1 **Do coinfections maintain genetic variation in parasites?**

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Host individuals are often infected with multiple, potentially interacting parasite species and genotypes. Such coinfections have consequences for epidemiology, disease severity and evolution of parasite virulence. As fitness effects of coinfection can be specific to interacting parasite genotypes, coinfections may induce high fitness variation among parasite genotypes. We argue that such interactions can be an important mechanism maintaining genetic variation in parasite traits such as infectivity and virulence. We also argue that such interactions may slow coevolutionary dynamics between hosts and parasites. This is because instead of depending only on host genotype, parasite fitness may be determined by average infection success across all coinfection scenarios.

Maintenance of genetic variation in host–parasite interactions

High within-population genetic variation in host resistance (see Glossary) and immune function [1-6], as well as in parasite infectivity [2, 4, 6-8], is commonly found across a broad range of host–parasite interactions. This seems paradoxical because such traits are important components of fitness, and heritable genetic variation in them should be eroded by natural selection [9]. From host perspective, strong selection on resistance to infections can be expected as parasites reduce host survival and fecundity [10]. Similarly, successful infection and within-host growth is required for positive fitness in parasites. Thus, selection should sweep the best gene variants to fixation in the host and parasite populations, and lead to low genetic variation in defense and infection related traits. This, however, is not what is observed [1-8]. The question is what maintains genetic variation in host and parasite traits?

Negative frequency-dependent selection has been discussed as one key factor maintaining genetic variation in host and parasite populations [11]. In coevolutionary models, parasites that are able to infect the most common host genotype(s) have the highest fitness, rendering a fitness advantage to rare host genotypes [12, 13]. This leads to fluctuating host

and parasite genotype frequencies known as the Red Queen dynamics (RQD, Box 1), which can maintain genetic variation in the interacting populations [13]. Being very difficult to study, RQD has only rarely been demonstrated in natural populations [14, 15]. However, a tight genetic specificity determining the outcome of infections, and thus opportunity for RQD, has been shown in many systems [2, 4, 16], suggesting that RQD might be more common than directly shown.

Many host and parasite traits are, however, not specific to the genetic identity of the interacting partners (e.g. nonspecific immune function) [17, 18]. Such traits evolve through selection on heritable additive genetic variance [3, 5] and call for other mechanisms than negative frequency-dependent selection to explain the maintenance of genetic polymorphism. For example, spatial and temporal heterogeneity of the environment shaping the outcome of host–parasite interactions is widely acknowledged to promote maintenance of genetic variation in host defenses against parasites [11, 19]. This is because genetic variation in host resistance often interacts with environmental variation so that genotype rank order for susceptibility to infections or immune function changes in relation to environmental conditions [i.e. different genotypes are favored by parasite-mediated selection in different environments; genotype-by-environment ($G \times E$) interaction; Figure 1] [20–23]. Studies investigating $G \times E$ interactions in determining host resistance have covered a range of environmental factors and discovered strong effects of primary environmental parameters [11, 19]. For example, in a study by Mitchell and coworkers [21], clone rank order of the aquatic crustacean *Daphnia magna* for infection by the bacterial parasite *Pasteuria ramosa*, as well as for fitness losses caused by the infection, changed dramatically depending on water temperature. Such strong $G \times E$ interactions indicate context dependent fitness variation among genotypes that can effectively maintain genetic polymorphism. How significant $G \times E$ interactions are in maintaining genetic variation in parasites is still largely unknown. So far,

genotype-specific responses of parasites to ambient temperature as well as to food quality for hosts in determining parasite performance have been reported in few systems [6, 8, 24-26].

In this opinion article, we review the recent research on genetic specificity in interactions among coinfecting parasites, and argue that fitness variation created by such interactions in variable coinfecting communities in nature may be one key mechanism maintaining genetic variation in parasite populations. Furthermore, we propose that genetically heterogeneous interactions with direct consequences on parasite fitness may influence also other evolutionary processes such as evolution of virulence and host–parasite coevolutionary dynamics. Research on evolutionary consequences of coinfections for parasite fitness and disease dynamics is already an important field in host–parasite research and we believe that this research direction has potential to address some of the key open questions in host–parasite evolutionary ecology.

