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A Population Biology Perspective on the Stepwise Infection Process of the Bacterial Pathogen *Pasteuria ramosa* in *Daphnia*

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Abstract

The infection process of many diseases can be divided into series of steps, each one required to successfully complete the parasite's life and transmission cycle. This approach often reveals that the complex phenomenon of infection is composed of a series of more simple mechanisms. Here we demonstrate that a population biology approach, which takes into consideration the natural genetic and environmental variation at each step, can greatly aid our understanding of the evolutionary processes shaping disease traits. We focus in this review on the biology of the bacterial parasite *Pasteuria ramosa* and its aquatic crustacean host *Daphnia*, a model system for the evolutionary ecology of infectious disease. Our analysis reveals tremendous differences in the degree to which the environment, host genetics, parasite genetics and their interactions contribute to the expression of disease traits at each of seven different steps. This allows us to predict which steps may respond most readily to selection and which steps are evolutionarily constrained by an absence of variation. We show that the ability of *Pasteuria* to attach to the host's cuticle (attachment step) stands out as being strongly influenced by the interaction of host and parasite genotypes, but not by environmental factors, making it the prime candidate for coevolutionary interactions. Furthermore, the stepwise approach helps us understanding the evolution of resistance, virulence and host ranges. The population biological approach introduced here is a versatile tool that can be easily transferred to other systems of infectious disease.

1. INTRODUCTION

In parasite–host interactions, the parasite must pass through a series of steps (or stages) to successfully complete its life and transmission cycle (Combes, 2001; Schmid-Hempel, 2011). It must encounter the host, enter it, survive the host's immune response, reproduce and release transmission stages. The stepwise nature of this infection process is well understood for many human, animal and plant infections and in some cases we even know the interacting genes for some of the steps (e.g. Dodds and Rathjen, 2010; Ferrandon, 2013; Gutjahr and Parniske, 2013; Lemaitre and Hoffmann, 2007; Nakajima and Akutsu, 2014; Sarker and Paredes-Sabja, 2012; Schulenburg et al., 2007; van Schie and Takken, 2014). Indeed, life and transmission cycles depicting the steps of the infection process have long been part of parasitology and infectious disease textbooks (Burnet and White, 1972; Cox, 1993). However, while we have clarified details about the infection processes for many diseases, we rarely look at these steps from a population biology perspective, which considers natural variation among host and parasite genotypes and how they are modified by the

environment (Dybdahl et al., 2014; Schmid-Hempel and Ebert, 2003). Estimates of variation in trait expression at given steps of the infection process are usually not included in pictures of life and transmission cycles. In most cases, such estimates do not even exist. Understanding natural variation is, however, essential to understanding how evolution and the environment shape infection processes.

Mechanistic and population biological approaches can yield very different conclusions about the expression and evolution of disease-related traits. A gene that plays a key role in the infection process, for example, is largely irrelevant for the evolution of the disease if all individuals share the same variant of the gene (Hueckelhoven et al., 2013). In contrast, genes with allelic variation segregating in a population may be exposed to selection and lead to an adaptive response (Schmid-Hempel and Ebert, 2003) even if their overall contribution to disease expression is small. Thus, knowing where genetic variation exists in the infection process would enable us to better understand the evolution of disease traits. Furthermore, nongenetic factors that cause variation in the expression of disease traits must also be considered, as they can influence the rate of adaptive evolution. Some steps of an infection process may stand out in being more prone to environmental variation such as climate, environmental stressors and competition among parasites. Other steps may be influenced by genetic interactions among host and parasite genotypes or even complex combinations of host, parasite and environmental factors.

Although we know little about the sources and consequences of differential variation in the individual steps of the infection process for most diseases, some studies have noted that such differences exist and are important. For example, the experimental manipulation of early steps in the infection process can reveal very different disease outcomes (Behrens et al., 2014; Dhondt et al., 2007; Martins et al., 2013). In part this can be attributed to variation in the contribution of genetic and environmental factors influencing trait expression during different steps (Martins et al., 2013; Wargo et al., 2012). Furthermore, steps in the infection process without genetic variation are less likely to evolve in response to our measures to control diseases and might therefore be good targets for therapy (He et al., 2014). This relates to the idea that parasite control strategies could be made 'evolution proof' by targeting genetically constrained infection steps, thereby preventing or delaying evolution of parasite resistance (Koella et al., 2009; Read et al., 2009).

One system for which we have a thorough understanding of the infection process is *Daphnia* and its bacterial parasite, *Pasteuria ramosa*

(Duneau et al., 2011; Hall and Ebert, 2012). Over the last two decades, studies of the ecology and evolution of this system have produced a detailed picture of the steps of the infection process within an environmental and evolutionary context. The system has become a model for the study of the ecology, evolution and coevolution of infectious diseases (Decaestecker et al., 2007; Ebert, 2008). In this review, we apply a population biology approach to this system, explicitly considering the sources of natural variation that influence the different steps of the infection process and how this variation affects disease expression. We examine, in turn, the effects of host genetics, parasite genetics, the environment and their interactions on each of the seven steps in the infection process. We highlight the developmental and phylogenetic constraints on these disease-related traits. Finally, we apply the insights of this analysis to issues regarding host and parasite evolution and coevolution, the genetics of disease expression and resistance, the evolution of host ranges and the evolution of virulence.

2. THE *DAPHNIA*–*PASTEURIA* SYSTEM

Pasteuria ramosa is a common bacterial endoparasite of *Daphnia* and related Cladocera, reported in Eurasia and North America (Andras and Ebert, 2013; Auld et al., 2012a; Goren and Ben-Ami, 2013; Green, 1974). In natural populations, it can reach a prevalence of 100% in adult hosts (Duncan and Little, 2007) and has strong fitness consequences, sterilizing hosts and reducing life expectancy (Ebert et al., 1996). It is therefore believed to play a major role in the ecology and evolution of its hosts (Auld et al., 2012a; Ebert, 2005). Research on this system is facilitated by the cyclic parthenogenetic reproduction of the hosts (Box 1), which allows clonal replication of host genotypes but also enables genetic crosses among clones (Luijckx et al., 2012). Because of its predominantly asexual mode of reproduction, research on *Daphnia* is carried out mostly with females. Therefore, unless mentioned otherwise, we report here results for females. *Pasteuria* can be cloned as well, and transmission stages (spores) can be kept frozen (Luijckx et al., 2011). The genetic characteristics of the parasite are most clearly seen in clones, as isolates (propagation of spores from field-collected infected hosts) often harbour multiple genotypes (Luijckx et al., 2011; Mouton et al., 2007) and therefore diminish the genetic resolution.

Box 1 The *Daphnia* model

Daphnia is a genus of planktonic freshwater crustaceans with a worldwide distribution. Adults are 1–5 mm in size and reach maturity in 6–12 days (at 20 °C) (Ebert, 1992). They grow throughout their life with a lifespan, under laboratory conditions, of 50–150 days. *Daphnia* reproduce primarily by means of cyclic parthenogenesis, i.e. produce mostly genetically identical daughters and sons, but can also reproduce sexually by producing haploid eggs that require fertilization by males. Sexual eggs require an obligate resting phase. The combination of sexual and asexual reproduction provides powerful means for genetic crossing designs, allowing the estimation of genetic and nongenetic variance components (Ebert et al., 1993). Under natural conditions, *Daphnia* undergoes sexual reproduction about once a year (Lampert, 2011).

In the past few years *D. pulex* and *D. magna* have become model systems in ecological genomics (Colbourne et al., 2011; Ebert, 2011; Smirnov, 2014), opening up new possibilities for combining functional and evolutionary genetics with ecology and epidemiology, in particular in the fields of ecotoxicology and environmental health. *Daphnia* is one out of 13 official model organisms for biomedical research in the National Institutes of Health, USA (http://www.nih.gov/science/models/).

3. STEPS OF THE INFECTION PROCESS IN THE *DAPHNIA–PASTEURIA* SYSTEM

The infection process of the *Daphnia–P. ramosa* system is well documented, and two decades of research allow us to present its major steps in detail. Although certain processes may happen in parallel, e.g. the parasite might have different routes to enter the host, or different components of the immune system may act in parallel to thwart the parasite, the major steps can be presented as a chain-like sequence. Processes occurring in parallel could affect the evolution of the system; however, we currently know too little to address this topic for the *Pasteuria–Daphnia* system. Here we use a seven-step sequence to map the infection process in this system, but the distinctions between steps are not always clear. Indeed some steps could be subdivided, or two steps may be clustered into one. Nevertheless, the model presented here has proven convenient to examine the underlying mechanisms. The number of steps for other systems may be different.

3.1 Step 1. Host encounter with parasite transmission stages

Infection processes begin with an encounter of host and parasite (Figure 1, Table 1). Hosts often reduce the chance of encounter by avoiding contact with infected hosts or avoiding locations where there is a greater likelihood of encountering parasite transmission stages. *Pasteuria* infects *Daphnia* hosts via environmental transmission stages (in the following called spores) encountered either in the water (free floating spores) or deposited in the sediment of ponds and lakes (Ebert, 2005; Ebert et al., 1996). Vertical and mixed mode transmission was never reported for any *Pasteuria* species (Ebert, 2013; Ebert et al., 1996). For the water-to-host route of transmission, *Daphnia* ingest *Pasteuria* spores along with food particles floating in the water while filter-feeding (Ebert, 2005; Smirnov, 2014), thus producing a conflict between the benefits of food uptake and the risk of infection (Hall et al., 2007). The higher the density of spores in the water, the higher the likelihood of infection, with the form of this density dependence being well approximated by mass action (Ben-Ami et al., 2008b; Regoes et al., 2003).

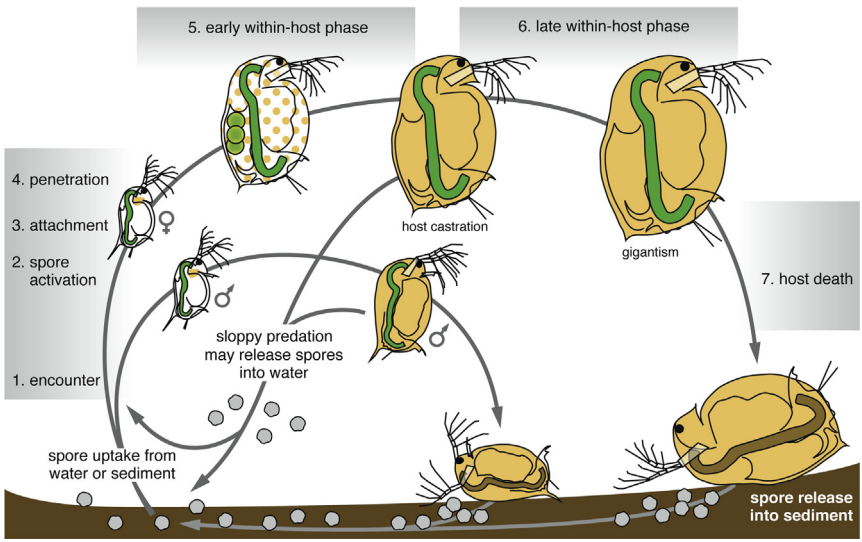

Figure 1 Schematic representation of the seven infection steps in the host (clockwise from the encounter step at the left). Encounter happens when spores filtered from the free water or the sediment come in contact with the host (step 1). Spores will then be activated by the host (step 2) and may attach to the gut wall (step 3). Attached parasites penetrate the gut wall (step 4) and enter the body cavity, where they multiply (steps 5 and 6). Eventually the host is killed by the parasite (step 7) and spores are released from the decaying cadaver. Both male and female *Daphnia* may become infected.

Even a single spore can cause an infection, although with very low likelihood (Luijckx et al., 2011). While experimental studies have primarily used well-mixed suspensions of spores in water to achieve controlled infections, in nature it is not clear how common the water-to-host route for transmission is. However, sloppy feeding by predators and water turbulence may indeed lead to spores being suspended in the water (Auld et al., 2014; Hall et al., 2010; Goren and Ben-Ami, 2015).

Under natural conditions, it is believed that infection most likely occurs via a sediment-to-host route when animals browse on and in the sediment surface and stir up particles that they filter from the water (Ebert, 2005; Horton et al., 1979). Spores are released from decaying host cadavers on the pond or lake floor, resulting in a clustered distribution of spores (Ebert, 2005). Experimental exposure of *Daphnia* to pond sediments frequently leads to infection, even when sediment from cores are used, which can be several decades old (Andras and Ebert, 2013; Decaestecker et al., 2002, 2004; Jansen et al., 2010). This sediment-to-host route has been linked to differences in host behaviour, which varies strongly among *Daphnia* genotypes (Decaestecker et al., 2002). Negatively phototactic *Daphnia* genotypes stay lower in the water column and tend to be found more in habitats with fish than in fishless habitats. They even move downward when fish kairomones are added to the water (De Meester, 1993, 1996; De Meester et al., 1995), a behavioural change that reduces their likelihood of encountering predatory fish. However, being closer to the pond sediments has costs in that it increases the likelihood of exposure to sediments and, thus, parasite spores. In contrast to the water-to-host route, transmission via the sediment-to-host route is not density dependent, because the spore bank in the pond sediments, which accumulates over months and even years, decouples the current production of transmission stages from infection of new hosts and thus dampens epidemiological and evolutionary dynamics (Auld et al., 2014; Ebert et al., 1997). The combination of direct (from water) and indirect (via spore bank) transmission is expected to increase the long-term persistence of the parasite in a host population, as it expands the range of environmental conditions under which transmission is possible. This is analogous to the epidemiological dynamics of mixed-mode transmitted parasites (Ebert, 2013).

