Interactions among bacterial strains and fluke genotypes shape virulence of co-infection

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Most studies of virulence of infection focus on pairwise host–parasite interactions. However, hosts are almost universally co-infected by several parasite strains and/or genotypes of the same or different species. While theory predicts that co-infection favours more virulent parasite genotypes through intensified competition for host resources, knowledge of the effects of genotype by genotype (G×G) interactions between unrelated parasite species on virulence of co-infection is limited. Here, we tested such a relationship by challenging rainbow trout with replicated bacterial strains and fluke genotypes both singly and in all possible pairwise combinations. We found that virulence (host mortality) was higher in co-infections compared with single infections. Importantly, we also found that the overall virulence was dependent on the genetic identity of the co-infecting partners so that the outcome of co-infection could not be predicted from the respective virulence of single infections. Our results imply that G×G interactions among co-infecting parasites may significantly affect host health, add to variance in parasite fitness and thus influence evolutionary dynamics and ecology of disease in unexpected ways.

1. Introduction

Host organisms are almost invariably subject to infection by multiple parasite species and/or parasite genotypes [1–4]. Past decades of theoretical and empirical studies have demonstrated that co-infections can drive altered infection risk, disease dynamics, parasite community structure and evolution of virulence [1,5–12]. Perhaps most importantly, experimental studies suggest that pairwise co-infections with different strains of a single parasite species generally select for increased virulence (damage to the host) when the strains compete within a host ([11,13–15]; but see [16] for asymmetry in competitive success of co-infecting strains and [17] for outcomes of co-infection in different conditions of kin selection). Such interactions can take place as interference in host tissues or through competition for the same host resources [18–20]. Co-infection can also elicit cross-reactive immune responses [20,21], changing the efficiency of host defence and leading to changes in virulence of infection.

Despite the widespread nature of taxonomically diverse co-infections and the plethora of potential interactions, knowledge of the variation in disease outcomes in genotype combinations of different parasite species is limited. Indeed, the majority of research on co-infections has focused on interactions among strains or genotypes of a single parasite species. However, genotype by genotype (G×G) interactions between different, unrelated parasite species should not be ignored, because they are potentially important and predominate in host populations in the wild as well as in man-made environments. Parasitological surveys show that majority of free-living host individuals are not infected with one but several different species of parasites simultaneously [9,22]. These co-infecting species may differ in their modes of transmission, spatio-temporal

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dynamics, sites of infection in a host and in their expected influence on host fitness (virulence) in single infections. Correlative evidence from clinical studies suggests that associations between taxonomically distant parasite species can have dramatic effects for disease outcome. For example, helminths (parasitic worms) can negatively affect host resistance to causative agents of tuberculosis, malaria and AIDS (reviewed in [4]). Encountering such infections in different genetic combinations in a host may result in unpredictable outcomes of a disease, but experimental evidence supporting this prediction in well-replicated parasite combinations is severely lacking. Indeed, most of the evidence on $G \times G$ interactions between unrelated parasite species comes from pairwise infections using single strains (e.g. [23–27]), while there are no experimental studies using a broad range of replicated parasite strains or genotypes.

$G \times G$ interactions between parasites may have fitness consequences for the parasites as well as for the host. Fitness consequences of co-infections can be unpredictable when life cycle and epidemiology of each co-infecting parasite genotype are not coupled (i.e. particular genotypes interact only in one generation). If co-infecting parasites are also co-transmitted, the fitness of each genotype will have an epistatic component defined by the interaction with the other parasite genotype [28] and the interaction between the co-infecting genotypes may coevolve. However, in most cases, co-transmission is unlikely due to differences in ecology and epidemiology of the parasites. In other words, most co-infections with another parasite genotype add to the phenotypic variance in parasite fitness. An increase in phenotypic variance in fitness may still have consequences for evolutionary dynamics as it effectively reduces the heritable variance in fitness and slows the response to selection. Therefore, considering fitness variation due to co-infections is important for evolution of virulence, evolution of quantitative resistance and for coevolutionary dynamics.

