

ORIGINAL ARTICLE

Dissecting the genetic architecture of a stepwise infection process

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Abstract

How a host fights infection depends on an ordered sequence of steps, beginning with attempts to prevent a pathogen from establishing an infection, through to steps that mitigate a pathogen's control of host resources or minimize the damage caused during infection. Yet empirically characterizing the genetic basis of these steps remains challenging. Although each step is likely to have a unique genetic and environmental signature, and may therefore respond to selection in different ways, events that occur earlier in the infection process can mask or overwhelm the contributions of subsequent steps. In this study, we dissect the genetic architecture of a stepwise infection process using a quantitative trait locus (QTL) mapping approach. We control for variation at the first line of defence against a bacterial pathogen and expose downstream genetic variability related to the host's ability to mitigate the damage pathogens cause. In our model, the water-flea *Daphnia magna*, we found a single major effect QTL, explaining 64% of the variance, that is linked to the host's ability to completely block pathogen entry by preventing their attachment to the host oesophagus; this is consistent with the detection of this locus in previous studies. In susceptible hosts allowing attachment, however, a further 23 QTLs, explaining between 5% and 16% of the variance, were mapped to traits related to the expression of disease. The general lack of pleiotropy and epistasis for traits related to the different stages of the infection process, together with the wide distribution of QTLs across the genome, highlights the modular nature of a host's defence portfolio, and the potential for each different step to evolve independently. We discuss how isolating the genetic basis of individual steps can help to resolve discussion over the genetic architecture of host resistance.

KEYWORDS

defence cascade, genetic architecture, host–pathogen interactions, infectious disease, *Pasteuria ramosa*, resistance, virulence

1 | INTRODUCTION

Infectious diseases are predicted to be a powerful selective force that shape the genetic architecture of their hosts (Karlsson, Kwiatkowski, & Sabeti, 2014; Prugnolle et al., 2005; Scanlan et al.,

2015). Through a combination of linkage and association studies, polymorphisms associated with the defence against infection have been identified in a diverse array of organisms. A common finding of these studies is that the capacity to fight infection is often under the control of a relatively simple genetic architecture (Casanova,

2015; Hill, 2012; Wilfert & Schmid-Hempel, 2008). In *Drosophila*, for example, a large proportion of the heritable variation in viral resistance can be accounted for by a single polymorphism (Cogni, Cao, Day, Bridson, & Jiggins, 2016; Magwire et al., 2012), while in plants, gene-by-gene interactions often explain most variation in pathogen resistance (Thompson & Burdon, 1992; Young, 1996). Yet this stands in contrast to the quantitative genetic nature of other complex traits, such as morphological traits and life-history traits, where many alleles of small effect are expected to contribute to genetic variability (Hill, Goddard, & Visscher, 2008). These contrasting results have fuelled debate as to whether the genetic basis of susceptibility to infection is inherently different from that of other complex traits or noncommunicable diseases (Casanova, 2015; Hall, Bento, & Ebert, 2017; Hall & Ebert, 2013; Hill, 2012), with important consequences for the evolution of these traits (as first raised by Haldane, 1949). Yet how well do we understand the complete genetic architecture of how a host can defend against pathogen attack?

Beginning with the initial contact between a host and pathogen, and ending with the transmission of the pathogen to a new host, are multiple mechanisms that interact to determine a host's susceptibility to infection (i.e., defence portfolios: van Baalen, 1998; Schmid-Hempel & Ebert, 2003; Hall et al., 2017; Lievens, Perreau, Agnew, Michalakis, & Lenormand, 2018). A simplified genetic basis is often predicted for steps that occur early in the infection process, whereby having a resistance allele may completely block infection, commonly referred to as "qualitative resistance." This response is typical of the all-or-nothing interaction between many plant species and various pathogens and is often linked to processes involved in the recognition of a pathogen-produced elicitor (Corwin & Kliebenstein, 2017; Flor, 1971; Frank, 1994; Thompson & Burdon, 1992). In contrast, the subsequent components of a host's defence cascade (i.e., secondary lines of defence) are more likely to reflect multiple minor alleles and environmental influences. Here many different pathways contribute to the control of pathogen proliferation, such as the host immune system, the availability of resources that a pathogen might exploit, and the general vigour or quality of a host, giving rise to a continuous distribution of possible infection outcomes known as "quantitative resistance" (Corwin & Kliebenstein, 2017; Krebs, Routtu, & Ebert, 2017; de Noij & van Damme, 1988; Poland, Balint-Kurti, Wissler, Pratt, & Nelson, 2009). A particular defence mechanism may not be completely effective, but instead relate to the ability to recover from infection or limit the deleterious fitness costs associated with the growth of a pathogen (see also tolerance; Råberg, Graham, & Read, 2009).

Therefore, not all components of the infection process necessarily conform to the same genetic architecture and as a consequence the influence of each step on host and pathogen co-evolution will vary (reviewed by Hall et al., 2017). Qualitative resistance steps, for example, are commonly associated with gene-for-gene and matching allele models of host–pathogen co-evolution (Lambrechts, Fellous, & Koella, 2006; Salathé, Kouyos, & Bonhoeffer, 2008). Owing to the intense selection that results from a host's ability to completely block a pathogen, these types of mechanisms lead to rapid selection for

either universally resistant host and infective pathogen genotypes (gene-for-gene model, unless costs are invoked), or the negative frequency-dependent selection of host and pathogen alleles (matching allele model). In contrast, the contribution of many more minor alleles to quantitative resistance steps enables this type of resistance to evolve to any intermediate level (Gandon & Michalakis, 2000; Nuismer & Dybdahl, 2016), over longer timescales. Theory, however, also predicts that multiple steps of infection can combine to influence the evolutionary dynamics of hosts and pathogens (Agrawal & Lively, 2003; Fenton, Antonovics, & Brockhurst, 2012; Nuismer & Dybdahl, 2016). Depending on the costs of different resistance mechanisms for a host, either one single step can dominate the co-evolutionary process, regardless of the genetic basis of subsequent steps in the infection process (Agrawal & Lively, 2003; Fenton et al., 2012), or correlated changes in host alleles can occur across functionally independent steps (Fenton et al., 2012).

Despite the importance of individual infection steps and their genetic basis for host–pathogen co-evolution, empirically detecting the unique genetic and environmental signatures of each step remains challenging as events that occur earlier in the infection process can easily overwhelm any step that follows. Take, for example, the changes in host survival that are commonly used as a metric of host susceptibility. As an end point of the entire infection process, variability in survival following pathogen exposure will depend on the ability of a pathogen to first enter the host, then establish infection, proliferate and finally terminate the infection (see discussions in Hall et al., 2017; Schmid-Hempel & Ebert, 2003). If one step in this cascade is influenced by all-or-nothing resistance alleles, then all subsequent estimates of host susceptibility, including survival, will reflect the variation imposed by this major-effect gene. This ability of large-effect loci, acting early in the infection process, to dominate the genetic signals of subsequent infection steps is recognized in the plant breeding literature (Corwin & Kliebenstein, 2017; Poland et al., 2009; Roux et al., 2014), but the relative contributions of qualitative and quantitative resistance mechanisms in animal populations is rarely considered (but see Gates, Valletta, Bonneaud, & Recker, 2018).