Coinfections as a source of fitness variation in parasites

In nature, individual hosts are often simultaneously infected with multiple parasite species and genotypes that interact [27, 28]. Since hosts represent the primary environment for parasites, the presence of coinfecting parasite species/genotypes can potentially modify their fitness the same way as the above-mentioned environmental factors alter the performance of host genotypes. In fact, coinfections have recently been shown to induce high variation in the relative fitness of parasite genotypes through genetically specific interactions among coinfecting partners [29-32].

For example, *Diplostomum* (Trematoda) parasites infecting freshwater fishes are typically observed in multiple-species [33] and in multiple-genotype [34] infections. In this system, interactions between genotypes of two species, *Diplostomum pseudospathaceum* and *Diplostomum gasterostei*, have a strong genetic basis that modifies the relative infection

success of *D. pseudospathaceum* (but not of *D. gasterostei*) genotypes between single and multiple infections [30, 31]. This alters the rank order of *D. pseudospathaceum* genotypes for infectivity depending on the presence/absence of *D. gasterostei* in the same fish individuals. Thus, the effect of coinfections on the relative fitness of parasites and maintenance of genetic polymorphism can have similar effects to the $G \times E$ interactions affecting host resistance. The effect of coinfections on the infection success of *D. pseudospathaceum* is, however, dose dependent, and observed in experimental exposures only when a high exposure dose (100 infective stages of each parasite species per fish) is used [31]. With a lower exposure dose (50 infective stages of each parasite species per fish), the effect of coinfections appears even more complex. In low exposure dose, the effect of coinfection on parasite performance becomes dependent on the genetic identity of both interacting partners, and the relative fitness of *D. pseudospathaceum* genotypes varies also among different multiply infected host individuals [31]. Since fish are most likely exposed to low numbers of parasite larvae simultaneously, the latter scenario probably dominates in this system in the wild. Furthermore, the effect of coinfections in changing the fitness rank of *Diplostomum* parasites is not limited to interactions between closely related taxa. In a recent study, Louhi and coworkers [32] showed that interactions with Flavobacteria strains also modify the infection success of *Diplostomum* genotypes in a strain-specific manner. These results suggest that although the infectivity of *Diplostomum* parasites seems genotype specific, there is ample variation in the relative success of different genotypes that depends on coinfections.

In addition to *Diplostomum* parasites of fish, the competitive ability of strains of the protozoan *Eimeria tenella* in double-genotype infections in chickens has been shown to be difficult to predict from their performance in single-genotype infections [29]. To our knowledge, the only test on the potential of coinfections to maintain genetic diversity in natural parasite populations comes from a tick-transmitted bacterium *Borrelia afzelii* infecting

bank voles. In this system, Andersson and coworkers [35] demonstrated that coinfections with multiple bacterium strains are more common than expected by chance, and most importantly, that strains that are antigenically more different are also more strongly associated [35]. Such an effect may maintain genetic polymorphism and take place if increased heterogeneity of infection leads to less effective host defenses [36, 37]. Taken together, the above examples suggest that genetically specific interactions in coinfections can create high fitness variation in parasites. This can be potentially very important in maintaining genetic polymorphism in natural parasite populations that experience high between-host variation in the genetic composition of coinfecting parasite communities (i.e. different host individuals are infected with different parasite species and genotypes). This is because such effects can create fitness variation among parasites in a very small scale (among host individuals) compared to other environmental factors (e.g. ambient temperature) that have similar effects on parasites across numerous hosts in a shared locality.

