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Reciprocal Interaction Matrix Reveals Complex Genetic and Dose-Dependent Specificity among Coinfecting Parasites

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ABSTRACT: Understanding genetic specificity in factors determining the outcome of host-parasite interactions is especially important as it contributes to parasite epidemiology, virulence, and maintenance of genetic variation. Such specificity, however, is still generally poorly understood. We examined genetic specificity in interactions among coinfecting parasites. In natural populations, individual hosts are often simultaneously infected by multiple parasite species and genotypes that interact. Such interactions could maintain genetic variation in parasite populations if they are genetically specific so that the relative fitness of parasite genotypes varies across host individuals depending on (1) the presence/absence of coinfections and/or (2) the genetic composition of the coinfecting parasite community. We tested these predictions using clones of fish eye flukes *Diplostomum pseudospathaceum* and *Diplostomum gasterostei*. We found that interactions among parasites had a strong genetic basis and that this modified genetic variation in infection success of *D. pseudospathaceum* between single and multiple infections as well as across multiply infected host individuals depending on the genetic identity of the coinfecting *D. gasterostei*. The relative magnitude of these effects, however, depended on the exposure dose, suggesting that ecological factors can modify genetic interactions between parasites.

Keywords: host-parasite interactions, multiple infections, *Diplostomum pseudospathaceum*, *Diplostomum gasterostei*, *Oncorhynchus mykiss*, Trematoda.

Introduction

High within-population genetic variation in host resistance (e.g., Alexander et al. 1993; Grosholz 1994; Kurtz and Sauer 1999; Carius et al. 2001; Koskela et al. 2002; Cotter et al. 2004; Salvaudon et al. 2005; Laine 2007) and parasite infectivity (e.g., Lively 1989; Henter 1995; Carius et al.

2001; Kaltz and Shykoff 2002; Laine 2007; Vale and Little 2009) is often observed across a broad range of natural host-parasite interactions both in animal and plant systems. This seems paradoxical because natural selection is expected to erode genetic variation in fitness-related traits by eliminating the least fit alleles from populations (reviewed by Roff 1997). Classically, heterogeneity of the environment (e.g., temperature, resource availability) is considered as an important factor contributing to the maintenance of genetic polymorphism (reviewed by Hedrick 2006), and it has recently been suggested also to be important for maintenance of genetic variation in host-parasite interactions (reviewed by Lazzaro and Little 2009). This is because genetic variation in host resistance often interacts with environmental variation; that is, different host genotypes are most resistant in different environments (G × E interaction; e.g., Blanford et al. 2003; Price et al. 2004; Mitchell et al. 2005; Seppälä and Jokela 2010). Thus, pathogen-mediated selection may favor different host genotypes under different environmental conditions, which promotes maintenance of genetic polymorphism in host populations.

The role of environmental variation in maintaining genetic variation in parasites is, however, still generally poorly understood (but see Fels and Kaltz 2006; Laine 2007; Vale and Little 2009; Bryner and Rigling 2011). Since hosts represent the parasites' primary environment, a potentially important environmental factor modifying their fitness is the presence of coinfecting parasites. In fact, individual hosts are often simultaneously infected by multiple parasite species (e.g., Holmes and Price 1986; Valtonen et al. 2001) and genotypes (reviewed by Read and Taylor 2001) that interact (e.g., Lello et al. 2004; de Roode et al. 2005; Koskela et al. 2006; Staves and Knell 2010; Laine 2011). Furthermore, because the genetic composition of the coinfecting parasite community is typically variable across multiply infected host individuals (i.e., dif-

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ferent hosts are infected by different parasite species and genotypes), we propose that the outcomes of interactions among parasites could show high variation, potentially contributing to the maintenance of genetic variation. However, this is possible only if these interactions are genetically specific so that the relative fitness of parasite genotypes varies depending on the presence or absence of coinfections and/or the genetic composition of the coinfecting parasite community.

We tested these hypotheses using *Diplostomum* (Trematoda) eye flukes of fish. *Diplostomum* parasites are common in several freshwater fishes, and multiple-species (Valtonen and Gibson 1997; Rellstab et al. 2011) and multiple-genotype (Rauch et al. 2005) infections are the rule in nature. In this study, we used *D. pseudospathaceum* (called *Diplostomum spathaceum* in our earlier studies [Seppälä et al. 2007, 2009] but revealed to be *D. pseudospathaceum* using genetic analysis [Louhi et al. 2010]) and *D. gasterostei* (synonym *Diplostomum baeri*). We estimated variation in parasite fitness by assaying their infection success, which is an important fitness related trait. Natural populations of these parasite species show high among-genotype/isolate variation in their infectivity to fish (Seppälä et al. 2007, 2009; Rauch et al. 2008; Voutilainen et al. 2010; Karvonen et al. 2012). Furthermore, these parasites interact in fish so that the infection success of *D. pseudospathaceum* is modified in coexposures (Seppälä et al. 2009). Interestingly, the effect of interaction is highly variable suggesting a complex genetic or ecological interaction between these parasite species (Seppälä et al. 2009).