Negatively phototactic *Daphnia magna* clones have a higher infection rate than *Daphnia* that remain higher in the water, and addition of fish kairomones cause not only a downward movement of the *Daphnia*, but also an increase in infection rates (Decaestecker et al., 2002). These differences

Table 1 Description of steps in the infection process of *Pasteuria ramosa* in *Daphnia*

Step	Description of process	Trait(s) for which phenotypic variation was studied	Potential for the host to evolve resistance	Key references
1. **Encounter** with parasite spores	Filter-feeding host comes into contact with nonmotile parasite spores that either rest in pond sediment or float in the water	Behavioural differences among hosts influence the likelihood of encountering spores	Avoidance behaviour may evolve, which is functionally linked to predator avoidance and foraging	Decaestecker et al. (2002)
2. **Spore activation**	Spore activation upon physical/chemical contact with the host before ingestion. Shedding of outer spore shell (exosporium) releases activated spore	No variation in spore activation observed among *Daphnia* clones and species	Avoidance of activation is unlikely to evolve: There is no genetic variation among hosts or parasites genotypes	Duneau et al. (2011)
3. **Attachment** of activated spores	Activated spores are ingested by the host and attach to the gut wall	Attachment to host gut wall varies with host and parasite genotype	Impeding attachment	Duneau et al. (2011) and Luijckx et al. (2013)
4. **Penetration**	Penetration into the host's body cavity. This takes about 12 h. Host moulting within 12 h post attachment prevents penetration	Likelihood that the parasite penetrates the gut wall depends on host moulting	Moulting is developmentally/ phylogenetically constrained, preventing change in moulting rate. Variability in permeability of gut wall may be possible	Duneau and Ebert (2012b)

5. **Early within-host phase**	The host immune system interacts with the invading parasite	Parasite clearance, spore counts and development, castration, host body size	Clearance of parasite; reducing parasite growth and development	Hall and Ebert (2012), Hall et al. (2013), and Ben-Ami et al. (2010)
6. **Late within-host phase**	Phase of chronic infection	Spore counts, host fecundity, castration relief, host body size	Reducing parasite growth; castration relief	Hall and Ebert (2012), Ben-Ami and Routtu (2013), and Mageroy et al. (2011)
7. **Host death**	Hosts die 30–70 days after infection. The cadaver breaks open and releases the environmentally resistant parasite endospores	Time to host death	None	Hall and Ebert (2012), Jensen et al. (2006), Ben-Ami et al. (2008a), and Ben-Ami and Routtu (2013)

Mentioning of quantitative estimates were collected from experiments conducted at 20 °C. Higher temperatures accelerate these processes. Compare to Figure 1.

were not due to differential susceptibility of the *Daphnia* genotypes, but only to the higher exposure of the negative phototactic clones to the sediment-borne spores. Genetic variation for phototactic behaviour is believed to be a quantitative genetic trait and the interaction between *Daphnia* clones and kairomones highlights that the encounter step is subject to genotype × environment interactions (Table 2) (De Meester, 1989; Routtu and Ebert, 2015). Besides the correlation between phototactic behaviour and infection, the actual uptake of spores from the sediment has so far not received attention. Disregarding phototactic tendencies, *Daphnia* individuals may differ in their propensity to dig into the sediment surface and thus to influence the encounter rate with spores. A negative phototactic clone with a low propensity to dig may enjoy the combined benefits of protection from fish predation and parasitism.

In summary, exposure to free-floating parasite spores in the water is unavoidable for the filter-feeding hosts (Hall et al., 2007), whereas exposure to spores in pond sediments depends on host behaviour. While the former process is density dependent, transmission in the latter type of parasite encounter is density independent, with important consequences for the start and the spread of epidemics (Ebert et al., 1997). Variation among host clones for encounter rates varies strongly among populations (local adaptation), seems to have a quantitative genetic basis, and is prone to genotype by environment interactions.

3.2 Step 2. Activation of dormant parasite spores

Once a dormant parasite comes into contact with a potential host, it must become active. This process has been well documented in fungal pathogens of plants and animals and in spore-forming bacteria and has been shown to require specific triggers associated with the host (Hu et al., 2014; Jaronski, 2010; Paredes-Sabja et al., 2014). *Pasteuria* spores can rest dormant for decades in pond sediments (Decaestecker et al., 2004), but within minutes of coming into contact with a potential host, the nearly spherical spores shed their exosporium and assume a disc-like shape with a thick central body (Duneau et al., 2011) (Figure 2). Only this activated spore can attach to the host (see next step). How activation is induced is not yet known, but requires some form of interaction between spore and host. *Pasteuria* activation alone does not induce vegetative outgrowth of the germ tube (germination), which happens only after the activated spores attach to a susceptible host (see penetration step below).

Experiments with different combinations of parasite and host clones and species under various environmental conditions have revealed that *Pasteuria* spore activation is largely nonspecific (Duneau et al., 2011). Any tested host genotype within species belonging to the family Daphniidae, whether susceptible or resistant, was found to activate spores of *P. ramosa*, while a more distant arthropod, a filter-feeding mosquito larvae, did not (Duneau et al., 2011) (Table 2). Thus, the activation signal seems phylogenetically conserved. Possibly, the costs for the host of evolving a defence against activation are so high that a mutant doing so could not spread. Furthermore, spores have been activated under a variety of test conditions, e.g. at different temperatures, in well-fed and starved hosts, in male and female hosts and in conventional and microbiota-free hosts (Duneau et al., 2011) (M. Sison-Mangus et al., in prep.), suggesting that activation is insensitive to environmental conditions.

Hardly anything is known about the mechanism of activation. Preheating *Pasteuria* spores to 99 °C does not prevent subsequent activation at room temperature, suggesting that the activation process does not depend on proteins or on the viability of the spores, which are rendered incompetent by exposure to temperatures above 70 °C (Metzger, 2014). Under laboratory conditions, activated spores have a lifespan of under 24 h (S. Gygli, unpublished data), unless they are frozen (King et al., 2013), but they remain infectious after passing through the gut of susceptible or resistant *Daphnia* (King et al., 2013).

In summary, *Pasteuria* spores shed their exosporium upon receiving a phylogenetically conserved trigger from *Daphnia* and closely related Cladocera. Since neither ecological conditions nor host or parasite genotype measurably influence spore activation, the host has little room for an evolutionary adaptation at this step that would reduce the likelihood of infection.

3.3 Step 3. Attachment of activated spores

Attachment of parasite cells to host tissue is important in many infectious diseases and often requires specific adhesion molecules (Adamu et al., 2013; Benhamed et al., 2014; Doran et al., 2013). In many systems, the contact zone between bacterium and host epithelium marks the host's first line of defence and is the subject of anti-adhesion therapy research (Krachler and Orth, 2013). In *Pasteuria*, host attachment is an important step in the infection process, as variation in this step explains most of the overall variation in the entire infection process, as we elaborate in section 4 of this article.

Table 2 Variation in disease trait expression at different steps of the infection process

Step	1. Encounter	2. Spore activation	3. Attachment	4. Penetration	5. Early within-host phase	6. Late within-host phase	7. Host death	1–7. All steps
Traits measured	Likelihood of contact with spores	Change in spore phenotype while being activated	Attachment of activated spore to host gut wall	Penetration of germ tube through host gut wall	Likelihood of infection, spore counts, host and parasite life history traits	Spore counts, host and parasite life history traits	Time to parasite-induced host death	Likelihood of infection, host and parasite life history traits
Form of phenotypic variation observed	Quantitative	None	Binary (0/1)	Linked to moulting cycle*	Quantitative	Quantitative	Quantitative	Quantitative
Evidence for genetic and environmental contribution to trait variation								
Host genetic variation, H_G (among host clone variation)	Yes	No	Yes	No	Yes	Yes	Yes	Yes
Within (H_{GW}) versus between population (H_{GB}) genetic variation	$H_{GW} < H_{GB}$	No	$H_{GW} > H_{GB}$	No	?	?	?	?
Parasite genetic variation, P_G (among parasite clone variation)	No	No	Yes	No	Yes	Yes	Yes	Yes

Impact of the environment, E	Yes	No	No	Yes	Yes	Yes	Yes	Yes
$H_G \times P_G$	No	No	Yes	No	Yes	No	No	Yes
$H_G \times E$	Yes	No	No	No	Yes	Yes	No	Yes
$P_G \times E$	No	No	No	No	Yes	No	No	Yes
$H_G \times P_G \times E$	No	No	No	No	No	No	No	Yes
Host maternal environmental effect	?	No	No	No	Yes	Yes	Yes	Yes
Host status								
Host sex	Yes	No	No	Yes	Yes	Yes	No	Yes
Host age	Yes	?	?	Yes	Yes	Yes	No	Yes
Constraints on trait evolution								
Cost of resistance	Yes	NA	No	NA	?	?	NA	NA
Phylogenetic/ developmental constraint	?	Yes	?	Yes	?	?	?	NA

"Yes" indicates evidence for significant contribution to phenotypic variation; "No" and "?" indicate no current evidence or unknown, although a contribution may be discovered in future experiments; NA, not applicable; For references see Table 1 and main text.
Times given in the inner circle are approximate estimates.
* Processes influencing the moulting frequency of Daphnia (e.g. faster at higher temperature, slower at larger body size) influence the penetration process.

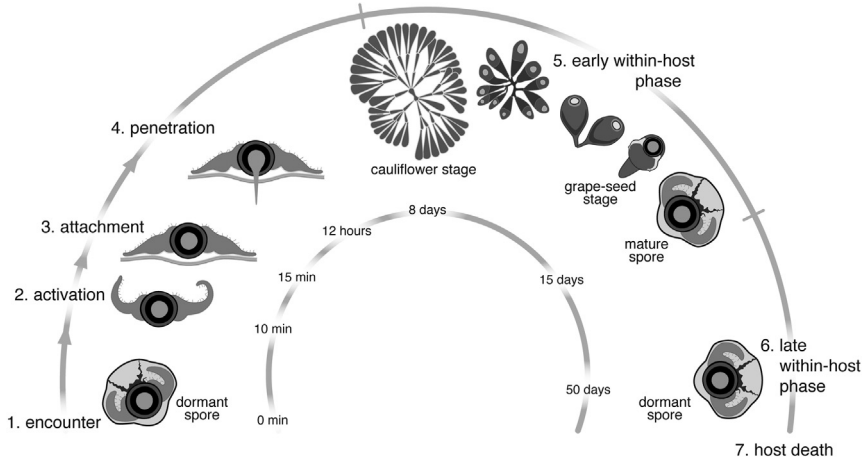

Figure 2 Schematic representation of *Pasteuria* development during the seven infection steps (in clockwise order, beginning with the encounter step at the left). The host encounters the dormant spore, enclosed in the exosporium (step 1). Upon activation the exosporium is shed (step 2) and the activated spore attaches to the gut wall (step 3). The attached parasite penetrates the gut wall (step 4) and soon starts to produce cauliflower-like stages (step 5), which break into smaller and smaller fractions, until each branch represents a single grape-seed stage spore, which further develops into a mature spore. During the late within-host phase (step 6), the host's entire body cavity becomes filled with mature, dormant spores, which are released into the environment upon host death (step 7).

Activated spores of *Pasteuria* need to attach to the cuticle of the foregut wall (the oesophagus) to cause infection. Failure to do so terminates the infection process and the activated spores are quickly degraded (Duneau et al., 2011). Fluorescently labelled *Pasteuria* spores can be readily observed in the living host when they adhere to the oesophagus wall of the transparent host. The attachment is so strong that spores are not dislodged by mechanical disturbance, such as food passing through the oesophagus. The attachment process observed in the *Daphnia* system is similar to the attachment process of *Pasteuria penetrans* to its juvenile nematode host (for a review see Davies, 2009). In both systems, attachment is to a chitin-containing cuticle of endodermal origin. Since the area of the tissue to which *Pasteuria* spores can attach to their host is small, interference competition among spores is likely, potentially influencing parasite evolution during host exposure to multiple parasite genotypes.

A particularly interesting feature of *P. ramosa* attachment to *Daphnia*, is that for a given combination of host and parasite genotypes, activated spores

either attach or do not (Duneau et al., 2011), resulting in a binary form of variation. In laboratory and natural populations, most of this variation is explained by pronounced genetic host–parasite interactions (Andras and Ebert, 2013; Duneau et al., 2011; Luijckx et al., 2011). Genetic crosses between *D. magna* clones with different susceptibility to attachment by different *Pasteuria* clones have revealed that attachment (susceptibility) is recessive but strongly influenced by the presence of closely linked interacting loci (epistasis) (Little et al., 2006; Luijckx et al., 2012, 2013; Metzger, 2014). Observed patterns of host–parasite interactions show a signature of a matching allele model, whereby a single allele substitution can reverse the infection patterns of different parasite clones (Luijckx et al., 2013). Variation in attachment among hosts (resistotypes) and parasite (infectotypes) varies widely within and between *Daphnia* populations (Andras and Ebert, 2013; Luijckx et al., 2014), suggesting evolutionary processes at work, which maintain genetic diversity (see section on coevolution below).

A further interesting feature of the attachment step is that environmental conditions (e.g. temperature, well-fed or starved hosts, host crowding) as well as host sex and age do not affect attachment (Duneau et al., 2011) (Table 2). Even host microbiota, which have been shown in other systems to influence host–parasite specificity (Koch and Schmid-Hempel, 2011) and generally influence *Daphnia* biology (Qi et al., 2009; Sison-Mangus et al., 2015), do not interfere with the attachment process: replacing the natural microbiota of resistant and susceptible *D. magna* clones did not influence the results of attachment tests (M. Sison-Mangus et al., in prep.) (Table 2).

It is not understood how the high genetic specificity in the attachment process arises from the interaction between host and parasite genes. Proteins are likely involved on the side of the parasite, as spores heat treated to about 70 °C or above lose their ability to attach (Metzger, 2014). This has also been supported in studies of spores of *P. penetrans* attaching to their nematode host (Davies, 2009; Freitas et al., 1997). For both systems, it is believed that collagen-like proteins, expressed in large numbers on the surface of the activated spores, play a central role for attachment of *Pasteuria* (Davies, 2009; McElroy et al., 2011; Mouton et al., 2009; Schaff et al., 2011). Genes coding for these proteins are found in high abundance and variability in the *P. ramosa* genome (McElroy et al., 2011; Mouton et al., 2009). The molecules and pathways involved in attachment in the host are not known, but the chromosomal region for one locus has been mapped with the help of a quantitative trait loci (QTL) mapping panel (Routtu and Ebert, 2015; Routtu et al., 2014).