Partitioning the infection process into different components holds the key to resolving the genetic architecture of the entire defence cascade and consolidate our understanding for how different defence components evolve (see also Davis, Meconcelli, Radek, & McMahon, 2018; Duneau et al., 2017; Lievens et al., 2018). In this study, we dissect the genetic architecture of a stepwise infection process using a bacterial pathogen of the water-flea *Daphnia magna*; one of the few model systems with enough knowledge and experimental tools available to do so. Contributing to disease in this system are multiple steps, beginning with the initial contact between the host and pathogen that occurs during feeding (Table 1). Each step varies in its sensitivity to genetic and environmental sources of variation, and occurs at a different time point during infection (reviewed by Ebert et al., 2016). Infection success, for example, depends largely on the all-or-nothing opportunity for a pathogen to attach to the host oesophagus, penetrate and enter the host (Duneau, Luijckx, Ben

Infection step	Characteristics	Traits assessed herein
1. Exposure	Behavioural differences among hosts can influence the likelihood of encountering spores via filter-feeding.	
2. Activation	Spores activate and release outer spore shell (exosporium) upon contact with the host. No genetic variation detected in <i>Daphnia</i> clones or species so far.	
3. Attachment	Activated spores attach to gut wall of oesophagus. A host can impede attachment. Varies with host and pathogen genotype. No nongenetic variation detected in <i>Daphnia</i> clones so far.	A. Attachment of the pathogen to the host oesophagus (i.e., host susceptibility, yes or no)
4. Penetration	Attached spores penetrate into the body cavity and haemolymph of the host.	
5. Early within-host	The host immune system interacts with the pathogen to prevent infection. <i>Pasteuria</i> acts to establish a successful infection and castrate the host. The host can attempt to clear infection or mitigate the loss of reproduction by investing in early reproduction before castration acts (i.e., fecundity compensation).	B. Proportion of replicates for each susceptible genotype in which infection established (i.e., infection success) C. Size of first clutch (i.e., fecundity compensation).
6. Late within-host	Late phase of chronic infection. A host can act to prevent the complete control of reproduction (i.e., castration relief) and limit gigantism.	D. Adult body size (i.e., gigantism) E. Proportion of replicates that temporarily regained control of reproduction (i.e., castration relief).
7. Termination	A host dies from infection 30–70 days after exposure. <i>Daphnia</i> clones vary in the length of time that an infection is maintained and the total amount of offspring they can produce until they die.	F. Lifespan and infection duration G. Lifetime fecundity

Note: The broad characteristics of the steps are described as well as the trait measurements used in this study for each component that shows genetic variability in the QTL mapping panel.

TABLE 1 A summary of the steps involved in the infection process of *Pasteuria ramosa* in *Daphnia magna* as previously outlined by Ebert et al. (2016, see references therein)

Ami, Laforsch, & Ebert, 2011; Luijckx, Ben Ami, Mouton, Pasquier, & Ebert, 2010), followed by more quantitative steps related to the capacity of the pathogen to establish in the host's body cavity (Hall & Ebert, 2012; Michel, Ebert, & Hall, 2016). The severity of disease is in turn influenced by the intensity and efficiency of parasite-mediated host fecundity suppression, the control of pathogen growth, and the mitigation of any reduction in survival imposed by the pathogen (Clerc, Ebert, & Hall, 2015; Hall & Mideo, 2018; Michel et al., 2016; Vale & Little, 2012).

Here, we use a quantitative trait locus (QTL) approach to identify the chromosomal regions underlying the components of the defence cascade, from the initial contact between the host and pathogen, to the transmission of the pathogen at host death. Work on the highly specific step underlying host susceptibility has already revealed that this attachment step is under the control of a limited number of regions in the host genome (Bento et al., 2017; Luijckx, Fienberg, Duneau, & Ebert, 2013; Metzger, Luijckx, Bento, Mariadassou, & Ebert, 2016), consistent with the idea that the

genetic architecture of this step is unlike other complex traits. Yet, the other components of the infection process remain uncharacterized. By focusing only on susceptible hosts, we experimentally exclude the qualitative variation induced by the attachment step and expose the genetic variation in traits being influenced by subsequent steps of the infection process. We discuss the modular nature of the multistep defence cascade and the evolutionary consequences of having a step-specific genetic architecture; this topic has been the target of a recent review (Hall et al., 2017), but has rarely been approached using an empirical data set—but see Vale and Little (2009) and Lievens et al. (2018) for an interesting decomposition of pathogen fitness at the phenotypic level.

2 | MATERIALS AND METHODS

The host, *Daphnia magna* Straus, is a cyclically parthenogenetic crustacean that lives in brackish and freshwater habitats, from lakes to

small seasonal pools. The two clones used in the QTL panel are from a pond near Munich, Germany (genotype ID: linb1), and a rock pool in southwest Finland near Tvärminne (genotype ID: Xinb3). *Pasteuria ramosa* Metchnikoff 1888 is a gram-positive bacterial pathogen of *Daphnia*, found throughout the Northern Hemisphere (Ebert et al., 2016). Initial contact with the pathogen takes place via the filter feeding of the infectious *Pasteuria* spores, after which the pathogen proliferates to fill the entire body cavity with spores, and host reproduction consequently ceases (i.e., castration). Transmission occurs exclusively horizontally via the release of several million spores after the death of infected hosts (for an epidemiological model of this system see Hall & Mideo, 2018). The genotype of *P. ramosa* (genotype ID: C19) used here was isolated from an infected *D. magna* collected from a pond in Gaarzerfeld, Germany, and has been characterized in earlier studies (Clerc et al., 2015; Hall & Ebert, 2012; Luijckx et al., 2010; Michel et al., 2016).

Throughout the experiment, all animals were maintained in two climate-controlled incubators (16:8 hr light-dark, $20 \pm 0.5^\circ\text{C}$), with both the incubator and shelf position rearranged daily to avoid positional effects. To account for the increased food requirements of growing *Daphnia*, daily food supply increased from 0.5×10^6 algal cells of *Scenedesmus obliquus* per animal at day 2, to 1×10^6 , 2×10^6 , 2.5×10^6 , 3×10^6 and 5×10^6 algal cells per animal per day on days 4, 7, 9, 11 and 13, respectively.

2.1 | Experimental lines and genetic map

The experimental lines used in this study originated from a QTL mapping panel first established by Routtu, Jansen, Colson, Meester, and Ebert (2010). The panel uses an F_2 -intercross design, whereby two inbred parental clones were crossed and the resulting F_1 clone in turn self-crossed (equivalent to self-fertilization) to generate F_2 lines with recombinant genotypes. The two parental clones differ in several phenotypic characteristics (Roulin et al., 2013; Routtu & Ebert, 2015; Routtu et al., 2014), but most notably the linb1 clone (genotype AA) is completely resistant to *P. ramosa* genotype C19, whereas the Xinb3 clone (genotype BB) is susceptible. Segregation of susceptible genotypes within the broader F_2 population was approximately one-quarter—allowing phenotypic data on the severity of disease to be collected using the susceptible genotypes of the mapping panel. The first-generation linkage map utilized 214 F_2 lines genotyped at 109 microsatellite markers. This core panel was extended with a nonrandom set of F_2 clones, all being susceptible (allowing attachment), that were selected from a larger set of F_2 clones. The combined core and extended F_2 panel included more than 353 F_2 clones that were than genotyped at 1,324 single nucleotide polymorphism (SNP) markers (see Routtu et al., 2014 for details on the SNP sequence tags).

2.2 | Experimental infection trials

For each of 353 F_2 lines of the extended panel (Routtu et al., 2014), we first scored the attachment of fluorescently labelled

spores (C19 genotype) to the oesophagus of the hosts (following Duneau et al., 2011). Four individuals were assessed per host genotype, with a susceptible genotype being defined as one in which at least one replicate was positive for “attachment” (but note that none of the replicates within a genotype varied in their attachment status, an observation made earlier by Duneau et al., 2011). Subsequently, the 189 *Daphnia* clones that were identified as susceptible were used in an infection trial to characterize the outcomes of pathogen exposure. For each of the F_2 clones, we began by raising females individually in 100-ml jars for two generations to randomize maternal effects. From the third clutches of the second-generation females, six randomly chosen daughters were exposed to the pathogen, and three additional individuals chosen to act as unexposed controls ($189 \text{ clones} \times [6 \text{ exposed} + 3 \text{ controls}] = 1,701 \text{ animals}$).