In this study, we examined the potential role of coinfections in maintaining genetic variation in parasites by testing whether the observed variation in the outcome of coinfections (Seppälä et al. 2009) has a genetic basis. We measured the infection success of *D. pseudospathaceum* genotypes in single-genotype exposures and in coexposures with different *D. gasterostei* isolates using a reciprocal infection matrix (we did not measure the infection success of *D. gasterostei* because it was not affected by coexposure with *D. pseudospathaceum* in our earlier study; Seppälä et al. 2009). This experiment reveals whether the outcome of coexposure is determined by genetic identity of *D. pseudospathaceum*, *D. gasterostei*, or both ($G \times G$ interaction), which is important in determining under which conditions coinfections could maintain genetic variation. Interactions between parasites can promote maintenance of genetic polymorphism if coinfections change the relative fitness of parasite genotypes (1) between single and multiple infections and/or (2) across different multiply infected hosts depending on the genetic identity of the coinfecting parasite. Furthermore, we examined the effect of exposure dose, which is largely determined by ecological factors, on the outcome of interactions among parasites. This is rel-

evant as wild fish are typically exposed to variable doses of parasite transmission stages. We predicted that dose effects may modify the nature of genetic specificity of the interactions through induced host immune responses.

Material and Methods

Study Organisms

Trematodes of the genus *Diplostomum* have three-host life cycles (see Williams 1966; Chappell et al. 1994). Parasites mature in the intestine of fish-eating birds and reproduce sexually. Their eggs are released to water with birds' feces and hatch into free-swimming miracidia larvae. Miracidia of *Diplostomum pseudospathaceum* infect mainly *Lymnaea stagnalis* snails (Louhi et al. 2010), whereas *Diplostomum gasterostei* infects mainly *Radix balthica* and *Myxas glutinosa* snails (Karvonen et al. 2006). Parasites penetrate into snail gonads, where they develop into sporocysts and multiply asexually. Sporocysts produce thousands of free-swimming cercariae larvae that leave the snail and seek a fish. Because multiplication of *Diplostomum* parasites in snails is asexual, all cercariae originating from a single miracidial infection represent one genetically identical clone. Cercariae of both parasite species infect fish by penetrating the gills and skin and migrating to the eyes, where they develop into metacercariae. Metacercariae of *D. pseudospathaceum* locate themselves in the lenses whereas *D. gasterostei* locates itself in the vitreous body (Williams 1966; Karvonen et al. 2006), which could be one potential reason why only *D. pseudospathaceum* was affected by coexposure in our previous study (i.e., only *D. pseudospathaceum* migrates through the organ inhabited by the other species; see Seppälä et al. 2009). For successful completion of the life cycle, an infected fish has to be eaten by a piscivorous bird.

We collected naturally infected snail hosts of the parasites (*D. pseudospathaceum*: *Lymnaea stagnalis*; *D. gasterostei*: *Radix balthica*) from Lake Konnevesi (62°37'N, 26°21'E) in Finland. We separated snails infected with *D. pseudospathaceum* and *D. gasterostei* from snails infected with other parasite species by observing the morphology and behavior of released cercariae (see Karvonen et al. 2006). We used only *D. pseudospathaceum* cercariae from snails infected with one parasite genotype. We verified this by genotyping 20 cercariae from each snail using polymorphic microsatellite markers (Reusch et al. 2004) as described in Louhi et al. (2010). All clones derived from different snail individuals represented unique multilocus genotypes. We did not genotype *D. gasterostei* cercariae because microsatellite markers are not available for this species. Therefore, it is possible that some *R. balthica* snails released more than one parasite genotype. However, since

all infections in the snails originated from sexually produced miracidia, we know that *D. gasterostei* cercariae derived from different snail individuals were genetically distinct parasite isolates and therefore suitable for testing the responses of *D. pseudospathaceum* genotypes to genetically varying coinfections. Furthermore, the possible “noise” in the data caused by multiple-genotype infections in *D. gasterostei* infected snails would lead to a conservative error. This is because if multiple infections were common, the probability of detecting differences in the outcome of interaction among parasites would be reduced because among-isolate variance in *D. gasterostei* would be confounded with within-isolate variance (see Luijckx et al. 2011). Before the experiment (see below), we fed all the snails ad lib. with fresh lettuce for 4 days to minimize the possible variation in quality of released cercariae that could arise from differences in the snails’ physiological condition (see Seppälä et al. 2008).