In summary, the attachment step is characterized by a very high specificity of host–parasite genotype combination without environmental factors being involved. The strong host–parasite interactions show a binary pattern of variation (attachment or not), making it a strong candidate for a step that may undergo host–parasite coevolution.

3.4 Step 4. Host penetration

After attaching to the host, the parasite must enter the host cell or body. Bacteria often use different secretion systems to achieve penetration, while plant fungal pathogens grow hyphae into the host tissue (Cossart and Helenius, 2014; He, 1998; Naglik et al., 2011; Underwood, 2012). Although the process of host cuticle penetration for *P. ramosa* has not yet been described, for the related pathogen *P. penetrans* it was suggested that the nematode's cuticle is locally dissolved by an enzymatic process and that a germ tube penetrates the cuticle of the hosts (Dickson et al., 2009; Sayre and Wergin, 1977). Through this tube the parasite injects its sporoplasma into the host body. *P. ramosa* may use a similar mechanism for penetration.

The *Daphnia* oesophagus is part of the foregut and therefore of ectodermal origin (as is the hindgut, but not the midgut). Therefore, when the *Daphnia* moults, it also sheds the oesophagus lining and any attached spores (Duneau and Ebert, 2012b). When moulting occurs within 12 h of spore attachment (at 20 °C), the host sheds the attached spores with the carapace, and has a high likelihood of escaping infection (Duneau and Ebert, 2012b). Moulting is essential for growth and development in arthropods and in *Daphnia* continues throughout life. Juvenile *Daphnia* moult about every 36–48 h, adults every 3–4 days at 20 °C and at higher temperatures, moulting is more frequent (Bottrell, 1975). Thus, a considerable proportion of the attached spores are lost before penetration. Although, moulting seems to be a mechanism that reduces the likelihood of disease progression, there is so far no evidence that the host can alter this developmentally and phylogenetically constrained mechanism to further reduce infections (Table 2) (Duneau and Ebert, 2012b).

An alternative unexplored mechanism by which *Daphnia* may achieve resistance is by altering the thickness or strength of the cuticle thereby reducing the likelihood of penetration. A strengthening of the cuticle has been observed in other systems, conferring resistance against pathogens (Cotter et al., 2008; Dubovskiy et al., 2013) and may play a role in the *Daphnia* system as well.

In summary, the attached spores penetrate the host's gut epithelium and enter the body cavity. This process takes several hours, giving the host a chance to repel the parasite by moulting. So far no variation has been observed for this process among host or parasite genotypes.

3.5 Step 5. Early within-host phase

After a parasite enters the host, the actual disease develops. In most cases, the parasites begin to proliferate and cause disease-specific harm to the host. At the same time, the host's immune defence may be activated and, in some cases, eliminate the parasite. The mechanistic basis of host immune defence has been well explained for vertebrates, plants and some invertebrates, but little is known for less-studied taxa, like the lower crustaceans. This is true for the *Daphnia*—*Pasteuria* system, where we know a lot about disease progression and ecological immunity, but next to nothing about the molecular processes at work.

Once *Pasteuria* enters the host, it undergoes a rather unusual series of developmental stages resulting in the production of the mature endospores (Box 2, Figure 2). A clear and early external sign of infection is host castration, i.e. females stop producing eggs. We call this time period the within-host 'phase' (as opposed to step), as, in contrast to the other steps, it takes a considerably longer time — about 50 days from penetration to death, although the length of this period is highly variable (Ben-Ami et al., 2008a; Jensen et al., 2006). The within-host phase cannot yet be easily divided into clearly separated steps. However, because most experimental studies terminate infection experiments after 20—30 days, we define the early within-host phase as the first 25 days, and the following period until host death as the late within-host phase. Day 25 marks the approximate halfway point from infection to host death. As we gain more knowledge, this somewhat arbitrary classification may be replaced with a more meaningful biological classification. For example, the time until the first mature endospores are observed (about 15—18 days post infection) could be used as a biomarker for the early within-host phase. However, to make maximal use of the available information, we use here the halfway point to divide the within-host phase into two parts.

After the parasite enters its host, it replicates in the host's body cavity and muscle tissue. For approximately the first 7 days post-penetration (at 20 °C), the bacteria are not detectable by light microscopy. Thereafter, they are easily distinguished as large, multicellular vegetative structures, referred to as the 'cauliflower stage' (up to 15 μm diameter, Box 2, Figure 2), and later

Box 2 Development of *Pasteuria ramosa*

Individual *Pasteuria* cells undergo remarkable morphological development during the infection process (Figure 2). There is no evidence of growth, reproduction or development outside the host. The life cycle begins when the resting endospore comes into contact with a host and sheds its exosporium (activation step). The activated spore (i.e. the spore without the exosporium) has a spherical central body with a ring of parasporal fibres (peripheral fibres, or perisporium) around its circumference, forming a disc-like structure (also described sombrero-like). The activated spore attaches to the host cuticle in the gut (attachment step), and from here penetrates the host's body cavity (penetration step). In *P. penetrans* (but not yet investigated in *P. ramosa*) where host penetration was studied in more detail (Dickson et al., 2009), the spore seems to produce a germ tube (germination) that penetrates the cuticle and hypodermis of its nematode host. Penetration seems to be achieved by an enzymatic process (Dickson et al., 2009). The attached spore and parasporal fibres remain outside, while bacterial cells penetrate into the host.

It is not yet known what happens to the bacterial cells in the first few days after entering the host's body cavity, but after 5–8 days, the parasite is observable by light microscopy in infected hosts, appearing as floret- or cauliflower-like microcolonies up to 15 μm diameter. These microcolonies are composed of a dichotomously branched septate mycelium, which fragment into branch-like structures after the colonies reach a critical size. Peripheral cells (terminal hyphae) of the microcolony expand and give rise to sporangia. Branches with peripheral sporangia continue to grow and fragment into branchlets of quartet, triplet and doublet configurations, with the sporangia attached to each other at the pointed ends. At the rounded end, each sporangium has a small central refractile body visible by light microscopy that will develop into the actual endospore. Eventually, branchlets develop into single teardrop or grape-seed like sporangia. These sporangia and their endospore continue maturation, and eventually, about 14–18 days in the infection, assume the more spherical structure of mature spores. Mature spores, the transmission stage of the bacterium, are nonmotile, have a diameter of about 5–6 μm and are composed of an environmentally resistant exosporium surrounding the endospore. Early during the within-host phase, the development of the parasite cells is synchronous; later during the infection, cauliflower stages can be found again, and eventually all developmental stages are present concurrently. The number of mature spores increases and accumulates until the host's death, when they are released in millions from the dead host.

by their characteristic spores. The typical symptoms of a *Pasteuria* infection are parasite-induced sterilization (castration) and enhanced body growth (gigantism). Castration is sometimes detectable as early as 10 days post infection. Other traits of interest during the early within-host growth phase are the proportion of infected hosts, the fecundity of the infected hosts before parasitic castration, the host body growth and early parasite spore production (Coors et al., 2008; Hall and Ebert, 2012; Hall et al., 2013; Vale and Little, 2012).

Some disease symptoms previously attributed to the early within-host phase (e.g. reduced infection rate, host castration (Hall and Ebert, 2012)) may also be influenced by the penetration step (step 4), as the ease of penetration may determine the number and speed of parasite spores entering the host body cavity. This in turn may be influenced by experimental conditions. Furthermore, wounding of the host's gut wall during penetration may induce an immune response, with consequences for the subsequent within-host phase. So far, no study has tested whether the penetration step influences the expression of subsequent disease symptoms. A few experimental studies, however, applied different treatments only after penetration was complete (i.e. several days after penetration), revealing strong effects on host and parasite traits that had to be due to processes during the early within-host phase (Cressler et al., 2014; Ebert et al., 2004). Here we discuss traits expressed during the early within-host phase as part of step 5, but do not exclude the possibility that the penetration step may play a role in shaping disease expression during the within-host step.

To exclude the large variation caused by the attachment step, experiments were conducted using host and parasite genotypic combinations known to be 100% compatible, as assessed by the attachment of spores to the oesophagus. Assuming uninhibited penetration and no effective immune response, one can expect 100% infection rates for these cases. However, the proportion of hosts that progress to disease is often less than 100% depending on the treatments, which indicates parasite clearance and the elimination of the parasite by the host's immune defence (Hall and Ebert, 2012), although the mechanism for this is unknown. Such a reduction in infection rates is not observed in the late, 'chronic' within-host phase (see next step), as once a parasite has established itself in the host (as judged from the presence of disease symptoms), it is not cleared anymore (Hall and Ebert, 2012).

The *Daphnia*'s immune system is complex, involving melanisation and the typical immune pathways described in other arthropods (Brites et al., 2008; McTaggart et al., 2009; Metchnikoff, 1884), each of which may

contribute to control infections. The production of antimicrobial peptides has so far not been reported (McTaggart et al., 2009). Several studies have examined physiological and immunological responses expressed during the early within-host phase, but no strong effects were observed. Jansen et al. (2013) reported the largest number of differentially expressed genes 4 days post exposure to *Pasteuria* spores. Pauwels et al. (2011) reported that *Pasteuria* spore production in the first 21 days of infection is negatively correlated with phenol oxidase (PO) activity, but another study (Mucklow et al., 2004) reported that the PO activity of unexposed *D. magna* clones was not a good predictor of resistance. Auld et al. (2012b) reported that the number of phagocytic cells increased upon exposure and that this increase correlated positively with parasite dose. A report of effective immune priming 48 h after *Daphnia*'s exposure to noninfective *Pasteuria* spores (McTaggart et al., 2012) suggests that, even without attachment, the host may sense the parasite's presence. However, this finding seems to contradict the observation that a cellular response is only observed when parasite and host are compatible at the attachment step (Auld et al., 2012b). Taken together, these findings support the presence of a functional immune defence in *Daphnia*, but it is not clear if this immune defence is responsible for *Pasteuria* clearing, nor is it clear when during the infection process immune induction occurs.

Excluding variation at the attachment step, a number of studies have revealed pronounced effects of nongenetic factors influencing host and parasite disease traits during the early within-host phase, such as salinity, food quantity, food C:P ratio, fatty acid composition of food and the temporal distribution of feeding times (Cressler et al., 2014; Frost et al., 2008a,b; Hall et al., 2013; Schlotz et al., 2013). These studies suggest, that better conditions for the host are also better for the parasite. For example, better host nutrition results in higher host and parasite fitness estimates (Ebert et al., 2004; Vale et al., 2013) (for a review see (Tseng and Myers, 2014)). Another nongenetic factor influencing the outcome of disease is parasite exposure dose. Higher doses at exposure lead to more severe disease but also reduced parasite spore counts (Ebert et al., 2000b). In addition, the environmental conditions experienced by host mothers have strong effects on infection outcomes for the offspring (Ben-Ami et al., 2010; Frost et al., 2010; Hall and Ebert, 2012; Schlotz et al., 2013). Experiments including different host and parasite genotypes (all compatible at the attachment step) have also shown ample genetic variation in disease traits expressed during the early within-host phase and in some cases genotype/genotype (GxG) and

genotype/environment (GxE) interactions for parasite infection success, host fecundity before castration and early parasite spore production (Hall and Ebert, 2012).

One of the most important features of any individual is its age. Recent studies showed that *D. magna* of different ages differ in their susceptibility to *Pasteuria* infections. Izhar and Ben-Ami (2015) showed that after controlling for all other steps of the infection process, juvenile *D. magna* are more susceptible to *P. ramosa* than older females. This difference goes hand-in-hand with reduced parasite proliferation in older hosts, but does not change the time until host death. Furthermore, age at exposure played a strong role in mediating the outcome of within-host competition, with much stronger competitive exclusion being observed in hosts exposed at a higher age (Izhar et al., 2015). Given the strong variation in age structure of *Daphnia* populations over the course of a season, these findings have important consequences for the epidemiology and evolution of the system. For example, the number of new infections may be much higher in populations dominated by young *Daphnia* — as is typical in spring — than in mid-summer populations, where juveniles are much less common. On the other hand, competition among parasite genotypes would increase across the season.

Expression of disease symptoms depends strongly on host sex (Duneau et al., 2012). Spore counts at different time points across the infection period are substantially higher in females than in the much smaller males, even after correcting for body size. Both sexes suffer from fecundity reduction (sperm and eggs counts), but only female hosts show parasite-induced gigantism. As during these experiments, other steps of the infection process were controlled, these differences are primarily due to sex-specific effects at the within-host phase (Duneau et al., 2012).

Finally, the early within-host phase is a period where intense within-host competition takes place. High dosages of spores administered to hosts will result in strong within-host competition, resulting in the retarded development of the parasite's endospores (Ebert et al., 2000b). Consistent with this, within-host competition of different *Pasteuria* clones and isolates is largely determined during the early within-host phase (Ben-Ami and Routtu, 2013), although this effects varies with host age at infection (Izhar et al., 2015).

Many other experiments that did not explicitly exclude variation at the attachment step, reported environmental effects (direct and maternal effects) for diverse stressors, such as pesticide, food, predator kairomones and

temperature during the early within-host phase (for example Coors and De Meester, 2011; Coors et al., 2008; Cressler et al., 2014; Garbutt et al., 2014; Mitchell and Read, 2005; Stjernman and Little, 2011; Vale et al., 2008). Because environmental effects are absent in the attachment step (Table 2), it is reasonable to assume that these environmental effects are caused by factors acting on the early within-host step, not the attachment step.

In summary, the early within-host phase is a complex step of the infection process, with the traits being expressed during this period showing ample evidence for quantitative genetic variation, sensitivity to environmental conditions, and genotype by environment interactions. Parasite evolution may be shaped strongly by competition during the early within-host phase. Several immunological pathways may act in parallel during this phase, however, the processes governing immunity in *Daphnia* are still poorly understood.