Experimental studies providing most of the evidence for fitness variation generated by coinfections are, however, limited only to interactions between two coinfecting partners [29-32]. Although the described effects are strong, we propose that the role of coinfections in inducing fitness variation in natural parasite populations may be even larger. This is because within-host parasite communities of many host species consist of a much higher number of different parasite species and genotypes than what have been investigated experimentally [27, 34, 35, 38]. Furthermore, these communities typically show high variation among host individuals. Thus, interactions among coinfecting parasites are potentially very complex and variable in natural settings. Since fitness variation induced by them can be assumed to increase with the number of interacting partners (Box 2), coinfections may even become one of the most significant factors maintaining genetic polymorphism in parasites. Unfortunately, it is still largely unclear how common such heterogeneous interactions are in nature. This

would be important to understand both across various study systems and parasite traits to reveal the full potential of coinfection in maintaining genetic variation in parasite populations.

Implications for evolution of virulence

To our knowledge, studies on the genetic specificity in interactions among coinfecting parasites have largely focused on parasite infectivity (see above). Heterogeneous interactions could, however, also affect numerous other parasite traits. One of such traits that has also received wide interest in evolutionary ecological research is parasite virulence [32, 39]. In general, interactions among coinfecting parasites have been proposed to promote evolution of parasite traits that relate to high host exploitation, thus leading to high virulence [40, 41]. In single infections, evolution of host exploitation by parasites is predicted to arise from its positive and negative effects on different components of parasite fitness. Parasites need to exploit host resources for their within-host growth and reproduction in order to produce enough transmission stages to enable between-host transmission. High host exploitation rate, however, increases the mortality of their present hosts, thus reducing transmission opportunities (trade-off hypotheses) [42, 43]. Therefore, parasite fitness is often assumed to be highest at intermediate levels of virulence. In coinfections, however, parasites can compete for the same within-host resources. Several theoretical models predict parasites with the highest host exploitation rate to be the most successful in exploitation competition, which then leads to evolution towards higher optimal level of virulence when coinfections are common [44-46]. Despite of strong theoretical support, empirical evidence for coinfections selecting for increased virulence is, however, scarce [47-49].

In addition to exploitation competition, coinfecting parasites can interact through several other mechanisms such as interference and host immune responses [50-52]. When competition among parasites takes place through interference mechanisms rather than

exploitation, the predictions of the effect of coinfections on the evolution of virulence can change dramatically [40, 46, 51, 53]. This is because interference competition requires parasite traits that harm competitors such as production of toxins. Because within-host selection under interference competition can favor high investment in interference-related traits, and such traits are likely to be energetically costly to the producer (e.g. reduce growth and/or reproduction), parasite virulence may evolve towards lower optimal level in such systems. Therefore, it is highly important to understand under which kind of competitive interactions parasites occur in the wild, and if this varies across different host individuals depending on the coinfecting parasite community. For example, if parasites compete for common host resources exploitatively in multiple genotype infections, but by interference in multiple species infections, this can lead to considerable variation in which parasite traits are under selection in different host individuals, and thus modify selection on virulence [40, 53]. Such context dependence could favor different host exploitation strategies in single infections, in multiple-genotype infections, and in multiple-species infections. This variation could maintain genetic polymorphism in virulence in a parasite population and slow down its evolution towards the direction of the most dominant selection gradient.

The best evidence supporting this idea comes from mixed infections of two nematode species (*Steinernema* spp.) infecting insects [49]. These nematodes live in soil and have bacterial symbionts that enable parasitism. Nematodes release bacteria into their hosts, which increases virulence and enhances nematode reproduction after host death [54]. Interestingly, the interaction between the nematode and the bacteria is specific, nematode performance being reduced with nonnative bacteria [55]. These symbionts also produce toxins that kill other bacteria, thus enabling interference competition between coinfecting nematodes. The ability of bacterium strains to interfere with each other, however, varies greatly. This defines the competitive ability of nematodes and also selection on virulence in

multiple infections. When nematodes carry bacteria that do not release toxins, the most virulent nematode is the most successful [49]. However, nematodes carrying bacteria able to inhibit the bacteria of their competitor have a competitive advantage independently of their virulence [49]. Similar effects are proposed also in the fungus *Metarhizium anisopliae* that infects insects [48]. Under intraspecific competition, strains with the highest virulence show the highest competitive ability (i.e. the smallest reduction in performance when compared to single infections) [48]. In mixed-species infections with a nematode, *Steinernema feltiae*, less virulent strains of the fungus show least reduction in their performance [48]. This study, however, does not directly test the relationship between parasite virulence and fitness. Although the most virulent strains show the largest proportional reduction in their performance in mixed-species infections, their fitness may still remain higher than that of the less virulent strains that show smaller proportional reduction in performance.