We used juvenile (0+ years old) rainbow trout (*Oncorhynchus mykiss*) as a fish host in the experiment. We obtained the fish from a commercial fish farm where they had been reared in indoor tanks supplied with groundwater. This ensured that the fish had no previous experience of eye flukes or other helminth parasites.

Experimental Design

To produce parasite cercariae for the experiment, we placed six infected *L. stagnalis* and four infected *R. balthica* snails individually in glass jars containing 2 dL of water (17°C) and allowed them to shed cercariae for 6 h. We estimated the density of cercariae in each jar from 10 1-mL samples.

In the experiment, we measured the infection success of each *D. pseudospathaceum* genotype to fish in single-genotype exposures and in mixed-species coexposures by testing each genotype against each of the four *D. gasterostei* isolates separately (fig. 1). We used two exposure doses (100 and 50 cercariae of each parasite species per fish) in each exposure treatment to control for possible dose effects (fig. 1). This is important because exposure dose may, for example, determine the strength and type of host immune responses (see Bancroft et al. 2001; Bleay et al. 2007). We did not use a lower exposure dose than 50 cercariae of each parasite species per fish because the statistical power in such a treatment would have been low due to low infection levels (see analyses below).

In each treatment combination, we exposed 10 randomly selected fish to infection by placing the fish individually into containers with 0.5 L of water and cercariae for 30 min at 17°C. We distributed the cercariae into the containers using a microtiter pipette. We determined the volume of cercariae suspension needed based on its esti-

		Single-genotype exposures					
		<i>D. pseudospathaceum</i> genotype					
		1	2	3	4	5	6
Dose	100	10	10	10	10	8	10
	50	10	10	10	10	10	10

		Mixed-species coexposures					
		<i>D. pseudospathaceum</i> genotype					
		1	2	3	4	5	6
Dose 100	<i>D. gasterostei</i> isolate	1	10	10	10	10	10
	2	9	10	10	10	10	10
	3	10	9	10	9	9	10
	4	9	10	8	10	10	10

		Mixed-species coexposures					
		<i>D. pseudospathaceum</i> genotype					
		1	2	3	4	5	6
Dose 50	<i>D. gasterostei</i> isolate	1	10	10	10	10	10
	2	10	10	10	10	10	10
	3	10	10	10	10	8	10
	4	10	10	9	10	10	10

Figure 1: Experimental design used in the study showing different exposure treatments as well as replication (number of fish used in the analyses) in each treatment combination.

ated cercariae density (see above) and the used exposure dose. After the exposure, we transferred the fish to four 1400-L tanks, where we maintained the fish from different treatments separately in cages (40 cm × 40 cm × 40 cm). We killed the fish 48 h postexposure (parasites establish in the eyes within 24 h from exposure; Whyte et al. 1991) with an overdose of 0.01% MS 222 (Sigma Chemical, St. Louis). We measured the length (± 1 mm) of the fish and counted the number of *D. pseudospathaceum* parasites in fish eyes after dissecting them. We did not measure infection success of *D. gasterostei* because it was not affected by coexposure in our earlier experiment (Seppälä et al. 2009). Twelve fish died before the dissection or we could

not determine the parasite numbers in their eyes. We excluded these fish from the data (see fig. 1).

Statistical Analyses

To assess whether *D. pseudospathaceum* genotypes differed in their infectivity when not interacting with coinfecting parasites, we analyzed the variation in parasite infection success in single-genotype exposures using ANCOVA. In the analysis, we used the proportion of cercariae successfully infecting fish as a response variable, *D. pseudospathaceum* genotype (G_p , 6 genotypes) as a random factor, exposure dose (D, 2 doses) as a fixed factor, and fish length as a covariate. Since the interaction term between genetic identity of *D. pseudospathaceum* and exposure dose ($G_p \times D$ interaction) approached statistical significance in this model (table 1; $P = .066$), we further analyzed the variation in infection success separately for different exposure doses. We did this to examine whether parasite infectivity showed genetic variation in both exposure doses.