The infection trials began by placing newly born animals individually in 100-ml jars filled with 20 ml of artificial medium (Klüttgen, Dülmer, Engels, & Ratte, 1994; but modified after Ebert, Zschokke-Rohringer, & Carius, 1998). Three days later, each individual was exposed to either 10,000 spores of *P. ramosa* (six replicates), or to a control suspension (placebo; three replicates). On day 5, and every 3 days thereafter, hosts were transferred to fresh 100-ml jars filled with 80 ml of artificial medium. We monitored survival daily and counted offspring at each transfer event. Body size was measured 30 days after exposure to the pathogen using a stereo microscope. Infection success was established based on the overt visual signals of infectious disease (castration, gigantism, loss of transparency and reddish-brown colouring), with the average infection success of susceptible lines at 80%. All of the control animals remained uninfected.

2.3 | Characterizing the infection process

The infection process for the *Daphnia*–*Pasteuria* system can be broken down into seven distinct steps (Ebert et al., 2016). Table 1 describes the main characteristics of each step and indicates the traits that display genetic variation and how they can be partitioned and explored using the mapping panel used in this study. We were able to characterize the following binary outcomes at the level of an individual: (a) host susceptibility for each replicate of all 353 F_2 lines (i.e., attachment, yes or no); (b) infection success for the 189 clones that allow pathogen attachment (yes or no for each exposed individual); and (c) castration relief for infected animals (i.e., if a host reproduced again after a period of castration had taken place already). We also estimated the following quantitative traits for both control and infected animals: (d) the size of an animal's first clutch (i.e., fecundity compensation); (E) their adult body size (providing measures of infection-induced gigantism); (F) lifespan; and (vii) lifetime reproductive success. Where appropriate, we estimated the proportion of variation that was explained by the F_2 lines—equivalent to broad-sense heritabilities (H^2)—using a Bayesian Markov chain Monte Carlo (MCMC) framework using the R package MCMCglmm (Hadfield, 2010). Noninformative parameter expanded priors were used for

the random effect (the F_2 intercross line), and models were run for 200,000 interactions, with a burn in period of 25,000 and a sampling interval of 50.

For traits related to the change in host fitness when infected (Table 1, D–G: size of first clutch, adult body size, lifespan and lifetime reproductive success) we used two metrics to characterize the impact of disease on host fitness in terms of the difference between control and infected trait values. First, we estimated the difference between the control and infected values, which estimates the magnitude and direction of a host genotype's direct response to infection. This measure is potentially influenced by the general quality or vigour of the host lines. If disease results in a change in fitness that is equal for all host genotypes, for example, then the direct reduction in fitness will be perfectly correlated with the values for control animals. As a second estimate of the impact of disease, we extracted the residuals from a regression of infected values on the values of each clonal line's respective controls. This relative estimate for the impact of infection is uncorrelated with the quality or vigour of the control animals.

2.4 | QTL mapping

To identify QTLs contributing to variation in the above traits we performed Haley–Knott regressions and interval mapping, as implemented in the R package *qtl* (Broman & Sen, 2009), using the average trait values for each of the F_2 lines. The genome-wide significance level for an association between markers and phenotypes was established using 10,000 permutation tests with significant ($\alpha = 0.05$) and suggestive ($\alpha = 0.1$) QTLs identified at logarithm of odds (LOD) scores of 3.78 and 3.44, respectively. LOD (logarithm of odds) scores refer to the $\log_{10}$ likelihood ratio for the hypothesis that there is a QTL at the given marker versus the null hypothesis that there is no QTL in the genome. We began with a scan for multiple, noninteracting, QTLs using the *stepwise()* function of the *qtl* package. This procedure uses forward and backward search algorithms to identify a set of loci that contribute to trait variation, exposing loci that may have been masked by a major QTL, while controlling for the inclusion of extraneous loci (i.e., ghost QTLs) that can arise in single QTL scans. Beginning with this additive-only model, we then scanned for additional interacting QTLs, again using the *stepwise()* function, as part of a search for additional loci involved in epistatic interactions. The SNP sequence tags (from Routtu et al., 2014 and the Supplementary material therein) associated with the nearest marker to a QTL peak are presented in Table S1.

We applied binary trait models for the analysis of susceptibility (i.e., attachment, yes or no), and parametric models for all other traits, including infection success (%) and castration relief (%), where quartile normalization was first employed to meet the requirements of the *stepwise()* function. For each QTL, the effect size (assuming both additive and dominance), percentage of phenotypic variance explained by each QTL and interval estimates for the location of each QTL (1.8-LOD support, as suggested for an intercross; Broman & Sen, 2009) were estimated using the *fitqtl()* and *lodint()* functions,

respectively. Finally, we visualized the distribution of QTLs along the host genome using the *segmentsOnMap()* function of the *qtlTools* package (available from github.com/jtlovell/qtlTools). All statistical analyses were performed in R (version 3.5.0; R Development Core Team, available at www.r-project.org).

3 | RESULTS

3.1 | The genetics of preventing infection and castration

We focused first on three traits related to the probability of a host preventing the pathogen from entering the host, establish infection and controlling castration. At the level of the individual, each of these defence components is qualitative, describing the capacity of the host to block an action of the pathogen or not. Only the attachment step, however, perfectly correlated with the genotype of the host. Our results confirm previous studies that the ability of the pathogen to attach to the host oesophagus was an all-or-nothing affair (Bento et al., 2017; Duneau et al., 2011), with all individuals of a host recombinant line either allowing complete parasite attachment or not (Figure 1a). In contrast, variation in the subsequent success of the pathogen in establishing infection was more variable (Figure 1b), with the probability of all replicates of a susceptible host line becoming infected varying continuously between 0 and 1 (mean = 0.80; $SD = 0.21$). How efficiently a host was able to disrupt castration (castration relief) by the pathogen was also continuously variable (Figure 1c), with some lines able to regain control of reproduction briefly in later life, while others remained permanently castrated (mean = 0.39; $SD = 0.30$).

We characterized the genetic architecture of preventing infection and mitigating castration by searching for putative QTLs and estimating their effect sizes (Table 2). For the capacity of the pathogen to attach to the host oesophagus, we observed a single significant major QTL on linkage group 3 (Figure 2a) that accounted for 64% of the variation in susceptibility. The probability of infection establishing once the pathogen has attached (and presumably penetrated the host's oesophagus cuticle) was described by a single suggestive QTL (genome-scan-adjusted $p = .066$) located on linkage group 3 (Figure 2a), which accounted for a much smaller 8.7% of the variation in overall infection success. Finally, the likelihood of a host temporarily relieving castration was linked to two significant QTLs on linkage groups 1 and 10 (Figure 2a), explaining 16.2% and 9.4% of the variance, respectively. No epistatic interactions were detected for any of these traits.