Implications for host–parasite coevolution

In addition to the effects of genetically specific interactions among coinfecting parasites on evolution of specific parasite traits, such interactions can potentially have wider coevolutionary consequences on host–parasite interactions. The RQD theory describes parasite fitness as a specific match/mismatch among parasite and host genes that allow/prevent infection [12, 13] (Box 1). Here, the fitness of a parasite genotype is determined by the frequency of the matching host genotype, and the evolutionary dynamics are governed by frequency-dependent selection in the coupled host and parasite populations. Evolutionary dynamics determine the probability for the same gene combinations of the parasite and the host to meet in the next generation. Contrary to the RQD theory, quantitative genetic theory of phenotypic evolution that relies on additive genetic variance considers both

genetic and non-genetic variation [56]. However, quantitative genetics is applied rarely in studies on host–parasite coevolution [10, 57].

In the case of coinfections, empirical data that we reviewed above suggest that the fitness of each parasite genotype is affected by the genotypes of coinfecting partners. This is a recognized form of among-individual epistatic fitness variation where genes in another individual determine the value of one’s own genes [58]. However, the role of this epistatic component in determining the fitness of an individual parasite is largely unpredictable owing to high variation in coinfecting parasite communities among different hosts. This is because in horizontal transmission, coinfecting parasite genotypes are uncoupled in each generation and there are very few ecological and evolutionary processes that would secure them sharing a host in the next generation. Therefore, the effects of coinfection on fitness are unpredictable and selection is unlikely to operate on such variation.

Parasite life-history traits could, however, respond to stochastic fitness variation by favoring high reproductive output that ensures dispersion of produced offspring across multiple host individuals and thus coinfection scenarios. In such bet-hedging strategies [59, 60], the parasite bets against multiple coinfection scenarios by sending a high number of offspring to interact with other parasite species/genotypes in numerous host individuals. Selection favors parasite genotypes that have the highest average infection success across various interactions with other players. In other words, high number of transmission stages may be the best way to deal with unpredictability of fitness. Not surprisingly, many parasite life-cycles exhibit this type of reproductive effort [61]. Such strategies do not rely on specific coevolution between host and parasite, but parasite infection success can behave as a quantitative trait in which variation induced by coinfecting parasites can be seen as noise around the mean infection success which is predictable for each parasite genotype. At the

moment, however, we do not know how such unpredictable interactions among coinfecting parasites influence Red Queen type coevolutionary dynamics.

Concluding remarks

Simultaneous infections with multiple parasite species and genotypes are a rule in nature [27, 28]. Interactions among coinfecting parasites are acknowledged to contribute to many important ecological and evolutionary processes from epidemiological dynamics to evolution of virulence. Recent evidence shows that the outcome of interactions among coinfecting parasites can be highly specific to the genetic identity of the interacting partners [29-32, 35]. This induces high variation in the relative fitness of parasite genotypes between singly and multiply infected hosts, as well as among different multiply infected host individuals. The latter effect arises from the differences in the coinfecting communities among individual hosts. Moreover, these effects can be assumed to become stronger when the number of interacting parasites in a single host individual increases (Box 2.). Due to all these aspects we argue that interactions among coinfecting parasites are a key mechanism maintaining genetic variation in parasites.