To analyze variation in interactions among coinfecting parasites, we first calculated the relative infection success of *D. pseudospathaceum* parasites in coexposures when compared to their infectivity in single-genotype exposures. In other words, we scaled parasite infection success in coexposures to their infectivity in single-genotype exposures to get a response variable that reflects the change in parasite performance between the exposure types. To do this, we first calculated the expected infection success of *D. pseudospathaceum* for each fish individual in coexposure treatments using the ANCOVA model for parasite infection success in single-genotype exposures (see above). We used this model rather than the average infectivity of each parasite genotype in single-genotype exposures because fish length affected parasite infection success (table 1). We then calculated the relative infection success of parasites in coexposures by dividing the observed infection success (proportion of cercariae successfully infecting fish) in each fish individual by its expected infection success. This variable shows how much parasite performance increased (values larger than 1) or decreased (values between 0 and 1) in coexposures when compared to single-genotype exposures. We log transformed these values to transform the data from relative to additive for the analyses. We analyzed the variation in relative infection success in coexposures using an ANCOVA with genetic identity of *D. pseudospathaceum* (G_p , 6 genotypes) and *D. gasterostei* (G_G , 4 isolates) as random factors, exposure dose (D, 2 doses) as a fixed factor, and fish length as a covariate. When a statistically significant interaction between genetic identity of *D. pseudospathaceum* and exposure dose (i.e., $G_p \times D$ and/or $G_p \times G_G \times D$ interaction) was observed, we further analyzed the variation in relative infection success of the

Table 1: ANCOVA for the infection success of *Diplostomum pseudospathaceum* in single-genotype exposures

Source	df	MS	F	P
<i>D. pseudospathaceum</i> (G_p)	5	.087	1.527 ^a	.328
Dose (D)	1	.004	.066 ^a	.808
Fish length	1	.113	4.243	.042
$G_p \times D$	5	.057	2.139	.066
Error	105	.027		

Note: Factors are the genetic identity of *D. pseudospathaceum* (6 genotypes, random factor), exposure dose (2 doses, fixed factor), and fish length (covariate).

^a ($G_p \times D$) as the error term.

parasites in coexposures separately for different exposure doses. We did this to examine whether genetic specificity (i.e., main effect of G_p and/or $G_p \times G_G$ interaction) among interacting parasites was observed in both exposure doses.

The above analyses reveal the genetic specificity and its dose dependence in the interactions between the parasites, but do not estimate whether these interactions significantly modify the genetic variation in infectivity of *D. pseudospathaceum* among exposure treatments. To examine this, we first analyzed the variation in the absolute infection success of *D. pseudospathaceum* genotypes in different coexposure treatments (i.e., we did not scale parasite infection success to their infectivity in single-genotype exposures) using similar ANCOVA models as above. When general differences in the infection success of parasite genotypes (main effect of G_p) was observed in coexposures, we examined whether differences in their infectivity in single-genotype exposures with the same exposure dose significantly predicted this variation. We did this using a linear regression with the mean infection success of each genotype in mixed-species coexposures (averaged across *D. gasterostei* isolates, fish length-adjusted) as a dependent variable and the mean infectivity of the same genotypes in their respective single-genotype exposures (fish length-adjusted) as an independent variable. This analysis reveals whether the genetic variation in parasite infection success is consistent or modified between single exposures and multiple exposures. Accordingly, the interaction terms between the genetic identities of coinfecting parasites ($G_p \times G_G$ interaction) in the ANCOVAs reveal whether or not the genetic variation in parasite infection success varies across different multiply infected host individuals. Assumptions of the all analyses were fulfilled, and we performed them using IBM SPSS 19.0 (SPSS, Chicago) software.

Results

Our analysis did not show a significant main effect of parasite genotype in determining the infectivity of *D. pseu-*