3.2 | The genetics underlying the subsequent reduction in host fitness

Infection in this system is associated with an increase in early reproduction (i.e., fecundity compensation) and body size (i.e., gigantism), and up to an 85% reduction in overall fecundity (via castration) and almost 50% reduction in lifespan (Table 3). Our results reveal that

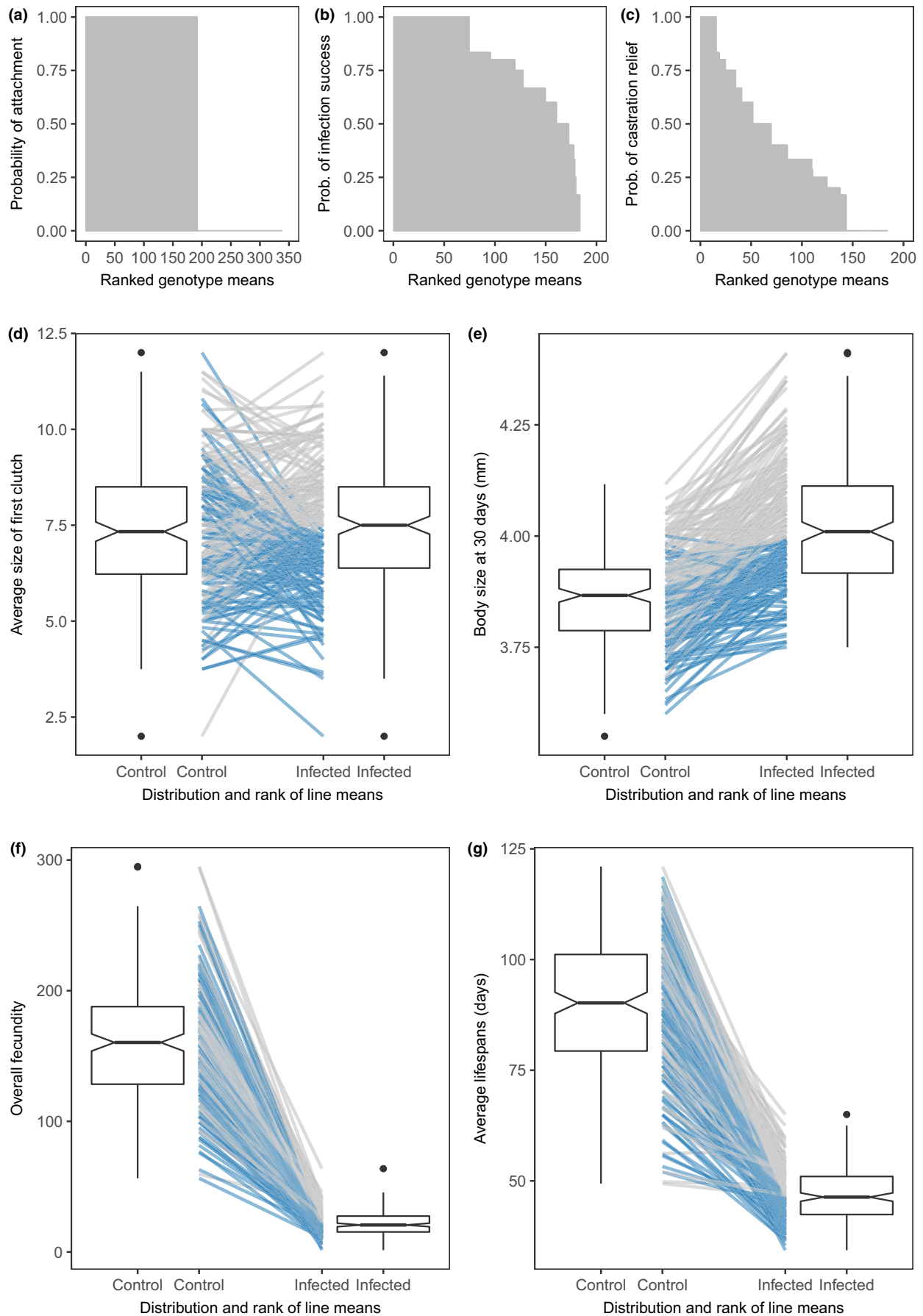


FIGURE 1 The distribution of genotypic means for each phenotype of interest. (a) The proportion of replicates that allowed the pathogen to attach to the oesophagus; (b) the proportion that became infected once the pathogen was able to attach to the host; (c) the likelihood that a host temporarily overcame castration, as estimated by the proportion of replicates that regained reproduction for a period later in life. Panels (d) to (g) compare the distribution and rank order of genotypic means of control animals and infected animals (for a comparison, grey lines represent the top 50%, and blue the bottom 50%, of genotypes as ranked by infected trait values) for all subsequent downstream metrics of host susceptibility. [Colour figure can be viewed at wileyonlinelibrary.com]

the magnitude and direction of these changes depends on the trait of interest and the genotype of the host (Figure 1d–g). On average, the size of the first clutch was hardly influenced by the infection (Figure 1d) and genotypic means between controls and infected animals were positively correlated ($r = .395$; 95% confidence interval [CI] = [0.251, 0.488]). Body size increased when infected (Figure 1e) and genotypic means between controls and infected animals were strongly and positively correlated ($r = .595$; 95% CI = [0.498, 0.677]). For both traits, there was no change in the overall expression of genetic variance when infected (Table 3, overlapping broad sense heritability, H^2 , CIs for control and infected). In contrast, for host fecundity and lifespan (Figure 1f,g), infection significantly reduced both mean fitness and the proportion of variation explained by host genotype (Table 3, nonoverlapping H^2 CIs for control and infected). Genotypic means between control and infected animals were only weakly and positively correlated (fecundity: $r = .264$; 95% CI = [0.132, 0.387]; lifespan: $r = .116$; 95% CI = [−0.022, 0.248]).

We used two different metrics to capture the genetic architecture underlying the impact of infection on host fitness. First, the direct impact of infection, as estimated by the absolute difference between controls and infected hosts, captures the capacity of a host to mitigate the damage of the pathogen, including underlying variation due to differences in genotype quality or vigour (Table 4 and Figure 2b). For this measure of resistance, we found two significant QTLs and one suggested QTL (genome-scan-adjusted $p = .064$) underlying the increase in body size, and nine significant QTLs underlying the reduction in host fecundity, that together accounted for 29.4% and 66.5% of the variability in each trait, respectively. For the reduction in host fecundity, the two QTLs on chromosome 3 (18.4 centimorgans [cM]) and 6 (58.3 cM) were found to interact with each other (LOD 10.0, 9.5% variance explained, $p < .001$). Next, the relative impact of infection, estimated via the residuals from a regression

of infected versus control trait values, describes the relative advantage of one genotype over other, after correcting for the general vigour of a genotype (Table 5 and Figure 2c). Collectively, we found three significant QTLs that explained 30.6% of the variation in relative body size, two significant QTLs that explained 20.1% of the variation in relative fecundity, and four significant QTLs that explained 33.9% of the variation in relative lifespan. No further interactions between these QTLs or other candidate loci were detected for any of these traits.

3.3 | Distribution and effect sizes of step-specific polymorphisms

The QTLs underlying a host's susceptibility at each stage of the infection process were found to be widely distributed across nine of the 10 linkage groups of the *Daphnia magna* mapping panel (Figure 3). Overall, there was very little colocalization of any QTLs for functionally distinct traits, outside a single region on linkage group 10 that was associated with variation in castration relief, relative lifespan, and both direct and relative changes in body size following infection. For both the changes in lifespan and fecundity following a successful infection, there was also no overlap between the loci detected using estimates for the direct impact of infection on host fitness (i.e., infection–control values), versus those relative metrics that controlled for variation in starting host vigour (i.e., residual estimates). For the loss of host fecundity, for example, this led to a total of 11 separate loci being detected that underlie variation in host fecundity following infection, relative to the uninfected controls. In contrast, for the increase in body size that occurs after infection, the QTL for both the direct and the relative change in body size colocalized to the same location, indicating that pathogen-induced gigantism has very little to do with the intrinsic variation in body size of the host genotype.

TABLE 2 Genomic positions and descriptive statistics for each of the putative QTLs underlying variation in the probability of a host preventing the pathogen from attaching, establishing infection, and controlling castration

LG	QTL (cM)	Nearest marker	95% CI	LOD	Additive (SE)	Dominance (SE)	% Variance
Attachment to host oesophagus							
3	157	Scaffold00288_965	154.0–161.2	74.9	4.059 (0.608)	−2.712 (0.809)	64.0
Success of the pathogen in establishing infection							
3	54.7	Contig14087_1482	40.0–152.6	3.6	0.374 (0.092)	0.168 (0.125)	8.7
Castration relief							
1	132.5	Scaffold02437_1388274	129.0–138.0	8.1	−0.431 (0.082)	−0.327 (0.116)	16.2
10	106.8	Scaffold01720_964	95.0–109.0	4.9	0.382 (0.081)	0.128 (0.116)	9.4

Note: For each QTL, the location (linkage group and location [cM]), nearest marker, 95% confidence interval of the QTL region, effect size (additive and dominance) and percentage phenotypic variance explained is shown.