In agricultural systems, intensive production units are often ideal for the spread of infectious diseases. For example, aquaculture production creates ‘hotspots’ for parasite interactions as several disease-causing agents thrive concurrently in high host densities, potentially causing interactions among a range of different parasite taxa and facilitating the spread of infectious diseases. Here, we explored virulence of co-infections in rainbow trout (Oncorhynchus mykiss) infected with two globally important parasites that co-occur in the wild and in aquaculture environments [29,30]: the bacterium Flavobacterium columnare, the causative agent of columnaris disease in fish [30], and the trematode fluke Diplostomum pseudospathaceum, causing cataracts and blindness in the eyes of fish [31,32]. Flavobacteria (Bacteroidetes) are Gram-negative rod-shaped bacteria that can be isolated directly from water and substrate surfaces, but also from fish where some species (F. columnare, F. psychrophilum, F. brancai) typically are pathogenic to a varying degree [33]. Infection of F. columnare can cause mortality among natural fish populations [34] as well as in aquaculture [35]. Infection takes place directly through contact with an infected fish or from contaminated water and results in epidermal disease on the skin and gills of fish. Tissue-degrading enzymes of the bacteria cause skin damage and gill necrosis, which lead to oxygen depletion, osmotic shock and death of fish. Epidemics can develop very rapidly in favourable temperatures (20–27°C) [30]. Flavobacteria are currently considered one of the most severe threats to aquaculture worldwide [35–37], and infections within the rearing units are treated with antibiotics.

Diplostomum flukes are ubiquitous macroparasites with a three-host life cycle that includes an avian definitive host, and invertebrate first intermediate host (aquatic snail) and a vertebrate second intermediate host (fish). Sexual reproduction among the adult worms in the bird gut produces eggs that are released into water, where they hatch to miracidia larvae to infect the snail. Within the snail, the parasite undergoes efficient asexual reproduction producing high numbers of clonal cercariae to infect the next host, fish, where they develop to the next larval stages (metacercariae) that are again transmittable to birds. Virulence of the infection in fish can be seen as acute mortality during cercarial invasion within a matter of hours or after some weeks as eye cataracts elicited by a chronic infection of the parasite metacercariae in the eye lenses [38]. Genotype–strain-specific co-infections of the two parasites can take place in the wild when fish are in proximity to infected snails that release high numbers of Diplostomum cercariae and at the same time pick up F. columnare from the water or from nearby infected fish. Co-infections are even more likely in aquaculture facilities that can harbour large numbers of infected snails [39,40] and high densities of fish positive for F. columnare [41].

We tested whether F. columnare strains and D. pseudospathaceum genotypes interact in fish during co-infection in a strain–genotype-specific manner and whether co-infection results in higher overall virulence (morbidity of fish that corresponds to mortality) and altered infection intensity of the fluke in the host compared to single-genotype infections. To achieve this, we conducted a cross-infection experiment with six F. columnare strains and five D. pseudospathaceum genotypes, using both single-strain and single-genotype infections, and a matrix of all possible strain–genotype combinations. We found higher overall virulence in co-infections but also a strong strain–genotype interaction so that virulence was increased in most co-infection combinations, but not in all. Interestingly, the infection success of the fluke genotypes was also altered in co-infections in a strain–genotype-specific manner. Our results indicate significant $G \times G$ interaction in co-infections of taxonomically distant parasite species.

2. Material and methods

(a) Bacterial cultures

Six F. columnare strains (1–6, electronic supplementary material, table S1) showing different levels of pathogenicity were used [42,43]. Bacterial strains were originally isolated from two fish farms or from the environment in 2007–2010 by standard culture methods using Shieh medium [44] and Shieh medium supplemented with tobramycin [45]. Different sampling locations, sampling times, sources of isolation (fish versus environment), ARISA groups (electronic supplementary material, table S1) and the different pathogenicity of the isolates [42,43] ensured that the strains had different genetic and ecological characteristics. Cultures were stored at −80°C with 10% glycerol and 10% fetal calf serum. Prior to the exposures, bacterial strains were grown overnight in 2 ml of Shieh medium, then enriched in 1:10 in fresh medium and incubated at 25°C with 150 r.p.m. agitation for 22 h. The optical density (A570) of the culture was measured with a spectrophotometer, and the corresponding colony forming units (CFU) were calculated.