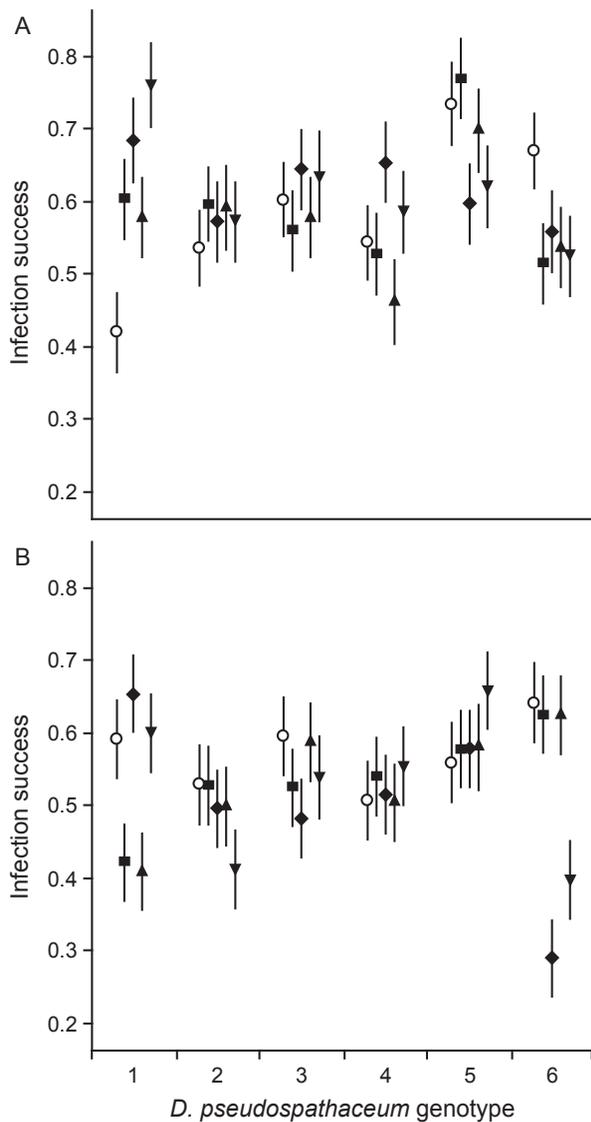


Figure 2: Infection success (fish length–adjusted mean \pm SE) of six *Diplostomum pseudospathaceum* genotypes in single-genotype exposures (open symbols) and in coexposures with four different *Diplostomum gasterostei* isolates (black symbols) measured as a proportion of parasites that successfully infected fish eyes. Each black symbol represents one *D. pseudospathaceum* genotype when tested against one *D. gasterostei* isolate (each symbol indicates one isolate). A and B refer to the two exposure doses (100 and 50 cercariae of each parasite species per fish, respectively) used in the experiment.

dospathaceum in single-genotype exposures (table 1). However, the interaction term between parasite genotype and exposure dose was close to statistical significance (table 1), which suggests that genetic variation in parasite infectivity may vary across exposure doses. Separate statistical models for each dose treatment revealed that this was be-

cause infectivity of *D. pseudospathaceum* genotypes differed in the high-exposure dose treatment (100 cercariae per fish; ANCOVA, $F_{5,51} = 4.410$, $P = .002$; table A1, available online; fig. 2A) but not in the low-exposure dose treatment (50 cercariae per fish; ANCOVA, $F_{5,53} = 0.818$, $P = .542$; table A1; fig. 2B).

In coexposures, *D. pseudospathaceum* showed genetic specificity in its responses to the presence of *D. gasterostei*. In other words, the relative infection success of *D. pseudospathaceum* in coexposures when compared to single-genotype exposures varied across different genotypes, as well as across different combinations of *D. pseudospathaceum* genotype and *D. gasterostei* isolate (table 2; fig. 2). These effects, however, depended on exposure dose as indicated by significant $G_p \times D$ and $G_p \times G_G \times D$ interactions (table 2). When the dose treatments were analyzed separately, in the high-exposure dose, *D. pseudospathaceum* genotypes showed general differences in their responses to coexposure that were independent of the genetic identity of the coinfecting partner (main effect of G_p ; ANCOVA, $F_{5,15} = 17.570$, $P < .001$; table A2, available online; fig. 2A). In the low-exposure dose treatment, *D. pseudospathaceum* genotypes did not show general differences in their responses to coexposures, but the outcome of interaction depended on the genetic identity of the both interacting parasite species ($G_p \times G_G$ interaction: ANCOVA, $F_{15,212} = 4.232$, $P < .001$; table A2; fig. 2B).

The observed genetic specificity in the interactions among coinfecting parasites modified the absolute infection success of *D. pseudospathaceum* genotypes (table 3; fig. 2). However, also this effect depended on the exposure dose as indicated by a significant $G_p \times G_G \times D$ interaction

Table 2: ANCOVA for the relative infection success of *Diplostomum pseudospathaceum* in coexposures with *Diplostomum gasterostei* when compared to the infectivity in single-genotype exposures

Source	df	MS	F	P
<i>D. pseudospathaceum</i> (G_p)	5	.325	1.211 ^a	.411
<i>D. gasterostei</i> (G_G)	3	.011	.180 ^b	.904
Dose (D)	1	.410	1.666 ^c	.259
Fish length	1	.104	5.513	.019
$G_p \times D$	5	.251	5.417 ^d	.005
$G_G \times D$	3	.041	.887 ^d	.470
$G_p \times G_G$	15	.064	1.373 ^d	.273
$G_p \times G_G \times D$	15	.046	2.459	.002
Error	421	.019		

Note: Factors are the genetic identity of *D. pseudospathaceum* (6 genotypes, random factor) and *D. gasterostei* (4 isolates, random factor), exposure dose (2 doses, fixed factor), and fish length (covariate).