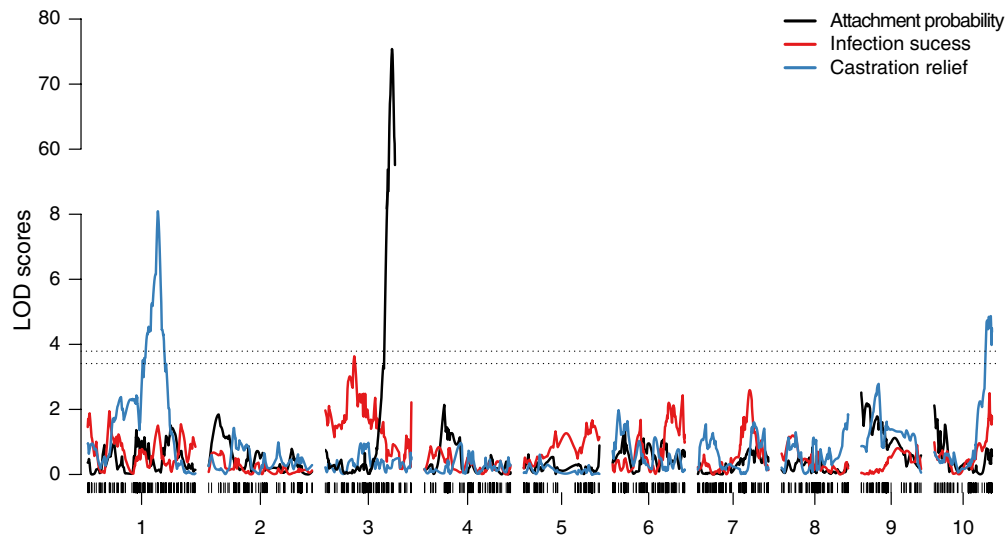
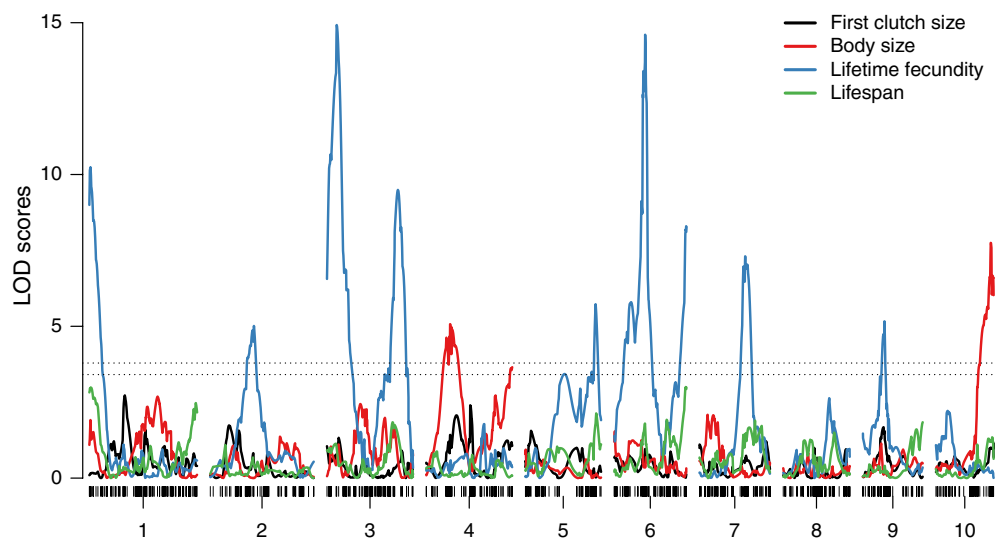
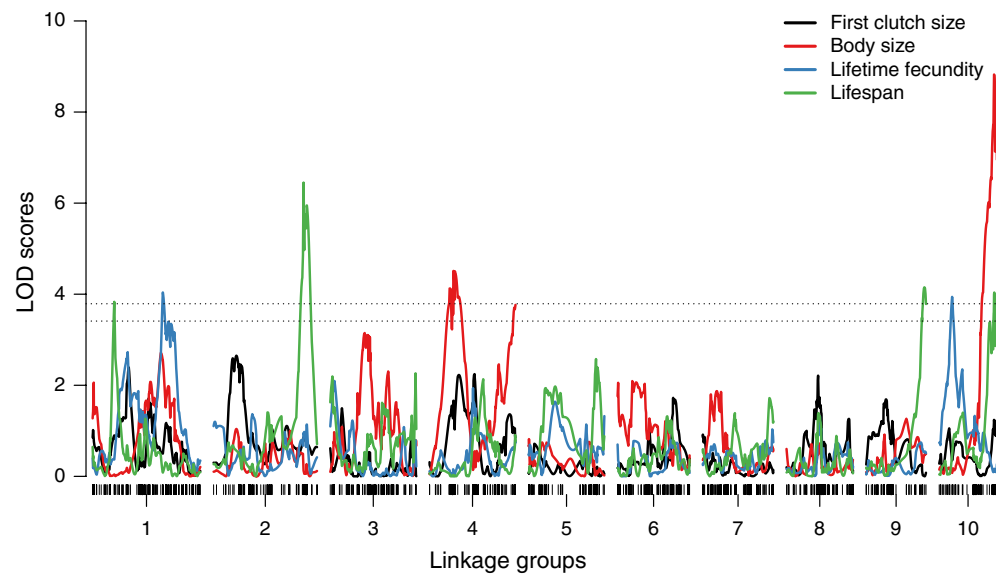
(a) Preventing infection and mitigating castration**(b) Direct impact of infection on host fitness****(c) Relative differences among infected individuals**

FIGURE 2 LOD scores along the 10 linkage groups for variation related to: (a) preventing infection and mitigating castration; (b) the direct impact of infection on host fitness; and (c) the relative differences amongst infected individuals. The direct impact metric was estimated as the difference between infected and control animals, while relative differences are the residuals after correcting for control vigour. Dashed horizontal lines indicate genome-wide thresholds for significant (LOD 3.78) and suggestive (LOD 3.44) QTLs. [Colour figure can be viewed at wileyonlinelibrary.com]

The additive and dominance effect sizes for each detected QTL are shown in their respective tables (e.g., Tables 2, 4 and 5) and the effect plots for the major QTL underling each trait are shown in Figure 4. Focusing on the major QTL identified for the quantitative resistance steps, we see that alleles from the Finnish panel parents (BB) are less likely to completely block infection (Figure 4a), lead to a 15% higher infection success in susceptible hosts (Figure 4b) and are associated with a 30% reduction in the host's ability to temporarily regain control of reproduction when castrated (Figure 4c). For subsequent quantitative steps of the infection process, many of the QTL (13 out of 19) reveal that individuals with alleles from the Finnish panel parents also perform worse when infected, remaining smaller on average (Figure 1d,g), and suffering a greater loss of fecundity when infected (both before and after controlling for vigour, Figure 1f,h). Genetic interactions between Finnish alleles at different locations further amplified the fitness costs of infection (Figure 4e). However, not all QTLs conform to the above patterns and a smaller number of QTLs were identified where Finnish alleles are associated with above average lifespan (Figure 4i) and were larger and more fecund when infected.