(b) Sampling and genotyping of flukes

Infected Lymnaea stagnalis snails ($n = 28$), intermediate hosts for D. pseudospathaceum, shedding clonal fluke larvae were collected...
from Lake Vuojärvi (62°24′54″ N, 25°56′14″ E), Finland, in June 2013. Fifteen larvae were collected from each snail and stored individually in Eppendorf tubes in 15 μl of water and frozen in −20 °C for subsequent microsatellite analysis to identify snails that were infected with one fluke genotype [46,47] (electronic supplementary material, table S2). Parasite DNA was extracted according to Criscione & Blouin [48]. Snails infected with one genotype were stored individually in 11 of water at 5.9 °C and fed ad libitum with lettuce for 9 days until the beginning of the experiment.

(c) Experimental exposures
Naive, uninfected juvenile rainbow trout (O. mykiss; age two months, average length ± s.e. 31.13 ± 0.10 mm) were obtained from a hatchery farm in Central Finland and maintained in aerated ground water with continuous water flow (17°C) for three weeks before the experiments. Fish were fed daily with commercial fish food pellets during the maintenance. Prior to the exposures, the water temperature was raised slowly to 25°C (2°C every second day) to allow fish acclimation to experimental conditions. Six freshly grown strains of F. columnare (1–6, see the electronic supplementary material, table S1) and clonally produced cercariae larvae of D. pseudospathaceum from five single-genotype infected snails (A–E; see the electronic supplementary material, table S2) were used in the fish exposures. Three hours prior to the exposures, the snails were placed individually in 2.5 dl of water (17°C), and allowed to produce cercariae. Cercarial density from each snail (fluke genotype) was estimated by counting five 1 ml subsamples from each container.

A pairwise infection design was then applied to test virulence and intensity of infection across the combinations. In the experiment, 15 rainbow trout were exposed individually to single bacterial strains (5 × 104 CFU ml−1), single fluke genotypes (50 cercariae/fish), or co-exposed to both bacteria and flukes, totalling 41 different treatment groups and 615 fish. The infection doses corresponded to those in natural conditions. For example, fish infected with F. columnare can emit bacterial concentrations that are orders of magnitude higher than those used here [49] and one infected snail can release thousands of D. pseudospathaceum cercariae per day [12,50]. All fish were haphazardly assigned to the different treatment groups (single exposure to F. columnare, single exposure to D. pseudospathaceum, co-exposure to both parasites). The set-up did not include untreated control groups as we were mainly interested in differences in virulence between different parasite combinations and very low mortality, if any, has typically taken place among unexposed fish in earlier studies [51]. The exposures and the consequent monitoring took place in small containers with 500 ml pre-aerated water (25.7°C). The fish were checked every hour for disease symptoms and morbidity. Morbid fish that had lost their natural swimming buoyancy and were lying on the bottom of the container and not responding to external stimuli but were still moving their gills were considered dead and were euthanized every hour. This gave an accurate estimate of time of death (see also [51,52]). The fish were immediately sampled for the presence of F. columnare on the skin and gills (by culture on Shieh containing tobramycin [45]), and dissected for D. pseudospathaceum in the eye lenses. Note that the establishment of D. pseudospathaceum in the eye lenses takes place within a few hours of exposure. The dissection protocol was used to determine the exact shape of the time-establishment relationship used in the estimation of differences in fluke abundance among the treatment groups (see below). The experiment was terminated at 31 h post-exposure when 82.3% of the fish had died. All surviving fish were subsequently euthanized and examined for bacterial and fluke infection as described above. Bacterial cultures confirming F. columnare infection were incubated at 22°C for 2 days and checked for the presence of bacterial colonies.