3.6 Step 6. Late within-host phase

In invertebrate taxa and plants, late infection stages are often chronic, lasting until host death. Such is the case for the late within-host infection phase of *P. ramosa*. During this phase, parasite spore production continues as before, leading to an intensive colouration of the host, showing various shades of yellow, red and brown (Ebert, 2005). The parasite is mainly seen in the form of mature spore stages that eventually fill the entire body cavity, although cauliflower and pre-spore stages (grape-seed stage, Box 2; Figure 2) can also be seen. When hosts have enough resources (i.e. sufficient food quantity and quality), *Pasteuria*-induced gigantism starts to become apparent shortly after hosts are effectively castrated (about 10–20 days post infection) (Cressler et al., 2014; Jensen et al., 2006) but is strongest during the late within-host phase. Clearing of infections has not been reported during the late phase of within-host growth, but is easily achieved with antibiotics (Little and Ebert, 2000). Antibiotic treatment of late-stage infections allows the host to reproduce again, suggesting that parasitic castration is not caused by physical destruction of the ovaries, but by physiological means. Consistent with this, during the late within-host phase, some hosts regain the ability to produce clonal offspring (Hall and Ebert, 2012; Mageroy et al., 2011; Schlotz et al., 2013), a trait called 'castration relief'. During castration relief, hosts produce one or a few, typically small, clutches about 25–40 days post infection (at 20 °C). In a comparison of five *P. ramosa* clones, the number of offspring produced during castration relief was shown to be negatively correlated with parasite spore production (Clerc et al., 2015). So far no

evidence has been found showing variation in castration relief among host clones. The mechanism for castration relief is not known, but it may be linked to the reduced physiological activity on the part of the parasite, whose physiologically inert endospores occurring at this phase exert less influence on the host.

Experiments have revealed that host genotype, parasite genotype and environmental effects (direct and maternal environmental effects) all strongly affect virulence and parasite spore production late in the infection process (Hall and Ebert, 2012; Schlotz et al., 2013; Vale et al., 2011). This is also true for castration relief (Hall and Ebert, 2012; Schlotz et al., 2013). Interestingly, these genetic and environmental main effects explain most variation in disease expression, while interaction terms between host and parasite genotypes or between genotypes and the environment seem much less influential (Hall and Ebert, 2012; Vale et al., 2011). Whether this pattern is typical for late-phase infections in general is not yet clear: unfortunately, most experiments terminate observations before the late phase is reached.

In summary, the late within-host phase is characterized by the chronic nature of the infection. The host seems to have no chance of eliminating the parasite, but may ameliorate the fitness cost of infection by castration relief. Genetic variation for disease-related traits is high throughout the within-host phase of infection, but genetic interactions seem to play less of a role.

3.7 Step 7. Host death and spore competence

As with many other invertebrate parasites, *Pasteuria* is an obligate killer, whose transmission stages are only released after host death (Ebert and Weisser, 1997). The time to host death and the quality of the parasite spores released are the key traits of interest here. Other traits, like host body size and spore counts, are considered in the late within-host phase.

Under optimal conditions, female hosts are killed by *Pasteuria* after 30–70 days (Ben-Ami et al., 2008a; Hall and Ebert, 2012; Jensen et al., 2006). For one host–parasite combination, it was found that an intermediate time to host death (about 50 days) resulted in the highest number of *P. ramosa* spores (Jensen et al., 2006). In natural ponds, the dying host most likely sinks to the bottom of the pond where it decays, releasing 0.5 to 20 million mature spores (Ebert et al., 2004). *Pasteuria* spores may also be released by infected hosts that die early from other causes, e.g. environmental stress (starvation, intoxication) and predation. Spore counts

increase from the first fully developed spores around 15–18 days post infection (at 20 °C) until death (Ebert et al., 2004; Hall and Ebert, 2012), although the rate of increase can vary considerably among genotypes (Clerc et al., 2015). It is not known if *Pasteuria* survives the gut passage of *Daphnia* predators, e.g. fish, but this seems likely, as this was shown for at least one fungal parasite (*Metschnikowia bicuspidata*) of *Daphnia* (Duffy, 2009). Spores may also be released into the free water as a consequence of sloppy feeding predators on infected hosts (Auld et al., 2014; Hall et al., 2010; Goren and Ben-Ami, 2015).

Time to parasite-induced host death differs among parasite genotypes infecting the same host clone and among host clones infected with the same parasite clone (Ben-Ami et al., 2008a; Ben-Ami and Routtu, 2013; Hall and Ebert, 2012; Izhar et al., 2015; Vale et al., 2013, 2011). Time to death also depends on environmental factors, such as food level, parasite spore dose, the presence of other parasites and temperature (Ben-Ami et al., 2011; Ebert et al., 2004, 2000b; Hall and Ebert, 2012; Vale et al., 2013, 2008, 2011). As was the case for the late within-host phase, interaction terms (G × G, G × E, G × G × E) tend to explain hardly any variation in time to death (Table 2). Time to *Pasteuria*-induced host death seems not to depend on host age at exposure or host sex: *D. magna* infected at different ages died after the same number of days (Izhar and Ben-Ami, 2015; Izhar et al., 2015) and males, which normally live about half as long as females, are killed about twice as fast as females by the parasite (Duneau et al., 2012).

After the death of the host, spores of *Pasteuria* are released into the environment. Spores as old as 30 years have been revived from sediment cores (Decaestecker et al., 2004). Nothing is known about genotypic or environmental effects on spore survival. However, experiments with spores collected from infected females kept under different feeding regimes have shown that the quality of spores may vary: spores from well-fed hosts were more virulent than spores from poorly fed hosts (Little et al., 2007). A similar effect was also found for a fungal parasite of *Daphnia* (Searle et al., 2015).

In summary, the parasite produces transmission stages that are only released when the host dies or is killed. The time to parasite-induced death depends strongly on host and parasite genetics and environmental factors, but not on interactions between these factors. Although mature spores are found in infected hosts as early as 15–18 days post infection, the parasite normally kills the host much later. Premature host death can contribute to parasite transmission.

4. USING THE STEPWISE MODEL TO ADDRESS EVOLUTIONARY QUESTIONS

Dividing the infection process into discrete steps allows us to more closely examine individual processes and how they are linked to functional aspects of the system. It also enables us to relate individual steps to evolutionary models, which are typically based on simplifying assumptions, such as simple genetics and no environmental effects. In this section of this review we address a number of questions using the stepwise approach to better understand the epidemiology and evolution of the system.

4.1 How much host variation can be explained by each step?

The overview in Table 2 illustrates the tremendous difference in the degree to which genetic and nongenetic factors shape trait variation during the steps of the infection process. Traits expressed during the first step and the last three steps (steps 1, 5, 6 and 7) show the typical signature of complex quantitative genetic traits: variation is quantitative, environmental factors influence trait expression and host and parasite genetic effects are apparent. In contrast, spore activation (step 2) seems not to be influenced by any known factor, while penetration (step 4) seems to be influenced only by environmental factors. In these two steps, variation appears limited by phylogenetic and developmental constraints. The spore attachment step (step 3) is governed by binary genetic variation, without any evidence for environmental effects. While this step-by-step consideration reveals the enormous diversity in the contribution of different steps to disease progression, it does not allow us to assess the relative importance of variation at each step. Here we ask, to what extent does the overall expression of a specific disease trait depend on variation at individual steps, and how does this variation influence the evolution of the trait?

As a first approximation, earlier steps tend to influence total variation of host traits more than later steps, as each step acts as a filter, reducing the possible variance of later steps. However, as the amount and distribution of variation among and within populations differs for each step, some steps may contribute more to the total variation than their position in the chain-like process would suggest. The list of reported significant effects on trait expression does not help us judge the relative importance of a given step, as these effects are typically assessed by reducing or even excluding variation at other steps. For example, testing which factors influence traits expressed

during the within-host phase only makes sense if host–parasite combinations are used that are compatible at the attachment step.

To illustrate how each step contributes to variation in disease, we focus here on the likelihood of infection, as we know most about this trait. Although it might seem that the first step, the host encounter step, would exert the strongest influence on the likelihood of infection, two factors reduce its impact: First, parasite variation is unlikely to contribute to variation in this step, as the parasite is passively waiting to be picked up by the filter-feeding host. Second, encounter depends in part on the spatial distribution of hosts and parasites. If parasite spores are homogeneously distributed in the water (free floating spores in the planktonic phase), no variation in encounter is expected, unless the intensity of filter feeding varies among host genotypes. In contrast, spores located in the pond sediments are more likely to be encountered by negative phototactic host clones (Decaestecker et al., 2002). However, as phototactic behaviour is known to show a signature of local adaptation in *Daphnia* (De Meester, 1993, 1996), it differs more between populations than within population (Table 2). Thus, the encounter step does little to explain overall variation in disease on a within-population level but may have a potentially high impact on variation globally. The second step, spore activation, does not contribute to the variation in infection success, because it appears to be a fixed trait common to all hosts.

The attachment step, however, shows particularly strong variation, both within and between populations, but without evidence for local adaptation for infection rate (Ebert et al., 1998; Luijckx et al., 2011). The hallmark of the attachment step is binary variation, caused by the strong host–parasite interactions, which may render combinations of host and parasite genotype incompatible (no attachment) (Luijckx et al., 2011). Approximately one-third of host clone–parasite clone combinations showed attachment (Luijckx et al., 2012), leaving more than two-thirds of the combinations incompatible. Incompatibility terminates the infection process and thus illustrates the strong filter effect of the attachment step.

The fourth step, penetration, has so far only been associated with variation caused by host moulting, which is more frequent in juveniles than in adults and at higher temperatures (Bottrell, 1975; Duneau and Ebert, 2012b). Given the variation in age and temperature, and no known variation among host and parasite genotypes, this step acts mostly as a random filter, reducing the number of parasites that reach the next step.

After excluding variation from the attachment step, strong variation during the early within-host phase (step 5) is due to both genetic and environmental effects. However, overall, the early within-host phase explains a much smaller proportion of the total variation in infection rate than the attachment step (step 3), as it can only contribute to variation in the subset of host–parasite combinations that have passed the earlier steps. As clearance does not seem to occur once the infection is established, the late within-host step does not influence infection success, but does influence the expression of host and parasite life history.

In summary, the filter-like nature of the stepwise infection process successively reduces the likelihood that later steps of the host defence machinery encounter the parasite. Thus, everything else being equal, selection for resistance is strongest at the earliest host steps. However, due to the specific biology of the *Daphnia–Pasteuria* system, we suggest that the attachment step (step 3) explains most variation in infection within a population and that selection would be strongest here. For other steps, e.g. host castration, this will be different, with the within-host phase playing possibly a stronger role. In a spatial setting, however, with different *Daphnia* populations showing divergent phenotypes due to local adaptation, steps that show spatial divergence (e.g. phototactic behaviour and thus encounter rate) may contribute more strongly to overall variation.

4.2 Genetic basis of disease expression

What is the genetic architecture underlying each step? So far, most genetic studies have focused on the attachment step. Breeding experiments and a QTL study with *D. magna* have revealed that resistance to parasite attachment is dominant, and that a few loci interact epistatically to produce an overall pattern, which seems always binary (Luijckx et al., 2011, 2012, 2013; Routtu and Ebert, 2015). So far, three closely linked loci have been hypothesised to be responsible for this pattern (Metzger, 2014). The genes responsible for attachment are not known, but comparative genomics, QTL studies, genome scans and transcriptome approaches are in progress (Decaestecker et al., 2011; McTaggart et al., 2009; Orsini et al., 2012; Routtu and Ebert, 2015; Routtu et al., 2010). The other steps with a signature of among clone variation (e.g. the encounter step and within-host steps) are all quantitative, complicating the identification of the underlying genes.

Currently, our limited evidence indicates that genes responsible for the variation in traits at different steps are independent of each other on a

genomic level. *D. magna* genotypes that differed strongly in their behaviour and thus in their propensity to encounter *Pasteuria* spores from sediment did not otherwise differ in resistance (Decaestecker et al., 2002). A sediment core study of *Pasteuria*—*D. magna* coevolution has proposed that selection shapes infectivity (probability of parasite establishment upon host encounter; presumably caused mainly by variation in the attachment step) and virulence (host fitness loss due to infection; presumably mainly due to variation during the within-host phases) differently, suggesting that they may be coded by different genes (Decaestecker et al., 2007).

Genes for resistance to *P. ramosa* seem also to be different from genes for resistance to other parasites. Evidence comes from the absence of correlations between the resistance to *Pasteuria* and resistance to other parasites, four microsporidian species, a virus and a fungus, suggesting that most variation in resistance is explained by different underlying genetic architectures (Auld et al., 2012a; Decaestecker et al., 2003; Ebert, 2008; Mucklow et al., 2004; Zbinden et al., 2008). Likewise, mapping resistance to *P. ramosa* and the microsporidium *Hamiltosporidium tvaerminnensis* in the same QTL panel, indicates a different genetic architecture underlying resistance to these two diseases: *P. ramosa* resistance showed a single strong QTL, while *H. tvaerminnensis* showed several weak QTLs and epistasis, without any co-localization of QTLs for the two parasites (Routtu and Ebert, 2015). However, minor QTL influencing resistance to both parasites may have gone undetected.

In contrast to the host, we know very little about the underlying genetics for disease-related traits in the unculturable *P. ramosa*. Proteomic and genomic analyses have suggested that collagen-like proteins (the bacterial version of collagen) may influence the attachment of *Pasteuria* genotypes (McElroy et al., 2011; Mouton et al., 2009). This hypothesis is supported by the fact that collagen-like genes seem to act as adhesins in pathogenic bacteria (McElroy et al., 2011; Mouton et al., 2009) and by studies on the nematode parasite *P. penetrans* (Davies, 2009). The family of collagen-like genes is vastly expanded in both *Pasteuria* species, far beyond what is found in any other fully sequenced bacterium, making it unusual among bacteria (Davies, 2009; McElroy et al., 2011). However, although collagen-like proteins may influence the attachment step, they are not candidates for variation observed in later steps of the infection process.

In summary, host genetic effects are seen at most steps, with the marked exception of the activation and the penetration step (Table 2). There is no evidence that genes with a function specifically relevant at one step influence disease expression at other steps. However, genetic independence is not the

same as evolutionary independence, as genes at different steps contributing to the expression of the same trait can be under selection together.