4 | DISCUSSION

How a host fights infection depends on an ordered sequence of events, beginning with the host's attempts to prevent a pathogen from attaching to and entering into the host, through to minimizing the damage caused during infection (Hall et al., 2017; Schmid-Hempel, 2011). To partition the step-specific genetic architecture along this infection process (see Table 1), we began by estimating the susceptibility of each host genotype during the first line of defence (attachment to the host oesophagus) and then used only hosts defined as susceptible at this step to assess the probability that an infection successfully established and the subsequent expression of disease. The resulting distribution of genotypic means for each step highlights how the evolutionary potential (i.e., genetic variability; Connallon & Hall, 2018) of any step in the infection process can vary. First, only the attachment step showed nearly no variation among replicates of the same clone, suggesting that variation in this step is completely accounted for by the host genotype (Figure 1a). All other traits, binary (Figure 1b,c) and quantitative (Figure 1d–g), showed variation among replicates of the same host genotype. Second, for steps related to the severity of disease, infection not only reduced host fitness, but also altered the expression of genetic variance, with broad-sense heritabilities for two traits (lifetime fecundity and lifespan) being drastically reduced among infected animals (Table 3). Third, control and infected trait values were positively

TABLE 3 Descriptive statistics describing the phenotypic and genetic variability for the estimates of traits underlying pathogen infection and exploitation

Trait	Mean	SD	H ²	95% CI
Unexposed controls				
Size of first clutch	7.449	2.607	0.140	0.079–0.220
Body size (mm)	3.862	0.133	0.592	0.531–0.650
Lifetime fecundity	159.025	56.766	0.500	0.428–0.567
Lifespan (days)	88.473	9.388	0.328	0.267–0.421
Infected animals				
Size of first clutch	7.277	2.867	0.183	0.125–0.260
Body size (mm)	4.021	0.169	0.653	0.600–0.709
Lifetime fecundity	22.140	14.732	0.227	0.154–0.289
Lifespan (days)	46.570	9.388	0.192	0.126–0.256

Note: Reported here is the phenotypic mean, standard deviation, and the posterior mode and 95% credible intervals for the proportion of variation that was explained by the F₂ intercross line—equivalent to broad-sense heritabilities (H²).

but, importantly, not highly correlated, allowing scope for variation in the susceptibility to infection to be determined by alleles linked to host vigour (i.e., genetic quality or condition), as well as alleles that are specific to variation amongst infected animals only (Figure 1d–g).

Most notable was the contrast between the genetic architecture of resistance related to the attachment of the parasite to the host oesophagus versus mitigating the symptoms of disease. Previously, a single QTL of large effect (50% variance explained) had been shown to underlie a continuous, but strongly bimodal, range of infection successes (Routtu & Ebert, 2015). Here we could partition infection success into one all-or-nothing qualitative resistance allele (Table 1, 64% variance explained, complete resistance) controlling the host's ability to prevent parasite attachment and a single minor quantitative resistance allele (Table 1, 9% variance, 15% reduction), determining the host's ability to prevent infection. This is consistent with the observation that overall infection success is governed by an at least two-step process (Hall & Ebert, 2012). Both of these loci were located on the same linkage group. In contrast, quantitative changes in host fitness, such as symptoms related to gigantism or the reduction in survival, were linked to many more alleles of lesser effect that

TABLE 4 Genomic positions and descriptive statistics for each of the putative QTLs underlying variation in the direct impact of infection on gigantism and parasite-induced reduction in host fecundity

LG	QTL (cM)	Nearest marker	95% CI	LOD	Additive (SE)	Dominance (SE)	% Variance
Absolute increase in body size (gigantism)							
4	45.3	Scaffold02806_1920	30.0–64.0	5.0	–0.012 (0.011)	–0.067 (0.015)	9.7
4	162.4	Scaffold03282_89227	126.0–162.4	3.7	0.044 (0.011)	–0.016 (0.015)	6.9
10	103.6	Scaffold02226_1046	99.0–109.0	7.8	–0.061 (0.010)	–0.012 (0.015)	15.4
Absolute reduction in host fecundity							
1	2.4	Scaffold01302_1996	0.0–10.0	10.3	20.421 (2.984)	–4.694 (4.428)	9.8
2	82.4	Scaffold00487_2907	68.0–89.0	5.0	12.060 (3.045)	–10.572 (4.366)	4.5
3	18.4	Scaffold01473_1263	15.0–24.0	14.9	18.267 (3.177)	–8.374 (4.468)	15.2
3	134.0	Scaffold01376_6699	127.7–142.0	9.5	–27.799 (5.556)	–2.308 (7.534)	9.0
5	132.5	Scaffold00099_954	129.5–137.0	5.7	13.267 (3.140)	–9.632 (4.355)	5.8
6	58.3	Contig11465_453	54.3–60.1	14.6	11.661 (3.196)	–8.775 (4.425)	14.6
6	135.9	Scaffold00744_888	132.4–136.0	8.3	16.800 (2.874)	5.302 (4.436)	8.3
7	86.3	Scaffold03263_403	81.0–96.0	7.3	11.241 (3.080)	–18.552 (4.397)	7.3
9	40.7	Scaffold01662_1345	34.5–44.0	5.2	1.043 (3.221)	20.461 (4.393)	5.2

Note: The direct impact of infection was estimated by the absolute difference between controls and infected animals at each F_2 line. For each QTL, the location (linkage group and location [cM]), nearest marker, 95% confidence interval of the QTL region, effect size (additive and dominance) and percentage phenotypic variance explained is shown.

TABLE 5 Genomic positions and descriptive statistics for each of the putative QTLs underlying variation in the relative impact of infection on gigantism and parasite-induced reduction in host fecundity and lifespan

LG	QTL (cM)	Nearest marker	95% CI	LOD	Additive (SE)	Dominance (SE)	% Variance
Relative differences in body size (gigantism)							
4	45.3	Scaffold02806_1920	28.0–66.0	4.5	–0.014 (0.011)	–0.059 (0.014)	8.4
4	162.4	Scaffold03282_89227	129.0–162.4	3.8	0.044 (0.011)	–0.020 (0.014)	6.9
10	103.6	Scaffold02226_1046	99.0–108.9	8.8	–0.065 (0.010)	0.007 (0.014)	17.5
Relative differences in host fecundity							
1	132.7	Contig09843_961	59.0–158.1	4.0	–3.176 (0.846)	–2.108 (1.201)	8.6
10	24.4	Scaffold01697_476	16.0–44.8	3.9	3.419 (0.891)	–2.754 (1.206)	8.3
Relative differences in host lifespan							
1	40.9	Contig12074_891	36.0–47.0	3.8	1.433 (0.546)	–2.655 (0.748)	6.7
2	170.0	Scaffold01490_2062	168.0–182.0	6.5	2.013 (0.510)	2.639 (0.510)	11.6
9	110.0	Scaffold01813_2915	102.0–112.9	4.2	–1.843 (0.544)	–1.758 (0.751)	7.2
10	104.0	Scaffold00075_1960	91.0–108.9	4.0	–0.719 (0.740)	2.989 (0.740)	7.0

Note: The relative impact of infection was estimated via the residuals from the regression of infected versus control trait values for each F_2 line. For each QTL, the location (linkage group and location [cM]), 95% confidence interval of the QTL region, effect size (additive and dominance) and percentage phenotypic variance explained is shown.

were distributed widely across the host genome (23 loci, all except linkage group 8).