(d) Statistical analysis
 Accelerated failure time (AFT) survival models (Weibull regressions) were fitted to the fish survival data including all fish that died and survived the experiment to obtain predicted survival times. The model predicts failure time (mortality rate) in specific treatments. In our application, failure time refers to virulence of the parasite combination, i.e. the model predicts hourly mortality rates each host is expected to experience in specific treatment combination. The best model for the co-exposure data included bacterial strain (CGB), fluke genotype (CGF), fish length and their interaction as covariates, whereas the best single-exposure model included only CGB or CGF as covariates, respectively. All survival analyses were computed with the open-source statistical package R, v. 2.15.2 using R-package ‘survreg’ [53]. Null model prediction was calculated assuming that strain/genotype-specific mortality rates in single infections were additive and independent. Mortality rates in single infections were estimated from experimental data with AFT survival models for each bacterial strain and fluke genotype, respectively (figure 1). These models predict...
null model prediction for expected virulence in each particular co-infection combination is calculated as a sum of the strain/genotype-specific mortality rates. The observed mortality rates are failure time predictions from the AFT survival model that included the interaction between bacterial strain and fluke genotype (figure 1 and table 1). In addition, we applied analysis of covariance (ANCOVA; IBM SPSS version 20.0) with an asymptotic contrast to data on fluke numbers in fish eyes to identify factors that affected infection intensity in single infections and in co-infections with the bacterial strains. To correct for variation in fluke exposure and establishment time between fish individuals showing different survival times, we used the residuals of the regression predicting infection intensity as a function of survival time as the response variable (see the electronic supplementary material, figure S1). Statistical testing was performed at the $p = 0.05$ level of significance (two-sided). Assumptions of the statistical tests were fulfilled.

### Table 1. Factors influencing survival of rainbow trout in co-infections with *D. pseudospathaceum* and *F. columnare*. AFT model was used to analyse survival of rainbow trout in co-infections of six bacterial strains ($G_B$) and five fluke genotypes ($G_F$). The best model included main effects of bacterial strains, fluke genotypes and fish length and their interactions (Model log-likelihood = $-1318$, d.f. = 59, $p < 0.001$, $n = 450$).

<table>
<thead>
<tr>
<th>source</th>
<th>d.f.</th>
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<th>residual d.f.</th>
<th>$-2 \times \text{LL}$</th>
<th>$p$-value</th>
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<tr>
<td>$G_B$</td>
<td>5</td>
<td>39.7</td>
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<tr>
<td>$G_F$</td>
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<td>22.2</td>
<td>439</td>
<td>2792</td>
<td>&lt;0.001</td>
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<tr>
<td>$G_B \times G_F$</td>
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<td>84.0</td>
<td>418</td>
<td>2686</td>
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<tr>
<td>fish length</td>
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<td>2770</td>
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<tr>
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<td>2682</td>
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<tr>
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<td>13.2</td>
<td>409</td>
<td>2669</td>
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<tr>
<td>$G_B \times G_F \times$ fish length</td>
<td>20</td>
<td>32.9</td>
<td>389</td>
<td>2636</td>
<td>0.035</td>
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### 3. Results

The results were consistent with multiple parasite infections producing higher virulence (figure 1), but also generating unpredictable strain by genotype variation in disease severity (figure 2; electronic supplementary material, figure S2). We found that fish host survival time was shorter in co-infections (mean median survival time predicted by an AFT model = 12 h) than in single exposures (baseline mean median survival time predicted by an AFT model: bacterial strains = 38 h; fluke genotypes = 68 h; figure 1). The observed mortality rate in the co-infection treatment was 1.8-fold higher than that expected based on the null assumption that the virulence of single infections is additive (figure 2). We also found statistically significant variation in virulence among different strain–genotype combinations (figure 2 and table 1; electronic...
supplementary material, figure S2). Survival of the fish in a single exposure to *F. columnare* strains was strain-specific (figure 1; AFT model log-likelihood = $-203.4$, d.f. = 5, $p < 0.001$; effect of bacterial strain, d.f. = 5, deviance residual = 33.6, residual d.f. = 83, $-2 \times LL = 406.78$, $p < 0.001$), while no genotype-specific survival was observed in the single exposures to *D. pseudospathaceum* (figure 1; d.f. = 4, deviance residual = 6.40, residual d.f. = 69, $-2 \times LL = 308.06$, $p = 0.172$).