^a ($G_p \times D$) + ($G_p \times G_G$) - ($G_p \times G_G \times D$) as the error term.

^b ($G_G \times D$) + ($G_p \times G_G$) - ($G_p \times G_G \times D$) as the error term.

^c ($G_p \times D$) + ($G_G \times D$) - ($G_p \times G_G \times D$) as the error term.

^d ($G_p \times G_G \times D$) as the error term.

Table 3: ANCOVA for the absolute infection success of *Diplostomum pseudospathaceum* in coexposures with *Diplostomum gasterostei*

Source	df	MS	F	P
<i>D. pseudospathaceum</i> (G_p)	5	.153	2.846 ^a	.178
<i>D. gasterostei</i> (G_G)	3	.007	.132 ^b	.935
Dose (D)	1	.636	175.746 ^c	.901
Fish length	1	.978	33.683	<.001
$G_p \times D$	5	.028	.523 ^d	.756
$G_G \times D$	3	.030	.544 ^d	.659
$G_p \times G_G$	15	.080	1.465 ^d	.234
$G_p \times G_G \times D$	15	.055	1.878	.024
Error	421	.029		

Note: Factors are the genetic identity of *D. pseudospathaceum* (6 genotypes, random factor) and *D. gasterostei* (4 isolates, random factor), exposure dose (2 doses, fixed factor), and fish length (covariate).

^a ($G_p \times D$) + ($G_p \times G_G$) - ($G_p \times G_G \times D$) as the error term.

^b ($G_G \times D$) + ($G_p \times G_G$) - ($G_p \times G_G \times D$) as the error term.

^c ($G_p \times D$) + ($G_G \times D$) - ($G_p \times G_G \times D$) as the error term.

^d ($G_p \times G_G \times D$) as the error term.

(table 3). When the dose treatments were analyzed separately, in the high-exposure dose, *D. pseudospathaceum* genotypes showed general differences in their infection success in coexposures that were independent of the genetic identity of coinfecting *D. gasterostei* isolate (main effect of G_p : ANCOVA, $F_{5,15} = 3.082$, $P = .041$; table A3, available online; fig. 2A). Infectivity of the same genotypes in single-genotype exposures, however, did not explain the observed variation (linear regression, $R^2 = 0.003$, $F_{1,4} = 0.011$, $P = .920$; fig. 3). In the low-exposure dose treatment, infection success of the parasites in coexposures varied across different combinations of *D. pseudospathaceum* genotype and *D. gasterostei* isolate ($G_p \times G_G$ interaction: ANCOVA, $F_{15,212} = 3.466$, $P < .001$; table A3; fig. 2B).

Discussion

Natural selection is expected to erode genetic variation in fitness related traits by eliminating the least fit alleles from populations (reviewed by Roff 1997). However, traits that determine the outcome of host-parasite interactions often show high genetic variation in nature (e.g., host resistance: Alexander et al. 1993; Grosholz 1994; Kurtz and Sauer 1999; Carius et al. 2001; Koskela et al. 2002; Cotter et al. 2004; Salvaudon et al. 2005; Laine 2007; parasite infectivity: Lively 1989; Henter 1995; Carius et al. 2001; Kaltz and Shykoff 2002; Laine 2007; Vale and Little 2009). Recently, heterogeneity of the environment has been acknowledged as a potential mechanism maintaining genetic polymorphism in host resistance (e.g., Blanford et al. 2003; Price et al. 2004; Mitchell et al. 2005; Seppälä and Jokela 2010), but its importance in maintaining genetic variation in par-

asites is still poorly understood (but see Fels and Kaltz 2006; Laine 2007; Vale and Little 2009; Bryner and Rigling 2011). In this study, we examined whether coinfecting parasites could introduce variation to the relative fitness of parasite genotypes through genetically specific interactions that modify infection success. By conducting experimental single-genotype exposures and mixed-species coexposures using *Diplostomum pseudospathaceum* and *Diplostomum gasterostei* eye flukes of fish, we found that interactions between these species were genetically specific and that they introduced significant variation to the infection success of *D. pseudospathaceum*. Furthermore, the observed effects depended on the exposure dose, which suggests that the ecological context of the actual exposure event can modify the genetic interactions determining parasite infection success.