While the relative contributions of qualitative and quantitative resistance loci are well described in the plant literature (Corwin & Kliebenstein, 2017; Poland et al., 2009; Roux et al., 2014), our results reinforce emerging findings that different types of genetic architecture underlie the fight against infection in animals. In the fly, for example, resistance to infection by the sigma virus has long

been shown to be influenced by a single locus of large effect, as measured by the binary response of exposed animals to a CO_2 paralysis test (Bangham, Knott, Kim, Young, & Jiggins, 2008; Bangham, Obbard, Kim, Haddrill, & Jiggins, 2007; Cogni et al., 2016; Magwire et al., 2012). Including more diverse phenotypes, however, such as changes in longevity, fecundity or virus titre (and also exploring new pathogens), is beginning to uncover many more minor effect loci (Cogni et al., 2016; Howick & Lazzaro, 2017; Palmer, Medd, Beard,

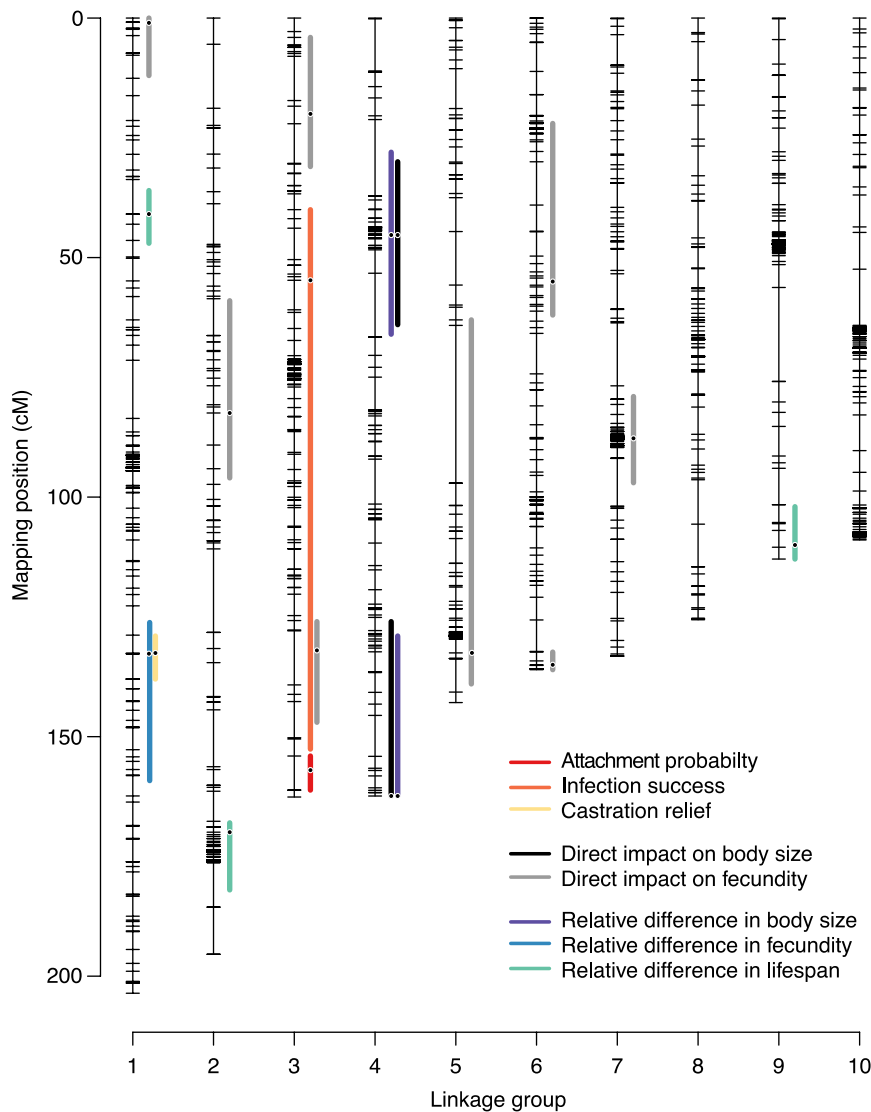


FIGURE 3 The distribution of significant and suggestive QTLs across the 10 linkage groups of *Daphnia magna*. Shown are the interval estimates for the location of each QTL based on the 1.8-LOD support. The map is based on the work of Routtu et al. (2014). [Colour figure can be viewed at wileyonlinelibrary.com]

& Obbard, 2018; Wang, Lu, & St Leger, 2017; but see Martins et al., 2014). Thus, the prediction that a host's susceptibility to infection may have a different genetic architecture to other complex traits need not apply to all components of disease, but rather, as shown here, to the very specific step that defines whether or not a pathogen has the ability to infect a host. Controlling for variation at the first line of defence against a pathogen, and the steps most probably related to qualitative resistance (see discussion in Hall et al., 2017), may further uncover a wealth of candidate loci involved not just in preventing infection. Indeed, mutagenesis studies have indicated that a broad spectrum of biological functions potentially underlie how infectious disease manifests in susceptible hosts (Lu, Wang, Brown, Euerle, & St Leger, 2015), suggesting that much of the complete genetic basis of host susceptibility and resistance remains to be uncovered.

Our results also point to a largely modular architecture in which a host's ability to fight infection is reinforced at multiple steps of the infection process, but each step remains genetically independent. The dominant QTLs for each trait, for example, showed that alleles from the German (AA) parent cause lower susceptibility to

infection, are more able to prevent a pathogen from establishing a successful infection and can mitigate the pathogen's control of reproduction, and as a result, any loss of fecundity is significantly curtailed (Figure 4); however, some QTLs do show the opposite pattern. For each of these traits, there was little evidence for QTL hotspots (Breitling et al., 2008), arising from either tight linkage or pleiotropy, except for QTL at the end of linkage group 10 that contributed to body size, castration, fecundity and lifespan (Figure 3). Epistasis effect sizes were also comparatively low, further reinforcing the largely independent effects of the qualitative and qualitative alleles underlying each metric of susceptibility—but see Hall and Ebert (2013) for why this is more a reflection of the genotypes used for the QTL panel, rather than a true assessment of additive versus epistatic variance in a population; and see also Metzger et al. (2016) and Bento et al. (2017).

The above contributions of quantitative and qualitative loci, together with the modular nature of a host defence portfolio, have two main implications for how host and pathogens might evolve. First, it explains how different steps of the infection process can potentially undergo divergent evolutionary trajectories within a