4.3 Evolution of resistance and its costs

Resistance, the host's ability to prevent or reduce parasite growth, is related to tolerance, where hosts minimize the fitness impact of the parasite but without the associated damage to the parasite (Raberg et al., 2007). The evolution of resistance and tolerance are driven by selection on the host to reduce the harmful consequences of infection. Any step during the infection process where the host shows genetic variation for the degree it is harmed by the parasite could contribute to the evolution of resistance and tolerance. As very little work has been done on tolerance in the *Daphnia*—*Pasteuria* system (but see Vale and Little, 2012; Vale et al., 2011), we will focus here on resistance. We suggested above that the genetic architecture for resistance is different across steps, with no current evidence of physical linkage. Nevertheless, partial resistance early in the infection process influences selection at later steps by modifying the parasite population composition and by reducing the number of parasites arriving at the later steps. In extreme cases, if one step evolves to prevent infection entirely, the following steps will not be exposed to the parasite and their variation for resistance may become neutral. Therefore, this indirect form of interaction among steps creates epistasis among the genes that act in different steps (Hall and Ebert, 2013).

Step-specific costs of resistance may modify this picture. Costs manifest as trade-offs between the fitness benefits of resistance and the fitness loss of having (constitutive) or using (inducible or deployment) resistance machinery (Schmid-Hempel, 2011). If resistance costs occur at several steps, resistance will more likely evolve at the step with the better cost—benefit ratio. So far we know little about the resistance costs expressed at different steps in the *Daphnia*—*Pasteuria* system. The encounter step presents a clear case of behavioural trade-offs between avoiding sediment-borne parasites and other fitness components such as reducing protection against fish (Decaestecker et al., 2002) and the opportunity to browse for food resources directly over the sediment (Ebert, 2005; Horton et al., 1979). In the attachment step, resistance comes at a cost of lost opportunity, because possessing a certain resistance allele precludes other alleles, such that resistance to a particular *Pasteuria* genotype may be traded-off against others (Luijckx et al., 2013). There is no evidence that resistance at the attachment step is resource intensive. In contrast, the within-host steps

(5 and 6) may show resistance costs, as immune defences may be resource intensive. Indeed, strong environmental effects are observed. However, studies on costs of resistance (excluding the encounter step) have yielded mixed results (Allen and Little, 2011; Jansen et al., 2011; Labbe et al., 2010; Little et al., 2002; Little and Ebert, 2001; Little and Killick, 2007). In hindsight, uncontrolled variation at the attachment step may have confounded some of these experiments, resulting in strong intra- and inter-experiment variation in detecting costs. No studies have yet examined resistance costs for *Pasteuria* at the within-host phase after excluding variation at all earlier steps.

Costs may also be paid as reduced resistance to other parasites, but this situation seems not to be the case in the *Pasteuria* system as discussed above (Auld et al., 2012a; Decaestecker et al., 2003; Ebert, 2008; Mucklow et al., 2004; Zbinden et al., 2008). Again, however, these experiments are inconclusive, as they did not control for variation at the attachment step.

Additionally, while the host genes of the attachment step seem specific to the interaction with *Pasteuria*, the within-host steps likely include components that are also functionally important in defending against other parasite species. As a consequence, in a parasite-rich environment, costly immune functions may be maintained. However, much remains to be done to understand how the *Daphnia* immune system functions against *P. ramosa* and other parasites.

4.4 Expression and evolution of virulence

Pasteuria has severe fitness costs for its host: a *Daphnia* infected as a juvenile loses 90–100% of its expected lifetime reproductive success, a young adult between 60% and 90% (Ben-Ami et al., 2008a, 2011; Decaestecker et al., 2005; Ebert et al., 2000a). Understanding the factors that influence the evolution and expression of parasite-induced harm in the host (mortality and morbidity = virulence) is a central issue in evolutionary parasitology (Poulin, 2007; Schmid-Hempel, 2011). *Pasteuria* has become a model system for the study of the evolution of virulence, in particular with respect of parasitic castration, gigantism and obligate host killing (Ben-Ami et al., 2008a, 2011; Ben-Ami and Routtu, 2013; Cressler et al., 2014; Ebert et al., 2004; Jensen et al., 2006). We have now a rather good understanding of the evolutionary process at work, with this system having pushed forward our insights into parasite-induced host castration and gigantism, a virulence syndrome known to have evolved several times independently in other host–parasite systems (Baudoin, 1975).

Only three steps, the encounter and the two within-host steps, contribute to the expression of virulence. As *Pasteuria* virulence is to some degree dose dependent, the encounter step plays a role for disease severity: higher exposure dose can lead to faster castration, more pronounced host gigantism and earlier host death (Ben-Ami and Routtu, 2013; Ebert et al., 2000b, 2004). However, since we lack theory and predictions for the evolution of virulence under conditions of variable exposure doses, studies on the evolution of *Pasteuria* virulence often avoid these effects by controlling dose (but see Ben-Ami and Routtu, 2013) and thus reduce the contribution of the encounter step to disease expression. Consequently, the host–parasite interactions during the within-host steps become the key players for the expression and evolution of virulence.

The within-host steps have a strong impact on the life history traits of both parasite (e.g. spore production, time to host death) and host (e.g. time to castration, castration relief, gigantism), with these traits all showing the signature of quantitative traits with genetic and environmental factors contributing to their variation (Tables 1 and 2). The different components contributing to virulence are correlated with each other, resulting in the typical *Pasteuria* virulence syndrome characterized by host castration, host gigantism and obligate host killing. Experimental work allowed to disentangle the different disease traits by manipulating the host and parasite material used and the experimental conditions (Ben-Ami et al., 2008a, 2011; Ben-Ami and Routtu, 2013; Coors and De Meester, 2011; Cressler et al., 2014; Ebert et al., 2004; Ebert and Weisser, 1997; Jensen et al., 2006; Little et al., 2008), thereby producing a rather clear picture about optimal disease expression in this system.

Evolutionary theory about parasitic castration (Baudoin, 1975; Ebert et al., 2004; O'Keefe and Antonovics, 2002; Obrebski, 1975) is based on the assumption of a zero-sum-game where host and parasite are competing for a fixed amount of resources, leading to a negative correlation between host and parasite resource allocation. The general idea is that castration serves the parasite by channelling resources away from host reproduction to serve the needs of the parasite. Since in the early phase of infection the parasite does not yet have the need for the large amount of resources liberated by host castration, it was suggested that parasite-induced host gigantism is beneficial for the parasite as it allows to store the excess resources liberated early during an infection until they can be used by the growing parasite later during infection (the temporal storage hypothesis, TSH) (Ebert et al., 2004). Under the TSH, it is expected that hosts will be killed when the parasite

cannot extract more resources from the gigantic host. While earlier models of castrating parasites predicted instantaneous and complete host castration (O'Keefe and Antonovics, 2002; Obrebski, 1975), the TSH predicts that castration starts when the parasite reaches a sufficient biomass to exert control over the host and that the age at castration is largely independent from the resource level because resources at early stages of the infection process are not yet limiting (Cressler et al., 2014; Ebert et al., 2004). Finally, taking coevolution into account, hosts are selected to resist castration as long as possible or if possible to reverse castration during the late within-host phase.

Work on the expression of virulence in the *Pasteuria*—*Daphnia* system is largely in agreement with the TSH, but some gaps in our knowledge are apparent. *Pasteuria* has high resource requirements, as the endospores eventually fill the host's body cavity entirely, reaching a substantial biomass. This supports the TSH's assumption of a host—parasite conflict over resources. The finding that environmental factors that reduce resource intake also reduce both host fecundity and parasite spore counts (Cressler et al., 2014; Ebert et al., 2004; Schlotz et al., 2013) further supports this assumption. During the early within-host phase, *Pasteuria* castrates its host and shortly later induces enhancement of host growth. Castration is not instantaneous, but starts only after 7—20 days post infection and depends on the combination of host and parasite genotype (Ebert et al., 2004). Castration is initially complete, but in some host—parasite combinations the hosts may resume reproduction (castration relief) during the late within-host phase. This trait is not predicted by parasite-centred models on the evolution of virulence, but can be explained by taking host evolution into account (Minchella, 1985). In agreement with the TSH, the host is killed by the parasite when host growth slows down, consistent with the suggestion that host death occurs when all available resources are used up.

Since models of the evolution of virulence are mainly concerned with parasites maximizing their fitness, symptoms expressed in the host must be related to parasite fitness components. For example, it was predicted that parasites should kill their host when the transmission potential for the parasite is maximal (Anderson and May, 1981, Ebert and Weisser, 1997). Indeed, for one sympatric *D. magna*—*P. ramosa* combination it was shown that parasite spore production peaked at the average time of host death (Jensen et al., 2006). Furthermore, fast castration and strong gigantism were shown to benefit the parasite, while both traits have obvious costs for the host (Ebert et al., 2004), giving support for the TSH. Cressler et al. (2014) compared the TSH to two alternative models

of resource allocation and the expression of virulence. By manipulating food levels, they supported the TSH by showing that gigantism, but not castration, correlates with food level and that the parasite is able to use energy put into host growth as a resource. Alternative models were not supported by this experiment (Cressler et al., 2014).

There is also evidence for the host being able to counteract the parasite by reducing its harm. Hosts that are infected early in life mature earlier and are thus able to produce more offspring before castration starts (Ebert et al., 2004). Each clutch a host is able to produce before castration starts has costs for the parasite in terms of reduced spore production, highlighting the conflict over the limited resources (Ebert et al., 2004). The same is true for castration relief expressed during the late within-host phase, which is beneficial for the host, but has high costs in terms of spore production for the parasite (Clerc et al., 2015; Hall and Ebert, 2012). Infections caused by different clones of *P. ramosa* differ strongly in the extent of castration relief observed (Clerc et al., 2015; Hall and Ebert, 2012). A consequence of this strong genetic variation is that traits influenced by it (e.g. host fecundity and spore production) show increased levels of genetic variation during the late within-host phase. For example, an assessment of genetic variation for host size, fecundity and spore production during the infection period across five *P. ramosa* genotypes found no variation expressed during the early, but strong genetic variation during the late within-host phase (Clerc et al., 2015).

The within-host phase is clearly the virulence-determining step in *Pasteuria* infections. This was also apparent in assessment of sex-specific virulence. Two particular features of the *Daphnia-Pasteuria* system allow for predictions regarding the evolution of sex-specific virulence. First, as *Daphnia* populations are strongly female biased (a consequence of mostly asexual reproduction) the parasite encounters many more female than male hosts. Second, the strong dependence of *Pasteuria* on the large amount of resources liberated by castration makes females the more profitable sex. From this it was predicted, that *Pasteuria* should adapt primarily to female hosts (Duneau and Ebert, 2012a; Duneau et al., 2012). Indeed, *P. ramosa* reveals sex-specific adaptive virulence (Duneau et al., 2012), with females being more exploited than males. Since variation at other steps was excluded, these differences are likely caused by differences during the within-host phase.

Finally, the evolution of virulence is believed to be strongly influenced by the rate at which hosts become infected by multiple host genotypes.

Multiple infections are expected to lead in the long term not only to an evolutionary increase in virulence, but also to an immediate plastic upregulation of virulence (Frank, 1996; van Baalen and Sabelis, 1995). Data from the *Pasteuria* system confirms this prediction, with multiple infections resulting in earlier host death and higher success of the more virulent parasite genotypes (Ben-Ami et al., 2008a, 2011; Ben-Ami and Routtu, 2013; Izhar et al., 2015). Since multiple infections are likely to increase when the exposure to the parasite increases, the exposure step can indirectly play an important role for the evolution of virulence.

In summary, the exposure step and the two within-host phases of the infection process determine the evolution and expression of virulence in the *Daphnia*—*Pasteuria* system. Models of the evolution of virulence tailored to castrating parasites agree with the findings from this system, making this one of the best understood systems in the field of virulence research. The role of host counter defences needs more attention both in empirical research and in coevolutionary models of virulence.

4.5 Host—parasite coevolution

Host—parasite coevolution refers to evolutionary changes in host and parasite populations that act as agents of natural selection on each other, causing adaptive changes in both antagonists. Several genetic models for host—parasite coevolution have been put forth, the most prominent being the selective sweep model, and coevolution by negative frequency-dependent selection (NFDS), also called Red Queen dynamics (Lively, 2010; Woolhouse et al., 2002). During selective sweep coevolution, mutants arise and spread in the population. Selection in this case is directional. Any beneficial mutant, regardless of its genetic background or of the gene it affects, can spread and may reach fixation. In contrast, coevolution by NFDS operates on a specific genetic architecture based on a few loci in the host and parasite and highly specific interactions between genotypes or alleles of the two antagonists (so-called matching allele matrices). Matching allele interactions can lead to NFDS, such that parasite genotype frequencies track the frequencies of the host genotypes they are able to infect (Lively, 2010). In this case, alleles at the host and parasite loci responsible for the specific interaction engage in potentially endless cycles of frequency changes. Coevolution by selective sweeps and by NFDS can act simultaneously at different genes in the genome, as long as recombination exists, which is the case for *Daphnia* and *Pasteuria*, although at irregular

intervals (Andras and Ebert, 2013; Lampert, 2011). The *Daphnia*—*Pasteuria* system is among the few systems with eukaryotic hosts where it is possible to conduct experiments that explore mechanisms and test hypotheses of coevolutionary models (reviewed in Ebert, 2008). Furthermore, the possibilities of tracing coevolutionary dynamics over decades by using material recovered from lake sediment cores make this an even more powerful system.

Most research on coevolution in the *Daphnia*—*Pasteuria* system focuses on coevolution by NFDS, where advances on the phenotypic and genetic level under both controlled and natural conditions have been made. The key genetic assumption of coevolution by NFDS — the matching allele model — has so far only been confirmed in the *D. magna*—*P. ramosa* system, where it is visible in the attachment step (Luijckx et al., 2013) (see above). The loci responsible for the attachment step are the likely sites for coevolution by NFDS in this system. As this step shows no sensitivity to environmental variation and explains most variation in resistance, selection is likely to be rather efficient at these loci.