Additionally, we propose that variation in interactions among coinfecting parasites may significantly alter the predictions of two key processes in host–parasite evolution, namely evolution of parasite virulence and coevolutionary dynamics. Classically, coinfecting parasites are assumed to interact through exploitation competition and selection to favor high virulence [44, 45, 47]. However, this prediction changes dramatically when parasites interact, for example, through interference [51]. Therefore, understanding how selection operates on virulence requires understanding the variation in coinfecting communities experienced by the whole parasite population. Specifically, we propose that it would be important to measure how variation in host exploitation/virulence covaries with parasite fitness across hosts

experiencing different parasite communities. This can be studied experimentally by manipulating the parasite community structure. If covariance becomes weak due to stochasticity introduced by the parasite community structure, it complicates predictions for evolution of virulence. If the covariance remains strong, predictions for evolution of virulence are robust. Furthermore, the RQD theory of host–parasite coevolution predicts parasite fitness to be determined by a match/mismatch of host and parasite genes [12, 13] (Box 1). Unpredictability in parasite fitness caused by coinfections may significantly modify such coevolutionary interactions. Due to commonness of coinfections, high variation in the outcome of interactions among coinfecting parasites, and high reproductive output of most parasite species, we propose that host–parasite coevolution may not rely solely on specific genetic interactions between parasites and their hosts, but selection operates on the average fitness of each parasite genotype across all coinfection scenarios. Thus, parasite infectivity could evolve as a quantitative trait. How genetic interactions among coinfecting parasites influence Red Queen type coevolutionary dynamics between hosts and parasites is, however, not tested. We argue there is a need to incorporate coinfections into future theoretical and empirical studies on host–parasite coevolution.

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299 **Glossary**

300 **Additive genetic variance:** the magnitude of the phenotypic variance that is due to additive
301 effects of genes (alleles do not affect the expression of each other). Additive genetic variance
302 determines the degree to which parents' mean phenotype is reflected to offspring.

303 **Coevolution:** reciprocal evolution of two or more interacting partners. Evolutionary change
304 in one organism alters the selective pressure on others and vice versa.

305 **Fitness:** the relative contribution of an individual to the gene pool of the next generation. In
306 other words, individual's capacity to survive and reproduce when compared to competing
307 individuals.

308 **Infectivity:** the ability of parasites to successfully invade and establish into their hosts.

309 **Natural selection:** differential survival and reproductive success of individuals due to
310 differences in phenotype (i.e. relationship between phenotypic and fitness variation).

311 **Frequency-dependent selection:** a type of selection in which the fitness of each
312 phenotype/genotype depends on its frequency relative to other phenotypes/genotypes in the
313 population.

314 **Parasite:** an organism that lives in or on another organism (host), and at least partly exploits
315 its resources from the host.

316 **Resistance:** the ability of hosts to prevent parasites from infecting them.

317 **Stochastic:** either wholly or partially randomly determined.

318 **Virulence:** degree of harm (i.e. reduction in fitness) caused to the host by a parasite.

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Figure 1. Hypothetical effects of environmental conditions on susceptibility to infection in two host genotypes (each host genotype is connected between the two environments using a reaction norm). (A) Environment affects the susceptibility of hosts to infection, but the effect is similar in both genotypes. (B) Environment affects the susceptibility of hosts to infection, and the effect differs between the genotypes (crossing reaction norms indicate significant genotype-by-environment interaction that changes the fitness rank).

Box 1

Red Queen hypothesis

Consider interacting host and parasite populations that are both genetically diverse. Also assume that the parasite has to precisely match the host genotype to be able to infect it. Otherwise host immune system will recognize and eliminate the parasite. In such a situation, parasites that can successfully infect the most common host genotypes in the population are most likely to complete their life-cycles and produce propagules to the next generation. Thus, they have the highest fitness and are favored by natural selection. These genotypes will become more frequent in the parasite population over time, thus increasing the risk of infection of the common host types. If these parasites are also virulent (i.e. reduce host fitness), this reduces the reproductive capacity of the common host genotypes. This gives them a selective disadvantage compared to the rare host genotypes that are not under strong selective pressure owing to parasitism. Over time, this difference in selection will drive the frequencies of common host genotypes down, and some previously rare host genotype(s) become common. The change in genotype frequencies in the host population in turn changes the selective pressure on parasites as genotypes that are able to infect the new common host types become favored by selection. The described coevolutionary interaction can lead to cycling of both host and parasite genotype frequencies in the populations (Figure I), thus maintaining genetic variation in both interacting partners. These cycles are, however, not identical, but show a time lag. This is because each partner of the interaction cannot immediately respond to the changes in the genotype frequencies of the other. Instead, a change in host/parasite genotype frequencies will change the selective pressure on parasites/hosts, which can then respond to this selection over time. The term “Red Queen dynamics” for these fluctuations has been adopted by Bell [62] from the Red Queen’s

comment to Alice in *Through the Looking Glass* [63]: “Now here, you see, you have to run as fast as you can to stay in the same place”.