In the high-exposure dose treatment (100 cercariae of each parasite species per fish), responses of *D. pseudospathaceum* to coexposures were specific to each parasite genotype, but did not depend on the genetic identity of the coinfecting *D. gasterostei*. This modified the absolute infection success of *D. pseudospathaceum* so that the infection success of parasite genotypes when compared to

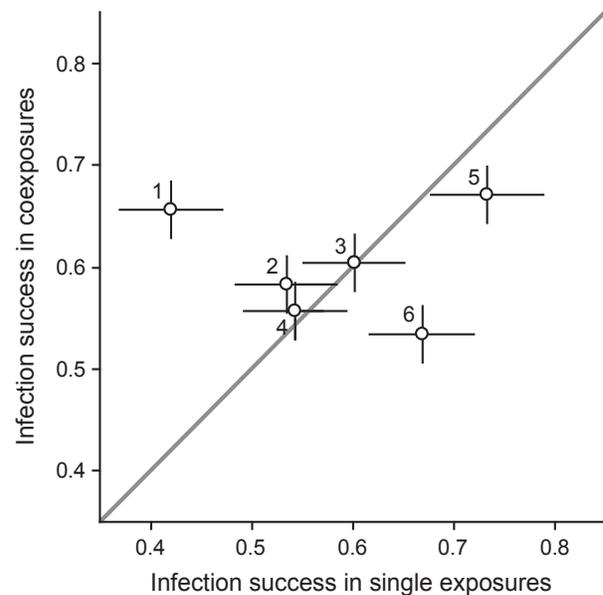


Figure 3: Infection success (fish length-adjusted mean \pm SE) of six *Diplostomum pseudospathaceum* genotypes in single-genotype exposures and in coexposures with *Diplostomum gasterostei* (averaged across different isolates) in the high-exposure dose treatment (100 cercariae of each parasite species per fish) measured as a proportion of parasites that successfully infected fish eyes. Gray line indicates equal infection success in both treatments, and numbers next to the error bars refer to the identity of different *D. pseudospathaceum* genotypes as in figure 2.

each other changed between singly and multiply infected host individuals. In the low-exposure dose treatment (50 cercariae of each parasite species per fish), the outcome of interaction depended on the genetic identity of the both parasite species. This modified the genetic variation in parasite infectivity across different multiply infected hosts. This kind of context dependence in infection success could erode most of the expected direct relationship between parasite genotype and fitness. Therefore, as variable environmental factors in general (see Gillespie and Turelli 1989; Schlichting and Pigliucci 1998), interactions among coinfecting parasites should promote maintenance of genetic variation by favoring different alleles of the parasites' genes depending on the presence/absence of coinfecting parasites and the genetic composition of the coinfecting parasite community within a host.

In studies examining interactions among coinfecting parasites, variation in exposure dose between single exposures and coexposures needs to be considered as a potential confounding factor as it is inevitable that either the total number of parasites or the relative dose of interacting partners differs between the treatments (the former is true in this study). This is because if parasite performance is dose dependent, differences between exposure treatments could simply reflect differences in the number of parasites the hosts are exposed to (Taylor et al. 1997). In our study, such dose effects could possibly confound the observed changes in parasites' infection success between single-genotype exposures and mixed-species coexposures in the high-exposure dose treatment. We, however, think this is unlikely because the infection success of *Diplostomum* cercariae in single-species exposures is known to be largely independent of the exposure dose within the range of doses used here (e.g., Brassard et al. 1982; Stables and Chappell 1986; Whyte et al. 1991). This is also the case for *D. pseudospathaceum* (Karvonen et al. 2003; this study) and *D. gasterostei* (A. Karvonen et al., personal observations, 2004). Verification of this, however, would have required additional treatments with higher-exposure doses in single-genotype exposures to be included in the experimental design. In the low-exposure dose treatment, the observed dependence of variation in the absolute infection success of *D. pseudospathaceum* genotypes from the genetic identity of coinfecting *D. gasterostei* cannot be confounded by potential dose effects because the exposure doses (both the total number of cercariae and the relative number of interacting partners) were the same across different combinations of coinfecting parasites. This allows us to interpret those results as interactions among parasites.

It is also important to note that each of the parasite genotypes/isolates used in this study originated from an individual snail host. Therefore, variation in the physiological condition of the snails could have affected the qual-

ity of released cercariae larvae and thus potentially modified their infection success. In fact, in our study system, starvation of snails is known to reduce parasite's reproductive rate and life span of produced cercariae (Seppälä et al. 2008), which is most likely due to limited amount of resources available for the parasite. As replicating each parasite clone across several snail individuals is impossible in our study system (clonal reproduction takes place only within snails, and larval stages infecting snails [miracidia] are produced through sexual reproduction), we aimed to reduce the possible variation in quality of the used cercariae due to effects of host condition by feeding all the snails ad lib. with fresh lettuce for 4 days before the experiment.