Host—parasite genetic interactions, a prerequisite for coevolution by NFDS, are also found in the early within-host phase (Table 1) (Hall and Ebert, 2012; Vale and Little, 2009), but the underlying genetic interaction matrix has not been studied. Since the amount of variation explained by this step is overall relatively low and its sensitivity to environmental factors high, it is not a good candidate for NFDS. Using host—parasite combinations that are fully compatible at the attachment step, will allow us in the future to explore how the host's immune response during the early within-host step coevolves with the parasite. Other steps of the infection process do not show genetic host—parasite interactions (Table 2), excluding them as candidates for NFDS.

A study of sediment cores from a Belgium pond, in which viable *P. ramosa* spores and *D. magna* resting stages were recovered from layers as old as about 25 years, provided the first evidence that their evolutionary interactions for infection were indeed highly dynamic over the observation period of about 25 years (Decaestecker et al., 2013, 2007). These results are consistent with the idea that differential infectivity, as caused by the attachment step, evolves by NFDS. The same experiments also suggested that traits resulting from interactions during the within-host phase, such as castration and production of parasite spores, are under directional selection, hinting that genes for these traits may have evolved by directional selection.

4.6 The evolution of host range

The host range refers to the genetic compatibility of a parasite with a range of different host species. Perfectly resistant hosts are not part of a parasite's host range. The filter function of the different steps can help to explain the evolution of host ranges by identifying the step where resistance occurs. Blocking of the parasite at any single step will exclude the host from the parasite's host range, no matter how permissible the other steps may be (Antonovics et al., 2013; Combes, 2001; Poulin, 2007). By examining individual steps of the infection process across a range of potential host species, one can test which steps contribute to shape the host range of the parasite.

Field surveys of *P. ramosa* have reported the parasite in several *Daphnia* species as well as in closely related genera, such as *Ceriodaphnia, Moina* and *Simocephalus* (Auld et al., 2012a; Goren and Ben-Ami, 2013; Green, 1974; Sayre et al., 1977; Stirnadel and Ebert, 1997). As no molecular analyses had been conducted, it was unclear if *Pasteuria* had a very broad host range or if cryptic host races existed, infecting only one or a smaller subset of host species. Infection experiments have indicated that *P. ramosa* can cause disease in host species different from the one it was isolated from, although rarely (Duneau et al., 2011) (F. Ben-Ami, unpublished data). A study testing *Pasteuria* isolates collected from natural *D. magna* and *D. longispina* infections with various clones of *D. magna, D. pulex* and *D. longispina*, found that the inability of *Pasteuria* to progress after attachment blocks disease progression, thus marking the penetration and/or the early within-host step as being primarily responsible for determining the host range of *Pasteuria* (Luijckx et al., 2014). The parasite causes disease only in the host species it was isolated from. Therefore, it is unlikely that *Pasteuria* evolving in one host species encounters and recombines with *Pasteuria* in another host species. Indeed the observed genetic divergence among the *D. magna* and the *D. longispina* derived *P. ramosa* (Luijckx et al., 2014) suggests that cryptic *Pasteuria* host races or even species exist.

5. CONCLUSIONS

Stepwise models of complex biological processes, such as sexual selection (pre- and postcopulatory selection), speciation (pre- and post-zygotic isolation), development (different life-history stages), cell division (two-step meiosis) and migration (migrant production, dispersal, establishment),

can provide a deeper understanding of the evolution of these processes by linking mechanisms to population processes. The breakdown of the infectious disease process into a series of steps is also not new (Burnet and White, 1972; Cox, 1993), but applying a population perspective to these steps to gain an evolutionary perspective has only rarely been undertaken (Combes, 2001; Schmid-Hempel and Ebert, 2003). Using this approach allows us to explore the contributions of natural genetic and environmental factors on variation at each step of the infection process and clarify the direct and indirect interactions that occur in the sequence of steps. Each step can be understood as a filter through which the parasite must pass. The step-specific variation determines how the filter acts: Steps that reduce the likelihood of the parasite passing on to the next step, reduce the number of hosts who encounter the parasite at the next step, which reduces the strength of selection for disease traits at the later steps. Interestingly, this reduction of the intensity of selection does not apply to the parasite, which has to pass through every step to conclude its life cycle and transmit to the next host. Besides the direct effect of the filters at each step, each filter influences the evolution at later steps by reducing the effective population size and thus making selection less efficient. Furthermore, filtering during the step-wise process has also consequences for the evolutionary dynamics of genes interacting across different steps. This is because earlier steps constrain later steps pleiotropically, by linking the filter function of one step to trait expression at later steps (Donohue, 2014). This effect has also emerged in models of host–parasite coevolution using two-step processes (Agrawal and Lively, 2003; Fenton et al., 2012).

The example of the *Daphnia–Pasteuria* system highlighted in this review reveals that variation at individual steps is due to a unique combination of factors (Table 2). Some steps have no variation while others are highly sensitive to host, parasite and environmental factors (Table 2). This knowledge provides a better mechanistic picture of how host–parasite interactions evolve. For example, identifying the variance components in the *Daphnia–Pasteuria* steps has revealed which step is the best candidate for explaining coevolution between the antagonists, which steps might carry the greatest cost of resistance, which steps limit the host range of the parasite and at which steps adaptive evolution is most likely to occur. In human and livestock systems, the same approach may further suggest which steps are best for therapy or vaccine development, namely those where the parasite is least likely to evolve resistance against our measures to control them. This has been suggested for *Helicobacter pylori* associated with human gastric

cancer by He et al. (2014), but is also discussed for other pathogens such as human immunodeficiency virus (Arfi et al., 2008), Picornaviridae (Koike, 2011), streptococci (Courtney et al., 2002) and several other bacterial pathogens (Koike, 2011).

By reducing the complexity of the infection process, we are also able to test the (often too simple) assumptions of mathematical infectious disease models, such as resource trade-offs, genetic architecture, and effects of environmental factors. Parasite models relating to the evolution of sex, for example, are often based on a matching allele model (Otto and Nuismer, 2004; Salathe et al., 2008). Close examination of *Pasteuria*'s infection process has shown that the attachment step is indeed based on a matching allele type model (Duneau et al., 2011; Luijckx et al., 2013), but its signature had previously been disguised by variation in other steps. Testing assumptions of evolutionary models is an important step towards closing the gap between empirical findings and theory.

The ideas and concepts presented in this review are not specific to the *Daphnia*–*Pasteuria* system but can be applied to any infectious disease, although the biology of the steps will differ from system to system, and the relative contribution of host, parasite and environmental factors will change. In the future, we may be able to compare stepwise accounts of the genetic and nongenetic contributions of different diseases and analyse them for common patterns.

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REFERENCES

Adamu, J.Y., Wawegama, N.K., Browning, G.F., Markham, P.F., 2013. Membrane proteins of *Mycoplasma bovis* and their role in pathogenesis. Res. Vet. Sci. 95, 321–325.

Agrawal, A.F., Lively, C.M., 2003. Modelling infection as a two-step process combining gene-for-gene and matching-allele genetics. Proc. R. Soc. Lond. Ser. B-Biol. Sci. 270, 323–334.

Allen, D.E., Little, T.J., 2011. Identifying energy constraints to parasite resistance. J. Evol. Biol. 24, 224–229.

Anderson, R.M., May, R.M., 1981. The population dynamics of microparasites and their invertebrate hosts. Philos. Trans. R. Soc. Lond. Ser. B 291, 451–524.

Andras, J.P., Ebert, D., 2013. A novel approach to parasite population genetics: experimental infection reveals geographic differentiation, recombination and host-mediated population structure in *Pasteuria ramosa*, a bacterial parasite of *Daphnia*. Mol. Ecol. 22, 972–986.

Antonovics, J., Boots, M., Ebert, D., Koskella, B., Poss, M., Sadd, B.M., 2013. The origin of specificity by means of natural selection: evolved and nonhost resistance in host-pathogen interactions. Evolution 67, 1–9.

Arfi, V., Riviere, L., Jarrosson-Wuilleme, L., Goujon, C., Rigal, D., Darlix, J.-L., Cimarelli, A., 2008. Characterization of the early steps of infection of primary blood monocytes by human immunodeficiency virus type 1. J. Virol. 82, 6557–6565.

Auld, S.K.J.R., Hall, S.R., Duffy, M.A., 2012a. Epidemiology of a *Daphnia*-Multiparasite system and its implications for the Red Queen. Plos One 7.

Auld, S.K.J.R., Edel, K.H., Little, T.J., 2012b. The cellular immune response of *Daphnia magna* under host-parasite genetic variation and variation in initial dose. Evolution 66, 3287–3293.

Auld, S.K.J.R., Hall, S.R., Housley Ochs, J., Sebastian, M., Duffy, M.A., 2014. Predators and patterns of within-host growth can mediate both among-host competition and evolution of transmission potential of parasites. Am. Nat. 184 (Suppl. 1), S77–S90.

van Baalen, M., Sabelis, M.W., 1995. The dynamics of multiple infection and the evolution of virulence. Am. Nat. 146, 881–910.

Baudoin, M., 1975. Host castration as a parasitic strategy. Evolution 29, 335–352.

Behrens, S., Peuss, R., Milutinovic, B., Eggert, H., Esser, D., Rosenstiel, P., Schulenburg, H., Bornberg-Bauer, E., Kurtz, J., 2014. Infection routes matter in population-specific responses of the red flour beetle to the entomopathogen *Bacillus thuringiensis*. BMC Genomics 15.

Ben-Ami, F., Ebert, D., Regoes, R.R., 2010. Pathogen dose infectivity curves as a method to analyze the distribution of host susceptibility: a quantitative assessment of maternal effects after food stress and pathogen exposure. Am. Nat. 175, 106–115.

Ben-Ami, F., Mouton, L., Ebert, D., 2008a. The effects of multiple infections on the expression and evolution of virulence in a *Daphnia*-endoparasite system. Evolution 62, 1700–1711.

Ben-Ami, F., Regoes, R.R., Ebert, D., 2008b. A quantitative test of the relationship between parasite dose and infection probability across different host-parasite combinations. Proc. R. Soc. B 275, 853–859.

Ben-Ami, F., Rigaud, T., Ebert, D., 2011. The expression of virulence during double infections by different parasites with conflicting host exploitation and transmission strategies. J. Evol. Biol. 24, 1307–1316.

Ben-Ami, F., Routtu, J., 2013. The expression and evolution of virulence in multiple infections: the role of specificity, relative virulence and relative dose. BMC Evol. Biol. 13, 97.

Benhamed, S., Guardiola, F.A., Mars, M., Angeles Esteban, M., 2014. Pathogen bacteria adhesion to skin mucus of fishes. Vet. Microbiol. 171, 1–12.

Bottrell, H.H., 1975. Generation time, length of life, instar duration and frequency of moulting, and their relationship to temperature in eight species of *Cladocera* from the river Thames, reading. Oecologia (Berlin) 19, 129–140.

Brites, D., McTaggart, S., Morris, K., Anderson, J., Thomas, K., Colson, I., Fabbro, T., Little, T.J., Ebert, D., Du Pasquier, L., 2008. The Dscam homologue of the crustacean *Daphnia* is diversified by alternative splicing like in insects. Mol. Biol. Evol. 25, 1429–1439.

Burnet, M., White, D.O., 1972. Natural History of Infectious Diseases. UK Cambridge Uni, Cambridge.

Clerc, M., Ebert, D., Hall, M.D., 2015. Expression of parasite genetic variation changes over the course of infection: implications of within-host dynamics for the evolution of virulence. Proc. R. Soc. B-Biol. Sci. 282, 20142820.

Colbourne, J.K., Pfrender, M.E., Gilbert, D., Thomas, W.K., Tucker, A., Oakley, T.H., Tokishita, S., Aerts, A., Arnold, G.J., Basu, M.K., Bauer, D.J., Caceres, C.E., Carmel, L., Casola, C., Choi, J.H., Detter, J.C., Dong, Q.F., Dusheyko, S., Eads, B.D., Frohlich, T., Geiler-Samerotte, K.A., Gerlach, D., Hatcher, P., Jogdeo, S., Krijgsveld, J., Kriventseva, E.V., Kultz, D., Laforsch, C., Lindquist, E., Lopez, J., Manak, J.R., Muller, J., Pangilinan, J., Patwardhan, R.P., Pitluck, S., Pritham, E.J., Rechtsteiner, A., Rho, M., Rogozin, I.B., Sakarya, O., Salamov, A., Schaack, S., Shapiro, H., Shiga, Y., Skalitzky, C., Smith, Z., Souvorov, A., Sung, W., Tang, Z.J., Tsuchiya, D., Tu, H., Vos, H., Wang, M., Wolf, Y.I., Yamagata, H., Yamada, T., Ye, Y.Z., Shaw, J.R., Andrews, J., Crease, T.J., Tang, H.X., Lucas, S.M., Robertson, H.M., Bork, P., Koonin, E.V., Zdobnov, E.M., Grigoriev, I.V., Lynch, M., Boore, J.L., 2011. The ecoresponsive genome of *Daphnia pulex*. Science 331, 555–561.

Combes, C., 2001. Parasitism: The Ecology and Evolution of Intimate Interactions. University of Chicago Press.

Coors, A., De Meester, L., 2011. Fitness and virulence of a bacterial endoparasite in an environmentally stressed crustacean host. Parasitology 138, 122–131.

Coors, A., Decaestecker, E., Jansen, M., De Meester, L., 2008. Pesticide exposure strongly enhances parasite virulence in an invertebrate host model. Oikos 117, 1840–1846.

Cossart, P., Helenius, A., 2014. Endocytosis of viruses and bacteria. Cold Spring Harb. Perspect. Biol. 6.

Cotter, S.C., Myatt, J.P., Benskin, C.M.H., Wilson, K., 2008. Selection for cuticular melanism reveals immune function and life-history trade-offs in *Spodoptera littoralis*. J. Evol. Biol. 21, 1744–1754.