Moreover, the infection intensity of *D. pseudospathaceum* (number of fluke metacercariae per infected fish) was significantly boosted upon co-exposure with the bacterium in 11 out of 30 combinations and was lower in two out of 30 combinations (figure 3). Thus, both the overall virulence and the intensity of infection of the fluke depended on the specific strain–genotype interaction between these parasites. Infection intensity did not differ among the *D. pseudospathaceum* genotypes in single infections (table 2). The majority of the fish (520 out of 537 = 96.8%; three fish were not sampled) that were exposed to the bacterial strains were positive for *F. columnare* infection in culture samples taken from the skin and/or gills. None of the 75 fish exposed only to flukes were infected with *F. columnare*. Two fish (0.4%) exposed to the flukes but dying during the first hour of the experiment remained uninfected (electronic supplementary material, figure S1).

### Table 2. Infection intensity of *D. pseudospathaceum* flukes in single infections and in co-infections with the bacteria *F. columnare*. Analysis of covariance of differences in infection intensity among five fluke genotypes ($G_f$) in (a), single infections and in (b), co-infections with six bacterial strains ($G_b$). Residuals of regression between infection intensity and time (number of flukes = 39.8 ± 48.0 × exp(−0.37 × time), $R^2 = 0.50$, $F_{1522} = 2866$, $p > 0.0001$) were used as a measure of infection intensity (see the electronic supplementary material, figure S1). Figure 3 shows the predicted average values for each fluke genotype–bacterial strain combination.

<table>
<thead>
<tr>
<th>source</th>
<th>MS</th>
<th>d.f.</th>
<th>$F$</th>
<th>p-value</th>
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<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>$G_f$</td>
<td>86</td>
<td>4</td>
<td>1.30</td>
<td>0.278</td>
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<tr>
<td>fish length</td>
<td>478</td>
<td>1</td>
<td>7.21</td>
<td>0.009</td>
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<tr>
<td>error</td>
<td>66.2</td>
<td>69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$G_b$</td>
<td>376</td>
<td>5</td>
<td>6.20</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$G_f$</td>
<td>987</td>
<td>4</td>
<td>16.25</td>
<td>&lt;0.001</td>
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<tr>
<td>$G_b \times G_f$</td>
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<td>1.89</td>
<td>0.012</td>
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<tr>
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<td>1</td>
<td>11.66</td>
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</tr>
<tr>
<td>error</td>
<td>61</td>
<td>419</td>
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</table>

### 4. Discussion

Co-infections may shape disease epidemiology and disease outcomes through interactions between the co-infecting parasite community and host immune responses (reviewed in [2,54–56]), thus underlying important processes in host–parasite ecology and evolution. Co-infections, however, are often unpredictable in the sense that in most cases it is difficult to predict which species, strains or genotypes of parasites will be interacting in a particular host. If the overall disease outcome in co-infections is dependent on the genetic identity of the co-infecting partners, this can create considerable variation in the overall virulence of infection among hosts within a population. Co-infections might thus add to the variation in disease outcomes making predictions of expected virulence difficult. For the co-infecting parasite genotypes, genotype-specific interactions with other parasites increase phenotypic variance in fitness suggesting that this additional source of fitness variation should be evaluated when considering evolutionary adaptation to host population.

In this paper, we conducted a co-infection trial using replicated bacterial strains and fluke genotypes to investigate whether $G \times G$ interactions between completely unrelated parasite species determine the overall virulence of infection. Our results revealed complex interactions associated with co-infections. We found that the overall virulence of infection (host mortality) and to some degree (11/30 cases) also the infection intensity of the flukes increased in co-infections.
At the same time, we found that different combinations of co-infecting bacterial strains and fluke genotypes showed marked variation both in overall virulence and in fluke infection intensity. This indicates significant strain–genotype interaction between these taxonomically unrelated parasite species during co-infection. These results significantly add to earlier investigations demonstrating higher virulence or higher competitive ability of more virulent strains in co-infections of strains or genotypes of one parasite species [11,13–15] or altered infection success of co-infecting genotypes of related parasite species [57,58]. More importantly, our data show that G × G interactions in parasites with completely different modes of transmission and strategies of host infection are possible and that the outcome of these interactions cannot be predicted from single-strain–genotype infections.