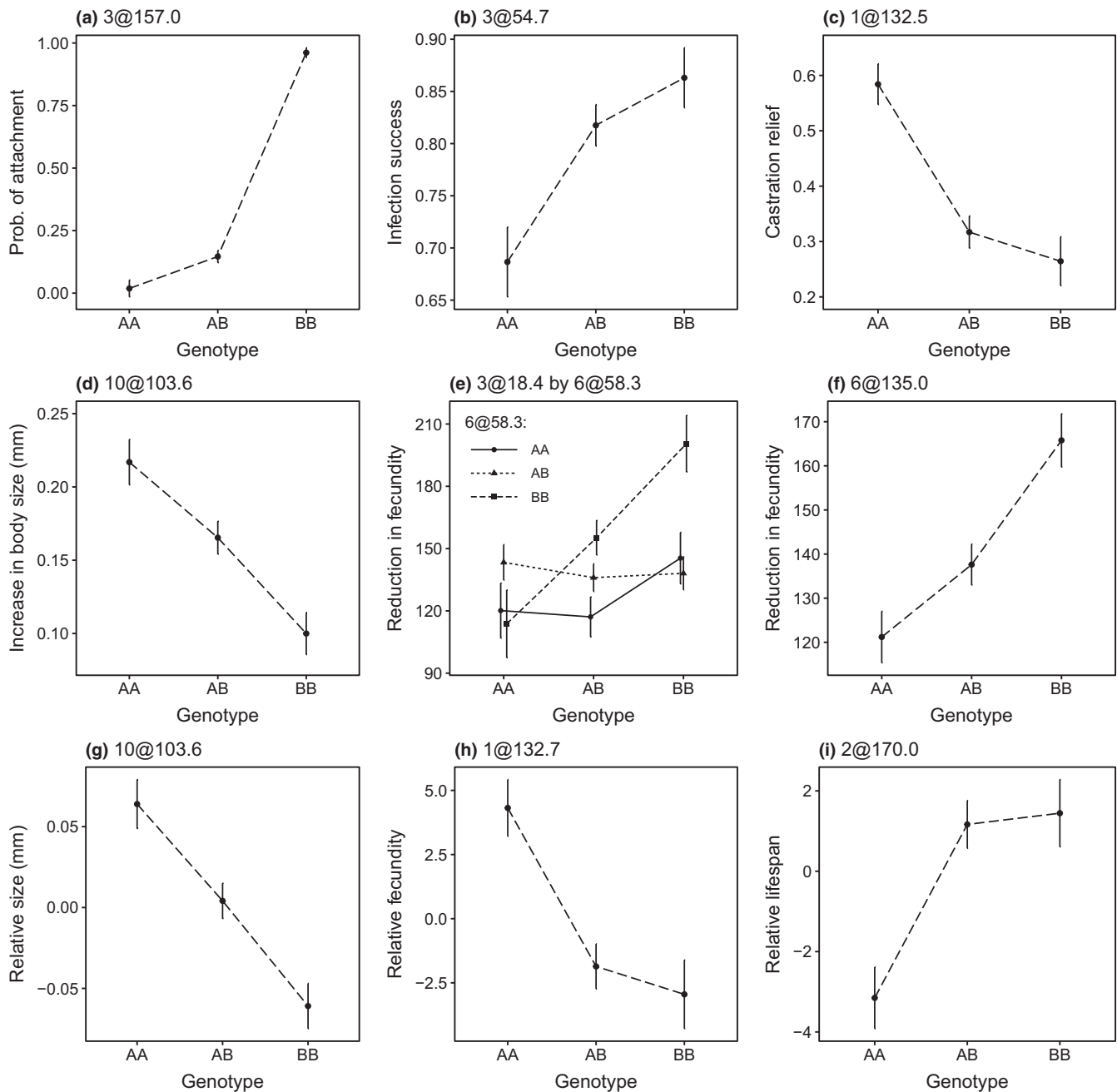


FIGURE 4 Phenotypic effect of the QTLs with the largest effect sizes. For each QTL, the linkage group and the map position (cM) are given (e.g., 3@157: linkage group 3 at position 157 cM). (a) Spore attachment to the host oesophagus; (b) infection success in susceptible hosts; (c) probability of temporary castration relief by the host; (d) the increase in body size; (e,f) the reduction in host fecundity that occurs when infected; and (g–i) the relative change in size, fecundity and lifespan, after controlling for host vigour. All error bars represent mean and standard errors. Genotypes are German (AA) and Finnish (BB) panel parents or heterozygotes (AB)

host population (Hall et al., 2017). In the same study system, for example, a time-shift experiment by Decaestecker et al. (2007) showed that infection rates fluctuated over evolutionary time in line with “Red Queen” dynamics, but changes in the severity of disease increased steadily. Our observation that infection rates are influenced by a limited number of qualitative resistance alleles of large effect (and see Routtu & Ebert, 2015; Bento et al., 2017), versus the genetically independent and quantitative architecture of other components of disease, helps to account for

this observation. Second, it reiterates how important the route of infection may be for a pathogen's evolution. With each point of entry into a host (i.e., ingestion, inhalation, via the skin or cuticle), a pathogen will probably encounter a unique suite of qualitative and quantitative resistance mechanisms that alter the very basis of host–pathogen genetic interactions. Although relating the type of host alleles that a pathogen interacts with to the route of infection has not yet been explicitly addressed, experimental evolution studies in the fly have manipulated the starting point

of infection in the host (injected vs. ingested) and observed divergent pathogen evolution (Martins, Faria, Teixeira, Magalhães, & Sucena, 2013). Pathogens infecting humans also appear to be more virulent if they infect via the skin rather than via inhalation or ingestion (Leggett, Cornwallis, & West, 2012).

Variation in host vigour or quality is likely to contribute to differences amongst hosts in their capacity to defend against a pathogen, particularly with respect to steps related to disease severity, but is rarely controlled for explicitly in QTL or association studies in animals. Such variation may be more pronounced in breeding panels that involve some degree of inbreeding, as is the case in our F_2 panel, where all F_2 lines are the result of selfing and have an inbreeding coefficient of 50%. In our system we were able to explore this issue because we can replicate the same genotype under different conditions and compare the contributions of loci to general quality differences between the host (i.e., direct impact of infection, Figure 1b) to those loci identified when the contribution of vigour was eliminated (i.e., relative impact of infection, Figure 1c). This comparison revealed variable results depending on the trait studied. For lifetime fecundity and lifespan, we found very little overlap when mapping QTLs with and without host vigour, suggesting that both the genetic quality of a host, and alleles that are specific to the response to infection, contribute to how severe the disease is. By not controlling for host vigour, however, many of the alleles specific to the infection process may be overlooked. In contrast, QTL analysis of body size resulted in the same two QTL peaks before and after the correction, indicating that host vigour does not seem to play a role in the gigantism symptom of disease in this system (i.e., larger hosts, in general, do not necessarily increase in size more when infected).

Finally, our study makes two assumptions that are important to consider both in light of our results and when attempting to partition the stepwise infection process in other systems. First, whenever variation at a single step of infection is controlled for, as we did here by phenotyping only individuals which allow attachment of the pathogen, then any loci that are in linkage disequilibrium with the QTL underlying this step (LD, due to physical linkage or coselection) will be hidden. In our study this bias is likely to be small, as the single major effect locus underlying host susceptibility lies on a relatively small 130-kb haplotype block at the very end of linkage group 3 (Bento et al., 2017), and as part of the F_2 intercross mapping design we experimentally induced recombination to break up LD amongst the parental alleles. Nevertheless, the potential for LD to mask the signal of additional loci within the susceptible fraction of host genotypes needs to be considered when mapping the individual steps of infection in this or other systems, particularly when using a genome-wide association approach in which recombination is not induced.

Second, our exploration of the genetic basis of the stepwise infection process is limited to the genetic variation exposed in response to a single pathogen genotype. Host–pathogen interactions are commonly characterized by strong genetic interactions between host and pathogen genotypes ($G \times G$ interactions), particularly with

regard to trait variation influenced by steps involving the all-or-nothing blocking of infection by the pathogen (Hall et al., 2017). The co-evolutionary history of a host and pathogen (novel vs. co-adapted) also shapes the expression of genetic variation, with the genetic variation in a host's susceptibility to a pathogen likely to be much higher when exposed to pathogens they normally encounter (Duxbury et al., 2019). Recent work by MacPherson, Otto, and Nuismer (2018) has shown that detection of loci underlying symptoms of disease are, as a result, highly sensitive to the types of host and pathogen genotypes included in a study. In the *Daphnia*–*Pasteuria* disease system, including more pathogen genotypes will likely uncover additional loci underlying each step of infection, particularly with regards to the highly specific attachment of the pathogen to the host oesophagus, and less strongly so to the later steps of infection (e.g., changes in fecundity and lifespan) where $G \times G$ interactions have been shown to be less pronounced or even absent (Hall & Ebert, 2012; Luijckx et al., 2010).

In summary, our study reveals how a host's capacity to defend against pathogen attack depends on a complex mix of qualitative and quantitative resistance loci. By partitioning a host's susceptibility to infection at multiple steps along the infection process, our results mirror those that are routinely found in the plant breeding literature, whereby an early step in the infection process is under the control of an all-or-nothing gene of large effect, followed by many more quantitative resistance loci that mitigate the ability of the pathogen to cause damage to the host. The general lack of pleiotropy and epistasis for traits related to different stages of the infection process, together with the wide distribution of QTLs across most linkage groups, highlights the modular nature of a host's defence portfolio, and the potential for each different step to evolve independently. Much like all mapping studies, extrapolation to allele frequencies in natural populations will provide the next challenge for assessing the complete genetic architecture of a host's defence portfolio and the stepwise infection process.

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AUTHOR CONTRIBUTIONS

M.D.H. conceived the study. J.R. and D.E. developed the QTL mapping panel. M.D.H., J.R. and D.E. designed the study. M.D.H. carried out the lab work, analysed the data and drafted the first version of the manuscript. All authors gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.tv2kb51>.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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