Courtney, H.S., Hasty, D.L., Dale, J.B., 2002. Molecular mechanisms of adhesion, colonization, and invasion of group A streptococci. Ann. Med. 34, 77–87.

Cox, F.E.G. (Ed.), 1993. Modern Parasitology. Blackwell, Oxford.

Cressler, C.E., Nelson, W.A., Day, T., McCauley, E., 2014. Starvation reveals the cause of infection-induced castration and gigantism. Proc. R. Soc. B-Biol. Sci. 281, 20141087.

Davies, K.G., 2009. Understanding the interaction between an obligate hyperparasitic bacterium, *Pasteuria penetrans* and its obligate plant-parasitic nematode host, *Meloidogyne* spp. Adv. Parasitol. Nat. Hist. Host–Parasite Interact. 68, 211–245.

De Meester, L., 1989. An estimation of the heritability of phototaxis in *Daphnia magna* Straus. Oecologia 78, 142–144.

De Meester, L., 1993. Genotype, fish-mediated chemicals, and phototactic behavior in *Daphnia magna*. Ecology 74, 1467–1474.

De Meester, L., 1996. Local genetic differentiation and adaptation in freshwater zooplankton populations: patterns and processes. Ecoscience 3, 385–399.

De Meester, L., Weider, L.J., Tollrian, R., 1995. Alternative antipredator defenses and genetic-polymorphism in a pelagic predator-prey system. Nature 378, 483–485.

Decaestecker, E., De Gersem, H., Michalakis, Y., Raeymaekers, J.A.M., 2013. Damped long-term host-parasite Red Queen coevolutionary dynamics: a reflection of dilution effects? Ecol. Lett. 16, 1455–1462.

Decaestecker, E., De Meester, L., Ebert, D., 2002. In deep trouble: habitat selection constrained by multiple enemies in zooplankton. Proc. Natl. Acad. Sci. U.S.A. 99, 5481–5485.

Decaestecker, E., Declerck, S., De Meester, L., Ebert, D., 2005. Ecological implications of parasites in natural *Daphnia* populations. Oecologia 144, 382–390.

Decaestecker, E., Gaba, S., Raeymaekers, J.A.M., Stoks, R., Van Kerckhoven, L., Ebert, D., De Meester, L., 2007. Host-parasite 'Red Queen' dynamics archived in pond sediment. Nature 450, 870–873.

Decaestecker, E., Labbe, P., Ellegaard, K., Allen, J.E., Little, T.J., 2011. Candidate innate immune system gene expression in the ecological model *Daphnia*. Dev. Comp. Immunol. 35, 1066–1075.

Decaestecker, E., Lefever, C., De Meester, L., Ebert, D., 2004. Haunted by the past: evidence for dormant stage banks of microparasites and epibionts of *Daphnia*. Limnol. Oceanogr. 49, 1355–1364.

Decaestecker, E., Vergote, A., Ebert, D., De Meester, L., 2003. Evidence for strong host clone-parasite species interactions in the *Daphnia* microparasite system. Evolution 57, 784–792.

Dhondt, K.V., Dhondt, A.A., Ley, D.H., 2007. Effects of route of inoculation on *Mycoplasma gallisepticum* infection in captive house finches. Avian Pathol. 36, 475–479.

Dickson, D.W., Preston III, J.F., Giblin-Davis, R.M., Noel, G.R., Ebert, D., Bird, G.W., 2009. Family Pasteuriaceae Laurent 1890. In: De Vos, P., Garrity, G.M., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H., Whitman, W.B. (Eds.), Bergey's Manual of Systematic Bacteriology, second ed., vol. 3. Springer, New York.

Dodds, P.N., Rathjen, J.P., 2010. Plant immunity: towards an integrated view of plant–pathogen interactions. Nat. Rev. Genet. 11, 539–548.

Donohue, K., 2014. Why ontogeny matters during adaptation: developmental niche construction and pleiotorpy across the life cycle in *Arabidopsis thaliana*. Evolution 68, 32–47.

Doran, K.S., Banerjee, A., Disson, O., Lecuit, M., 2013. Concepts and mechanisms: crossing host barriers. Cold Spring Harb. Perspect. Med. 3.

Dubovskiy, I.M., Whitten, M.M.A., Kryukov, V.Y., Yaroslavtseva, O.N., Grizanova, E.V., Greig, C., Mukherjee, K., Vilcinskas, A., Mitkovets, P.V., Glupov, V.V., Butt, T.M., 2013. More than a colour change: insect melanism, disease resistance and fecundity. Proc. R. Soc. B-Biol. Sci. 280, 20130584.

Duffy, M.A., 2009. Staying alive: the post-consumption fate of parasite spores and its implications for disease dynamics. Limnol. Oceanogr. 54, 770–773.

Duncan, A.B., Little, T.J., 2007. Parasite-driven genetic change in a natural population of Daphnia. Evolution 61, 796–803.

Duneau, D., Ebert, D., 2012a. Host sexual dimorphism and parasite adaptation. PLoS Biol. 10.

Duneau, D., Ebert, D., 2012b. The role of moulting in parasite defence. Proc. R. Soc. B-Biol. Sci. 279, 3049–3054.

Duneau, D., Luijckx, P., Ben-Ami, F., Laforsch, C., Ebert, D., 2011. Resolving the infection process reveals striking differences in the contribution of environment, genetics and phylogeny to host-parasite interactions. BMC Biol. 9, 11.

Duneau, D., Luijckx, P., Ruder, L.F., Ebert, D., 2012. Sex-specific effects of a parasite evolving in a female-biased host population. BMC Biol. 10, 104.

Dybdahl, M.F., Jenkins, C.E., Nuismer, S.L., 2014. Identifying the molecular basis of host-parasite coevolution: merging models and mechanisms. Am. Nat. 184, 1–13.

Ebert, D., 1992. A food independent maturation threshold and size at maturity in *Daphnia magna*. Limnol. Oceanogr. 37, 878–881.

Ebert, D., 2005. Ecology, Epidemiology and Evolution of Parasitism in Daphnia. National Library of Medicine (US), National Center for Biotechnology Information, Bethesda (MD). http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Books.

Ebert, D., 2008. Host-parasite coevolution: insights from the Daphnia-parasite model system. Curr. Opin. Microbiol. 11, 290–301.

Ebert, D., 2011. A genome for the environment. Science 331, 539–540.

Ebert, D., 2013. The epidemiology and evolution of symbionts with mixed-mode transmission. Annu. Rev. Ecol. Evol. Syst. 44, 623–643.

Ebert, D., Carius, H.J., Little, T., Decaestecker, E., 2004. The evolution of virulence when parasites cause host castration and gigantism. Am. Nat. 164, S19–S32.

Ebert, D., Lipsitch, M., Mangin, K.L., 2000a. The effect of parasites on host population density and extinction: experimental epidemiology with *Daphnia* and six microparasites. Am. Nat. 156, 459–477.

Ebert, D., Payne, R.J.H., Weisser, W.W., 1997. The epidemiology of parasitic diseases in *Daphnia*. In: Dettner, K., Bauer, G., Völkl, W. (Eds.), Vertical Food Web Interactions: Evolutionary Patterns and Driving Forces. Springer, Heidelberg.

Ebert, D., Rainey, P., Embley, T.M., Scholz, D., 1996. Development, life cycle, ultrastructure and phylogenetic position of *Pasteuria ramosa* Metchnikoff 1888: rediscovery of an obligate endoparasite of *Daphnia magna* Straus. Philos. Trans. R. Soc. Lond. Ser. B 351, 1689–1701.

Ebert, D., Weisser, W.W., 1997. Optimal killing for obligate killers: the evolution of life histories and virulence of semelparous parasites. Proc. R. Soc. Lond. Ser. B 264, 985–991.

Ebert, D., Yampolsky, L.Y., Stearns, S.C., 1993. Genetics of life-history traits in *Daphnia magna*: 1. Heritabilities in two food levels. Heredity 70, 335–343.

Ebert, D., Zschokke-Rohringer, C.D., Carius, H.J., 1998. Within and between population variation for resistance of *Daphnia magna* to the bacterial endoparasite *Pasteuria ramosa*. Proc. R. Soc. Lond. Ser. B 265, 2127–2134.

Ebert, D., Zschokke-Rohringer, C.D., Carius, H.J., 2000b. Dose effects and density-dependent regulation of two microparasites of *Daphnia magna*. Oecologia 122, 200–209.

Fenton, A., Antonovics, J., Brockhurst, M.A., 2012. Two-step infection processes can lead to coevolution between functionally independent infection and resistance pathways. Evolution 66, 2030–2041.

Ferrandon, D., 2013. The complementary facets of epithelial host defenses in the genetic model organism *Drosophila melanogaster*: from resistance to resilience. Curr. Opin. Immunol. 25, 59–70.

Frank, S.A., 1996. Models of parasite virulence. Q. Rev. Biol. 71, 37–78.

Freitas, L.G., Mitchell, D.J., Dickson, D.W., 1997. Temperature effects on the attachment of *Pasteuria penetrans* endospores to *Meloidogyne arenaria* race 1. J. Nematol. 29, 547–555.

Frost, P.C., Ebert, D., Larson, J.H., Marcus, M.A., Wagner, N.D., Zalewski, A., 2010. Transgenerational effects of poor elemental food quality on *Daphnia magna*. Oecologia 162, 865–872.

Frost, P.C., Ebert, D., Smith, V.H., 2008a. Bacterial infection changes the elemental composition of *Daphnia magna*. J. Animal Ecol. 77, 1265–1272.

Frost, P.C., Ebert, D., Smith, V.H., 2008b. Responses of a bacterial pathogen to phosphorus limitation of its aquatic invertebrate host. Ecology 89, 313–318.

Garbutt, J.S., Scholefield, J.A., Vale, P.F., Little, T.J., 2014. Elevated maternal temperature enhances offspring disease resistance in *Daphnia magna*. Funct. Ecol. 28, 424–431.

Goren, L., Ben-Ami, F., 2013. Ecological correlates between cladocerans and their endoparasites from permanent and rain pools: patterns in community composition and diversity. Hydrobiologia 701, 13–23.

Goren, L., Ben-Ami, F., 2015. To eat or not to eat infected food: a bug's dilemma. Hydrobiologia (in press).

Green, J., 1974. Parasites and epibionts of Cladocera. Trans. Zool. Soc. Lond. 32, 417–515.

Gutjahr, C., Parniske, M., 2013. Cell and developmental biology of arbuscular mycorrhiza symbiosis. Annu. Rev. Cell Dev. Biol. 29, 593–617.

Hall, M.D., Ebert, D., 2012. Disentangling the influence of parasite genotype, host genotype and maternal environment on different stages of bacterial infection in *Daphnia magna*. Proc. R. Soc. B-Biol. Sci. 279, 3176–3183.

Hall, M.D., Ebert, D., 2013. The genetics of infectious disease susceptibility: has the evidence for epistasis been overestimated? BMC Biol. 11, 79.

Hall, M.D., Vettiger, A., Ebert, D., 2013. Interactions between environmental stressors: the influence of salinity on host-parasite interactions between *Daphnia magna* and *Pasteuria ramosa*. Oecologia 171, 789–796.

Hall, S.R., Sivars-Becker, L., Becker, C., Duffy, M.A., Tessier, A.J., Caceres, C.E., 2007. Eating yourself sick: transmission of disease as a function of foraging ecology. Ecol. Lett. 10, 207–218.
Hall, S.R., Smyth, R., Becker, C.R., Duffy, M.A., Knight, C.J., MacIntyre, S., Tessier, A.J., Caceres, C.E., 2010. Why are *Daphnia* in some lakes sicker? Disease ecology, habitat structure, and the plankton. Bioscience 60, 363–375.
He, C., Chen, M., Liu, J., Yuan, Y., 2014. Host genetic factors respond to pathogenic step-specific virulence factors of *Helicobacter pylori* in gastric carcinogenesis. Mutat. Res. Rev. Mutat. Res. 759, 14–26.
He, S.Y., 1998. Type III protein secretion systems in plant and animal pathogenic bacteria. Annu. Rev. Phytopathol. 36, 363–392.
Horton, P.A., Rowan, M., Webster, K.E., Peters, R.H., 1979. Browsing and grazing by cladoceran filter feeders. Can. J. Zool. 57, 206–212.
Hu, D., Wang, C., Tao, F., Cui, Q., Xu, X., Shang, W., Hu, X., 2014. Whole genome wide expression profiles on germination of *Verticillium dahliae* microsclerotia. PLoS One 9.
Hueckelhoven, R., Eichmann, R., Weis, C., Hoefle, C., Proels, R.K., 2013. Genetic loss of susceptibility: a costly route to disease resistance? Plant Pathol. 62, 56–62.
Izhar, R., Ben-Ami, F., 2015. Host age modulates parasite infectivity, virulence and reproduction. J. Animal Ecol. 84, 1018–1028.
Izhar, R., Routto, J., Ben-Ami, F., 2015. Host age modulates within-host parasite competition. Biol. Lett. 11 (5), 20150131.
Jansen, M., Stoks, R., Coors, A., van Doorslaer, W., De Meester, Ln., 2011. Collateral damage: rapid exposure-induced evolution of pesticide resistance leads to increased susceptibility to parasites. Evolution 65, 2681–2691.
Jansen, M., Stoks, R., Decaestecker, E., Coors, A., Van de Meutter, F., De Meester, L., 2010. Local exposure shapes spatial patterns in infectivity and community structure of *Daphnia* parasites. J. Animal Ecol. 79, 1023–1033.
Jansen, M., Vergauwen, L., Vandenbrouck, T., Knapen, D., Dom, N., Spanier, K.I., Cielen, A., De Meester, L., 2013. Gene expression profiling of three different stressors in the water flea *Daphnia magna*. Ecotoxicology 22, 900–914.
Jaronski, S.T., 2010. Ecological factors in the inundative use of fungal entomopathogens. Biocontrol 55, 159–185.
Jensen, K.H., Little, T., Skorping, A., Ebert, D., 2006. Empirical support for optimal virulence in a castrating parasite. PLoS Biol. 4, 1265–1269.
King, K.C., Auld, S.K.J.R., Wilson, P.J., James, J., Little, T.J., 2013. The bacterial parasite *Pasteuria ramosa* is not killed if it fails to infect: implications for coevolution. Ecol. Evol. 3, 197–203.
Koch, H., Schmid-Hempel, P., 2011. Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. Proc. Natl. Acad. Sci. U.S.A. 108, 19288–19292.
Koella, J.C., Lynch, P.A., Thomas, M.B., Read, A.F., 2009. Towards evolution-proof malaria control with insecticides. Evol. Appl. 2, 469–480.
Koike, S., 2011. Early steps of picornavirus infection. Virus (Nagoya) 61, 183–191.
Krachler, A.M., Orth, K., 2013. Targeting the bacteria-host interface: strategies in anti-adhesion therapy. Virulence 4, 284–294.
Labbe, P., Vale, P.F., Little, T.J., 2010. Successfully resisting a pathogen is rarely costly in *Daphnia magna*. BMC Evol. Biol. 10, 355.
Lampert, W., 2011. *Daphnia*: Development of a Model Organism in Ecology and Evolution. Internat. Ecology Inst, Oldendorf/Luhe.
Lemaitre, B., Hoffmann, J., 2007. The host defense of *Drosophila melanogaster*. Annu. Rev. Immunol. 25, 697–743.
Little, T., Birch, J., Vale, P., Tseng, M., 2007. Parasite transgenerational effects on infection. Evol. Ecol. Res. 9, 459–469.