Mechanistically, increased virulence and infection intensity in co-infections could emerge as a consequence of direct facilitation of host invasion [59] or indirect interaction between the co-infecting partners through compromised host immune responses [12,60]. For example, in the present system, it is possible that co-infecting flukes, which penetrate the epithelium of the fish, may open a route for the bacteria to enter the host’s body resulting in faster and more aggressive progression of the disease. The simultaneous presence of two different parasite types could also result in overwhelming the host’s defence system. Indeed, a general collapse of the immune functions, such as a trade-off between the Th1 and Th2 arms of the innate immune defences in co-infections of microparasites and macroparasites [19,61,62], provide one possible explanation for the increase in virulence and infection success of the two parasites. Interestingly, in this particular system, co-infection, albeit beneficial for both parasites during the initial establishment in fish, may represent conflicting interests for the parasites later during the course of infection. For example, high virulence and rapid mortality of the fish host may be advantageous for F. columnare which can benefit from saprotrophic transmission [49], but deleterious for D. pseudospathaceum in terms of transmission failure. While this suggests that co-infection may be an evolutionary dead-end for D. pseudospathaceum, we nevertheless observed considerable variation in the outcomes of specific strain–genotype combinations. Furthermore, in natural conditions this issue may be greatly influenced by differences in the timing of the two infections, i.e. sequential host exposure, when one of the parasites infects first and launches an immune response in the host. These aspects form an interesting field for future research on the evolution of co-infections in terms of parasite transmission strategies, conflicts of transmission and mechanisms of host exploitation. Overall, similar mechanisms of facilitation in initial host establishment and disease progression and virulence could operate in most host–parasite systems, which typically include injuries to host epithelium or interaction with the host immune system at some point during the infection. However, details of these mechanisms in the present system, or in most other systems, are yet to be discovered.

Overall, these results are important as they demonstrate the complexity and dimensions of possible interactions on a scale of a community of co-infecting parasites. We argue that co-infections across different parasite taxa could shape perceived disease virulence broadly in natural and farmed environments where hosts are attacked by a diversity of parasites. If co-infections contribute significantly to parasite and host fitness, in other words, if parasite G × X interactions increase phenotypic variation in fitness, evolution of genotype- or strain-specific virulence should be evaluated not only across a range of possible host genotypes, but also across a range of possible co-infesting parasite genotypes. If true, this suggests that evolution of virulence may be shaped largely by ecological and evolutionary interactions among co-infecting parasite species sharing a host population. Therefore, the evolutionary scenario for virulence evolution may be much more complex than previously acknowledged. Similarly, estimating the relevant parameters for virulence evolution, for example, the outcome of within-host interactions and the transmission potential of the co-infecting partners [56], may rapidly increase in complexity in the context of co-infections.

From an applied perspective, predicting and managing infections under such co-infection scenarios may be challenging. For example, flavobacterial infections currently pose an increasing threat to fish health in aquaculture around the world [35–37], which emphasizes the importance of multiple co-occurring parasite infections in predicting the disease outcomes and virulence of infection. Such interactions in aquaculture may be further influenced by the prevailing conditions that favour more virulent strains (see [35] for results on flavobacteria) or changes in parasite dynamics following an increase in water temperature [41]. Although in most cases of co-infections, we might expect to see increased disease severity, more studies are needed to evaluate how G × G interactions among co-infecting partners affect the evolution of virulence in each interacting parasite population, particularly involving unrelated parasite taxa that frequently co-infect their hosts.

Ethics. All experiments were approved by Finnish Regional State Administrative Agency (license no. ESAVI/1375/04.10.03/2012) and they conform to the animal care legislation of Finland.

Data accessibility. All data analysed in this paper are publicly available from the Dryad: http://dx.doi.org/10.5061/dryad.21iq1.

Authors’ contributions. A.K., L.-R. and K.-R.L. designed and performed the experiments; J.J. and K.-R.L. analysed the results. K.-R.L. wrote the first draft of the manuscript, and all authors contributed substantially to revisions and approved the final version.

Competing interests. We declare we have no competing interests.

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