The reasons for the observed genetic specificity and its dependence on the exposure dose in interactions between *D. pseudospathaceum* and *D. gasterostei* are not fully understood. In general, interactions among coinfecting parasites can take place through direct competition (Patrick 1991; Selva et al. 2009; Staves and Knell 2010) and/or indirectly through host defenses (Adams et al. 1989; Råberg et al. 2006; Ezenwa et al. 2010; Laine 2011). In most study systems, separation between these mechanisms is difficult. In our system, however, interaction through direct competition is unlikely. This is because *D. pseudospathaceum* and *D. gasterostei* infect different parts of fish eyes (eye lenses and vitreous bodies, respectively), when competition for space and resources in the eyes should be weak or negligible. Furthermore, possible resource competition should be most intense during parasite growth and development, not during establishment, studied in this experiment. Similarly, although we cannot fully exclude the possibility that *Diplostomum* parasites interact directly while invading the fish and/or migrating to the eyes, such effects are also likely to be weak because the parasites can use the whole surface area of the fish for the invasion (Erasmus 1959; Chappell et al. 1994) reducing the probability of contact during establishment. Lack of direct competition among these parasite species is also supported by our previous study in which the effect of coexposure on the infection success of *D. pseudospathaceum* was not explained by the performance of the coinfecting *D. gasterostei* isolate as could have been predicted if the highly infectious isolates of one species were better in inhibiting the infection success of the other (Seppälä et al. 2009).

In this study, we used fish that had no previous experience of *Diplostomum* eye flukes. Furthermore, because we examined the fish 48 h postexposure, the fish did not develop acquired immune defense against the infections during the experiment (development of acquired immunity takes 3–4 weeks depending on water temperature; Aaltonen et al. 1994; Chappell et al. 1994). Thus, the observed interactions among parasites are likely to be mediated by the

innate immune defense of fish. This is possible because recent studies, including also *Diplostomum*-fish interaction, have revealed that parasite-genotype-specific host responses occur in the innate immune defense (Schmid-Hempel 1999; Carius et al. 2001; Rauch et al. 2006). Such responses could lead to genetic specificity in interactions between coinfecting parasites, because unique antigenic variation in each exposure combination can trigger different immune cascades that modify parasite infection success. Possible candidates for such molecular mechanisms include lectin-like receptors and natural antibodies, both of which are present in fish innate immune defense and can differentiate between parasite antigens (reviewed by Magnadóttir 2006). Similarly, the dependence of the interactions on the exposure dose may be related to the strength of fish immune responses during parasite invasion. In mammals, for example, parasite exposure dose can even determine the type of immune responses that are elicited (Bancroft et al. 2001; Bleay et al. 2007). Such dose-dependent changes could be very important in determining the interactions among coinfecting parasites. However, to our knowledge, such effects are poorly understood in fish immune system, the research limiting to studies investigating the protective effect of vaccines (LaPatra et al. 2000; Lorenzen et al. 2000). Nevertheless, also those studies have reported significant dose effects.

To conclude, our results indicate that interactions among coinfecting parasite species can show complex genetic specificity that is further modified by ecological context of the exposure event (i.e., exposure dose). Since the presence as well as the genetic identity of the coinfecting parasites can show high variation among host individuals in nature, such interactions may have important implications for parasite evolution by promoting maintenance of genetic variation. To our knowledge, our study is the first one demonstrating genetic specificity in the interactions among coinfecting parasites. This finding may have wide implications also in many other host-parasite interactions. This is because multiple infections are the rule in nature and variation in other environmental factors has been reported to interact with genetic variation in parasite traits across a broad range of different host-parasite systems including animal (Vale and Little 2009), plant (Laine 2007), fungus (Bryner and Rigling 2011), and protist (Fels and Kaltz 2006) hosts. Furthermore, coinfections with multiple parasite species and genotypes are suggested to play important roles in epidemiology, disease severity, and evolution of parasite virulence (e.g., Lello et al. 2005; de Roode et al. 2005). If genetically specific interactions observed here are common in nature, this can also complicate the predictions considering the role of coinfections in such ecological and evolutionary processes.

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Left, *Diplostomum pseudospathaceum* cercaria parasite. Photograph by Christian Rellstab. Right, rainbow trout infected with eye fluke. Photograph by Anssi Karvonen.