Little, T.J., Carius, H.J., Sakwinska, O., Ebert, D., 2002. Competitiveness and life-history characteristics of Daphnia with respect to susceptibility to a bacterial pathogen. J. Evol. Biol. 15, 796–802.
Little, T.J., Chadwick, W., Watt, K., 2008. Parasite variation and the evolution of virulence in a *Daphnia*-microparasite system. Parasitology 135, 303–308.
Little, T.J., Ebert, D., 2000. The cause of parasitic infection in natural populations of *Daphnia* (Crustacea : Cladocera): the role of host genetics. Proc. R. Soc. Lond. Ser. B 267, 2037–2042.
Little, T.J., Ebert, D., 2001. Temporal patterns of genetic variation for resistance and infectivity in a *Daphnia*-microparasite system. Evolution 55, 1146–1152.
Little, T.J., Killick, S.C., 2007. Evidence for a cost of immunity when the crustacean *Daphnia magna* is exposed to the bacterial pathogen *Pasteuria ramosa*. J. Animal Ecol. 76, 1202–1207.
Little, T.J., Watt, K., Ebert, D., 2006. Parasite-host specificity: experimental studies on the basis of parasite adaptation. Evolution 60, 31–38.
Lively, C.M., 2010. A review of red queen models for the persistence of obligate sexual reproduction. J. Hered. 101, S13–S20.
Luijckx, P., Ben-Ami, F., Mouton, L., Du Pasquier, L., Ebert, D., 2011. Cloning of the unculturable parasite *Pasteuria ramosa* and its *Daphnia* host reveals extreme genotype-genotype interactions. Ecol. Lett. 14, 125–131.
Luijckx, P., Duneau, D., Andras, J., Ebert, D., 2014. Cross-species infection trials reveal cryptic parasite varieties and a putative polymorphism shared among host species. Evolution 68, 577–586.
Luijckx, P., Fienberg, H., Duneau, D., Ebert, D., 2012. Resistance to a bacterial parasite in the crustacean *Daphnia magna* shows Mendelian segregation with dominance. Heredity 108, 547–551.
Luijckx, P., Fienberg, H., Duneau, D., Ebert, D., 2013. A matching-allele model explains host resistance to parasites. Curr. Biol. 23, 1085–1088.
Mageroy, J.H., Grepperud, E.J., Jensen, K.H., 2011. Who benefits from reduced reproduction in parasitized hosts? An experimental test using the *Pasteuria ramosa–Daphnia magna* system. Parasitology 138, 1910–1915.
Martins, N.E., Faria, V.G., Teixeira, L., Magalhaes, S., Sucena, E., 2013. Host adaptation is contingent upon the infection route taken by pathogens. PLoS Pathog. 9, e1003601.
McElroy, K., Mouton, L., Du Pasquier, L., Qi, W., Ebert, D., 2011. Characterisation of a large family of polymorphic collagen-like proteins in the endospore-forming bacterium *Pasteuria ramosa*. Res. Microbiol. 162, 701–714.
McTaggart, S.J., Conlon, C., Colbourne, J.K., Blaxter, M.L., Little, T.J., 2009. The components of the *Daphnia pulex* immune system as revealed by complete genome sequencing. BMC Genomics 10, 175.
McTaggart, S.J., Wilson, P.J., Little, T.J., 2012. *Daphnia magna* shows reduced infection upon secondary exposure to a pathogen. Biol. Lett. 8, 972–975.
Metchnikoff, M.E., 1884. Über eine Sprosspilzkrankheit der Daphniden. Beitrag zur Lehre der Phagocyten gegen Krankheitserreger. Virchows Arch. Path. Anat. Physiol. 9, 177–193.
Metzger, C.M.J.A., 2014. Specificity, Genetics of Resistance and Eco-immunology of *Daphnia* Microparasite Interactions. Basel University, Basel, Switzerland (Ph.D. thesis).
Minchella, D.J., 1985. Host life-history variation in response to parasitism. Parasitology 90, 205–216.
Mitchell, S.E., Read, A.F., 2005. Poor maternal environment enhances offspring disease resistance in an invertebrate. Proc. R. Soc. B-Biol. Sci. 272, 2601–2607.
Mouton, L., Nong, G., Preston, J.F., Ebert, D., 2007. Variable-number tandem repeats as molecular markers for biotypes of *Pasteuria ramosa* in *Daphnia* spp. Appl. Environ. Microbiol. 73, 3715–3718.

Mouton, L., Traunecker, E., McElroy, K., Du Pasquier, L., Ebert, D., 2009. Identification of a polymorphic collagen-like protein in the crustacean bacteria *Pasteuria ramosa*. Res. Microbiol. 160, 792–799.

Mucklow, P.T., Vizoso, D.B., Jensen, K.H., Refardt, D., Ebert, D., 2004. Variation in phenoloxidase activity and its relation to parasite resistance within and between populations of *Daphnia magna*. Proc. R. Soc. Lond. Ser. B-Biol. Sci. 271, 1175–1183.

Naglik, J.R., Moyes, D.L., Waechtler, B., Hube, B., 2011. *Candida albicans* interactions with epithelial cells and mucosal immunity. Microbes Infect. 13, 963–976.

Nakajima, M., Akutsu, K., 2014. Virulence factors of *Botrytis cinerea*. J. General Plant Pathol. 80, 15–23.

O'Keefe, K.J., Antonovics, J., 2002. Playing by different rules: the evolution of virulence in sterilizing pathogens. Am. Nat. 159, 597–605.

Obrebski, S., 1975. Parasite reproductive strategy and evolution of castration of hosts by parasites. Science 188, 1314–1316.

Orsini, L., Spanier, K.I., De Meester, L., 2012. Genomic signature of natural and anthropogenic stress in wild populations of the waterflea *Daphnia magna*: validation in space, time and experimental evolution. Mol. Ecol. 21, 2160–2175.

Otto, S.P., Nuismer, S.L., 2004. Species interactions and the evolution of sex. Science 304, 1018–1020.

Paredes-Sabja, D., Shen, A., Sorg, J.A., 2014. *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. Trends Microbiol. 22, 406–416.

Pauwels, K., De Meester, L., Decaestecker, E., Stoks, R., 2011. Phenoloxidase but not lytic activity reflects resistance against *Pasteuria ramosa* in *Daphnia magna*. Biol. Lett. 7, 156–159.

Poulin, R., 2007. Evolutionary Ecology of Parasites. USA Princeton University Press, Princeton.

Qi, W.H., Nong, G., Preston, J.F., Ben-Ami, F., Ebert, D., 2009. Comparative metagenomics of *Daphnia symbionts*. BMC Genomics 10, 172–189.

Raberg, L., Sim, D., Read, A.F., 2007. Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. Science 318, 812–814.

Read, A.F., Lynch, P.A., Thomas, M.B., 2009. How to make evolution-proof insecticides for malaria control. Am. J. Trop. Med. Hyg. 81, 165.

Regoes, R.R., Hottinger, J.W., Sygnarski, L., Ebert, D., 2003. The infection rate of *Daphnia magna* by *Pasteuria ramosa* conforms with the mass-action principle. Epidemiol. Infect. 131, 957–966.

Routtu, J., Ebert, D., 2015. Genetic architecture of resistance in *Daphnia* hosts against two species of host-specific parasites. Heredity 114, 241–248.

Routtu, J., Hall, M.D., Albere, B., Beisel, C., Bergeron, R.D., Chaturvedi, A., Choi, J.H., Colbourne, J., De Meester, L., Stephens, M.T., Stelzer, C.P., Solorzano, E., Thomas, W.K., Pfrender, M.E., Ebert, D., 2014. An SNP-based second-generation genetic map of *Daphnia magna* and its application to QTL analysis of phenotypic traits. BMC Genomics 15, 1033.

Routtu, J., Jansen, B., Colson, I., De Meester, L., Ebert, D., 2010. The first-generation *Daphnia magna* linkage map. BMC Genomics 11.

Salathe, M., Kouyos, R.D., Bonhoeffer, S., 2008. The state of affairs in the kingdom of the Red Queen. Trends Ecol. Evol. 23, 439–445.

Sarker, M.R., Paredes-Sabja, D., 2012. Molecular basis of early stages of *Clostridium difficile* infection: germination and colonization. Future Microbiol. 7, 933–943.

Sayre, R.M., Wergin, W.P., 1977. Bacterial parasite of a plant nematode: morphology and ultrastructure. J. Bacteriol. 129, 1091–1101.

Sayre, R.M., Wergin, W.P., Davis, R.E., 1977. Occurrence in *Moina rectirostris* (Cladocera: Daphnidae) of a parasite morphological similar to *Pasteuria ramosa* (Metchnikoff, 1988). Can. J. Microbiol. 23, 1573–1579.

Schaff, J.E., Mauchline, T.H., Opperman, C.H., Davies, K.G., 2011. Exploiting genomics to understand the interactions between Root-Knot nematodes and *Pasteuria penetrans*. In: Davies, K., Spiegel, Y. (Eds.), Biological Control of Plant-Parasitic Nematodes: Building Coherence between Microbial Ecology and Molecular Mechanisms, pp. 91–113.

Schlotz, N., Ebert, D., Martin-Creuzburg, D., 2013. Dietary supply with polyunsaturated fatty acids and resulting maternal effects influence host–parasite interactions. BMC Ecol. 13, 41.

Schmid-Hempel, P., 2011. Evolutionary Parasitology. UK Oxford University Press, Oxford.

Schmid-Hempel, P., Ebert, D., 2003. On the evolutionary ecology of specific immune defence. Trends Ecol. Evol. 18, 27–32.

Schulenburg, H., Boehnisch, C., Michiels, N.K., 2007. How do invertebrates generate a highly specific innate immune response? Mol. Immunol. 44, 3338–3344.

van Schie, C.C., Takken, F.L., 2014. Susceptibility genes 101: how to be a good host. Ann. Rev. Phytopathol. 52, 551–581.

Searle, C.L., Ochs, J.H., Caceres, C.E., Chiang, S.L., Gerardo, N.M., Hall, S.R., Duffy, M.A., 2015. Plasticity, not genetic variation, drives infection success of a fungal parasite. Parasitology 142, 839–848.

Sison-Mangus, M.P., Mushegian, A.A., Ebert, D., 2015. Water fleas require microbiota for survival, growth and reproduction. ISME J. 9, 59–67.

Smirnov, N.N., 2014. The Physiology of the Cladocera. Amsterdam Academic Press.

Stirnadel, H.A., Ebert, D., 1997. Prevalence, host specificity and impact on host fecundity of microparasites and epibionts in three sympatric *Daphnia* species. J. Animal Ecol. 66, 212–222.

Stjernman, M., Little, T.J., 2011. Genetic variation for maternal effects on parasite susceptibility. J. Evol. Biol. 24, 2357–2363.

Tseng, M., Myers, J.H., 2014. The relationship between parasite fitness and host condition in an insect–virus system. PLoS One 9, e106401.

Underwood, W., 2012. The plant cell wall: a dynamic barrier against pathogen invasion. Front. Plant Sci. 3, 85.

Vale, P.F., Choisy, M., Little, T.J., 2013. Host nutrition alters the variance in parasite transmission potential. Biol. Lett. 9, 20121145.

Vale, P.F., Little, T.J., 2009. Measuring parasite fitness under genetic and thermal variation. Heredity 103, 102–109.

Vale, P.F., Little, T.J., 2012. Fecundity compensation and tolerance to a sterilizing pathogen in *Daphnia*. J. Evol. Biol. 25, 1888–1896.

Vale, P.F., Stjernman, M., Little, T.J., 2008. Temperature-dependent costs of parasitism and maintenance of polymorphism under genotype-by-environment interactions. J. Evol. Biol. 21, 1418–1427.

Vale, P.F., Wilson, A.J., Best, A., Boots, M., Little, T.J., 2011. Epidemiological, evolutionary, and coevolutionary implications of context-dependent parasitism. Am. Nat. 177, 510–521.

Wargo, A.R., Kell, A.M., Scott, R.J., Thorgaard, G.H., Kurath, G., 2012. Analysis of host genetic diversity and viral entry as sources of between-host variation in viral load. Virus Res. 165, 71–80.

Woolhouse, M.E.J., Webster, J.P., Domingo, E., Charlesworth, B., Levin, B.R., 2002. Biological and biomedical implications of the co-evolution of pathogens and their hosts. Nat. Genet. 32, 569–577.

Zbinden, M., Haag, C.R., Ebert, D., 2008. Experimental evolution of field populations of *Daphnia magna* in response to parasite treatment. J. Evol. Biol. 21, 1068–1078.