential for strain-specific interactions between co-infecting parasite species has received only little attention in earlier research, interactions between HIV and co-infecting diseases being at the forefront of such studies (e.g. Perez-Ramirez et al. 1999; Capa et al. 2007).

Earlier studies have focused mainly on the interactions between conspecific parasite strains, where competitive ability of parasite strains in multiple-genotype infections has been shown to be difficult to predict from their performance in single infections (Nakamura et al. 1992; Hodgson et al. 2004). Findings reported in those studies are qualitatively similar to our results.

Our results suggest that the outcome of co-infections may have a strain-specific component that is difficult to predict. If such effects are common, then the fitness of a focal parasite genotype is partly determined by complex parasite genotype×host genotype×environment (here, parasite community) interactions (G×G×E). Therefore, interactions between parasite species could promote maintenance of genetic variation in interacting parasite populations, as different alleles of parasites’ genes may be favoured depending on the composition of the local parasite community within a host. Thus, selection gradients under which parasite infectivity evolves may be very complex as the ‘environmental template’ on which the population of parasite strains are tested contains not only the diversity of the host population (Lively 1989; Carius et al. 2001; Grech et al. 2006), but also the diversity of the populations of the other parasites. Such interactions can become very complex and difficult to predict especially when the number of interacting
parasite species and strains increases (Jackson et al. 2006). This calls for developing models of host–parasite evolution in a community genetic framework (Antonovics 1992). Interestingly, in our study, we found that for the strains of D. spathaceum, the interaction with the co-infecting parasite species significantly affected the infection success, but the same was not true for D. gasterostei. This suggests that the importance of interspecific interactions on parasite evolution may vary between parasite species. Furthermore, co-infection can be seen as an environmental effect with a potential genetic component. This is the case if the response of a certain parasite strain to co-infection depends on the genetic identity of a co-infecting parasite strain. In this study, however, this remains unclear because we did not test the infection success of each parasite strain against several strains of the other species. Much more research on the generality and importance of strain-specific species interactions is needed before we can fully understand the concepts affected by these findings.

In studies examining co-infections, potential dose effects need to be considered as it is inevitable that either the total number of parasites or the relative dose of interacting partners differs between exposure types (latter is true in this study). Dose effects may have a major role as the results of mixed infections may simply represent changes in the number of parasites host are exposed to (Taylor et al. 1997). More specifically, if infectivity of parasites is dose dependent, then the altered infection success in mixed exposures could be due to changed number of individuals of that parasite as half of the individuals in the exposure represent another species. In our study system, parasite infection success is known to be independent of the dose of exposure both in D. spathaceum (Karvonen et al. 2003) and D. gasterostei (A. Karvonen et al. 2004, personal observations) when doses similar to those used here are used. In other words, the proportion of parasites successfully establishing fish eyes is the same when fish are exposed to either 50 or 100 cercariae. This allows us to interpret the results as the effect of interactions between parasites.

Mechanisms through which interactions between Diplostomum parasites occur are unclear, but are likely to involve only indirect effects through host immune defence. This is because D. spathaceum and D. gasterostei infect different parts of fish eyes (Williams 1966; Karvonen et al. 2006), and thus they are unlikely to compete for any space or resources (see also Rauch et al. 2008). Furthermore, because the parasites migrate to the fish eyes within 24 h after exposure (e.g. Whyte et al. 1991), also direct interactions in organs other than the eyes are probably unlikely. Lack of direct competition between parasites was supported also by this study, because the effect of co-exposure on the infection success of D. spathaceum was not explained by the performance of co-infecting D. gasterostei strains.

In this study, we used fish that had no previous experience of eye flukes. Furthermore, because the experiment took only 12 days, the fish were unable to develop acquired immune defence against the parasites (development of acquired immunity takes three to four weeks depending on water temperature (e.g. Aaltonen et al. 1994)). Therefore, observed interactions between parasites are most likely due to innate immune reactions of fish. This is possible because recent studies, including a Diplostomum–fish interaction, have revealed that strain-specific host responses occur not only in acquired but also in innate immune defence (Schmid-Hempel et al. 1999; Carius et al. 2001; Rauch et al. 2006). Such responses could lead to specific interactions between different combinations of parasite strains observed in this study, because antigenic variation in each combination of parasite strains is unique. This can trigger different immune cascades, which may have different effects on parasite infection success. Possible candidates for such molecular mechanisms include lectin-like receptors and natural antibodies, both of which are present in innate immune defence of several fish species and can differentiate between different parasite antigens (reviewed by Magnadottir 2006). In this study, we chose to examine interactions between parasites using fish without acquired immunity because we were mainly interested in the general possibility of strain-specific interactions between co-infecting parasite species. However, experiments in which fish were exposed to different parasite species one after another could be interesting as in those exposures a wider variety of host immune mechanisms could be involved. Furthermore, such experiments would be relevant because in nature, Diplostomum parasites typically accumulate in fish over time (Marcogliese et al. 2001).

Also the mechanisms leading to asymmetry in the effect of co-exposure on D. spathaceum and D. gasterostei are not clear. It is possible that the infection success of D. gasterostei is unaffected by co-infecting parasites in general, or that the consistency in infectivity of D. gasterostei when compared with D. spathaceum is due to its lower general infectivity (the reasons for the difference in infectivity to rainbow trout are unclear, but it may be due to differences in host specificity (see Karvonen et al. 2006)). This could be if a response to co-exposure occurs only when parasite infectivity reaches a certain, relatively high level. This is possible because also in D. spathaceum, the response to co-exposure with D. gasterostei was mainly contributed by strains with the highest infectivity. Observed asymmetry could also be due to possibly faster establishment of D. gasterostei parasites as they need to reach only vitreous body of fish eyes whereas D. spathaceum is infecting eye lenses.

To conclude, our results suggest that the interactions between co-infecting parasite species can be specific to different combinations of interacting parasite strains. This may have important implications for maintenance of genetic variation in parasite traits as the species and genotype composition of the whole parasite community can provide a significant part of the environmental template on which the success of the specific strain is tested. In other words, between-species interactions provide an opportunity for fitness of a particular strain to have a significant component through the interactions with other species. Moreover, co-infections with multiple parasite species and strains are suggested to play an important role, for example, in epidemiology, disease severity and evolution of parasite virulence (e.g. Frank 1996; Lello et al. 2005; de Roode et al. 2005). Potential strain-specific responses may, however, modify also the outcomes of such ecological and evolutionary processes, thus emphasizing the need for further studies.
This research followed the laws and ethical guidelines of Finland.

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