Figure I. Hypothetical genotype frequencies of two host (A and B; black and gray solid lines, respectively) and two parasite (a and b; black and gray dashed lines, respectively) genotypes under coevolution. An exact match between the host and parasite genotypes is expected for infection (i.e. parasite a infects host A and parasite b infects host B).

Box 2

Fitness variation in relation to complexity of community

Additive effects of interactions

Consider a parasite genotype that infects its hosts either in single infections or in coinfections with any number of other parasite genotypes (same and/or other species). In single infections, fitness W of this parasite genotype is expected to be equal or larger than zero, and to follow any distribution with positive variance [i.e. $\mathbb{E}(W) > 0$ and $\text{Var}(W) > 0$]. In coinfections, interactions with other parasites additively increase or decrease its fitness and this effect has positive variance [i.e. $\text{Var}(I) > 0$]. For simplicity, assume that the interactions are not so strongly antagonistic that they would generally reduce the fitness of the focal parasite to zero, which is supported by empirical data [29-32].

Variance in the expected fitness of the focal parasite when coinfecting a host with k other parasites can be written using a rule for variance of the sum of variables (Bienaymé equality [64]), and is

$$\text{Var}(W + kI) = \text{Var}(W) + k\text{Var}(I).$$

This variance increases with the number of interacting partners if

$$\text{Var}(W) + k\text{Var}(I) < \text{Var}(W) + (k + 1)\text{Var}(I).$$

This simplifies to

$$0 < \text{Var}(I),$$

which is always true when interactions among coinfecting parasites are variable.

Multiplicative effects of interactions

Consider a similar focal parasite as above. Assume that interactions among parasites in coinfections have multiplicative effects, and that these effects follow any distribution with the minimum value of zero (interactions do not lead to negative fitness) and positive variance [i.e. $\text{Var}(I) > 0$].

Variance in the expected fitness of the focal parasite when coinfecting a host with k other parasites can be written using a rule for variance of the product of variables [65], and is

$$\text{Var}(WI^k) = [\text{Var}(W) + \mathbb{E}(W)^2][\text{Var}(I) + \mathbb{E}(I)^2]^k - \mathbb{E}(W)^2[\mathbb{E}(I)^2]^k.$$

The equation shows the effect of interactions among parasites on the variance in fitness of the focal parasite including both a ‘mixing’ term $\text{Var}(I)$ and a ‘scaling’ term $\mathbb{E}(I)$. More specifically, $\text{Var}(I)$ shows the effect of interactions on variance in parasite fitness due to variation in interactions, whereas $\mathbb{E}(I)$ only rescales parasite performance. $\mathbb{E}(I)$ cannot change the relative fitness of parasite genotypes and it can be removed from the equation by setting it to one. After that, the role of ‘mixing’ term $\text{Var}(I)$ can be estimated directly and the equation is written as

$$\text{Var}(WI^k) = [\text{Var}(W) + \mathbb{E}(W)^2][\text{Var}(I) + 1]^k - \mathbb{E}(W)^2.$$

This variance increases with the number of interacting partners if

447 $[\text{Var}(W) + \mathbb{E}(W)^2][\text{Var}(I) + 1]^k - \mathbb{E}(W)^2 < [\text{Var}(W) + \mathbb{E}(W)^2][\text{Var}(I) + 1]^{k+1} -$
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452 $[\text{Var}(W) + \mathbb{E}(W)^2][\text{Var}(I) + 1]^k - \mathbb{E}(W)^2 < [\text{Var}(W) + \mathbb{E}(W)^2][\text{Var}(I) + 1]^k [\text{Var}(I) +$
 453 $1] - \mathbb{E}(W)^2,$

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