

**INFECTION OUTCOMES UNDER GENETIC AND
ENVIRONMENTAL VARIATION IN A HOST-PARASITE
SYSTEM: IMPLICATIONS FOR MAINTENANCE OF
POLYMORPHISM AND THE EVOLUTION OF VIRULENCE**

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THESIS SUMMARY

Virulence (the harm to the host during infection) is the outcome of continuous coevolution between hosts and parasites. This thesis adds to a growing body of work on host-parasite interactions, and describes experiments that study the effects of variation in the genetic and the environmental contexts of infection. All of them focus on interaction between the planktonic freshwater crustacean *Daphnia magna* and a naturally occurring parasite, the spore-forming bacterium *Pasteuria ramosa*. I show that elevated minimum temperatures that facilitate parasite growth drive natural epidemics of this parasite. I also demonstrate that the expression of infection traits in *P. ramosa* is temperature-dependent in a genotype-specific manner [genotype-by-environment (GxE) interactions]. These GxE interactions could maintain polymorphism through environment-dependent selection. Next, I test if GxG interactions for infectivity can be altered by environmental variation (GxGxE interactions), and find that this trait is quite robust to thermal variation. Infectivity is also more important in determining parasite fitness relative to the production of transmission stages, highlighting the importance of considering natural infection routes, an aspect sometimes overlooked in studies of host-parasite systems. Another experiment under different food and temperature regimes showed evidence for environment-dependent virulence-transmission relationships, a fundamental component of virulence evolution models. Lastly, I show that variation in temperature does not increase the cost to the host of resisting infection.

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DECLARATION

I declare that all the work presented in this thesis is my own. Because scientific research is most successful when it is a collaborative endeavour, all the chapters were improved by the input of the many people I indicate below. I am deeply grateful to all of them. Unless mentioned otherwise, the conception, experimental design and execution of the experiments, the analysis of the data, and writing of the chapters, was done by me.

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Chapter 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Why are pathogen populations genetically variable? Under what conditions will pathogens evolve to cause more or less virulence? What drives the onset, duration, and severity of epidemics? How does environmental variation affect evolutionary processes and epidemiological outcomes? These questions illustrate the close link between ecology, evolution and epidemiology. Combining concepts from these fields will increase our understanding of host-pathogen interactions, their long-term evolution, and how shorter-term ecological processes may affect evolution. To this end, this thesis describes experiments that study the effects of variation in the genetic context (the genotypes of hosts and parasite involved in infection) and the environmental context (variation in abiotic factors such as food availability and temperature) in which infections occur. Each of the chapters that follow include an introduction detailing the relevance of such variation for the expression of host and parasite traits, the costs of infection, and the possible outcomes for coevolution and the evolution of virulence. Below I give a broad overview of these topics, concluding with an outline of the aims and a summary of the main results from each chapter. In chapter 2 I describe the model host-parasite system used in the experiments, *Daphnia magna* – *Pasteuria ramosa*.

1.1 What are the determinants of infectious disease?

1.1.1 The traditional paradigm: pathogen effects on infection

The causes behind the incidence and severity of infectious disease has driven many decades of research by workers across a range of disciplines, including medical microbiology (e.g. Jenner 1798; Koch 1880; Chermann et al. 1985), epidemiology (e.g. Koelle et al. 2005; Altizer et al. 2006), and evolutionary biology (Haldane 1949; Ewald 1994; Stearns and Koella 2008). Most obviously disease is caused by pathogens or parasites. Since Koch's seminal work identifying microbes as causal agents of infectious disease (Koch 1884), the prevailing mindset was to directly associate variation in the incidence and severity of disease with the presence of a "virulent" pathogen (Selander et al. 1987). Yet across all taxa, hosts vary in the way they respond to infection, even when challenged with the same pathogen, revealing that the outcome of disease is more complex than simply determining the source of the infection (reviewed in Lazzaro and Little 2008).

1.1.2 The disease triangle: host effects on infection

Plant pathologists were possibly the first to make this point in the 1960s with the so-called "disease triangle" (McNew 1960), where disease is seen as the outcome of a complex interaction between the pathogen, the host and the environment in which infection occurs (Scholthof 2007; Figure 1.1). This presented an important change from the "virulent pathogen" paradigm, to one that attempted to dissect all the relevant sources of the observed variation in disease incidence and severity. This period also coincided with the advent of several molecular tools that made the identification of genetic markers of disease resistance possible (reviewed in Hill 2001; Staskawicz 2001), increasing the ability to explore another

vertex of the disease triangle, the host. Variation in host responses to infection have been studied extensively in plants (reviewed in Staskawicz 2001), and to some extent in invertebrates (e.g. Carius et al. 2001; Lazzaro et al. 2006) and some vertebrate hosts (e.g. Sanjayan et al. 1996; Morris 2007). More recently, the availability of full genome data from human populations has permitted large-scale association studies between genetic variants and susceptibility to infectious diseases (e.g. W.T.C.C.C. 2007), and in the process these have uncovered tremendous genetic variation within human populations that is relevant for the establishment and progression of infections (Hill 2001). The emerging picture is that apart from the already acknowledged genetic variation in pathogen populations (Selander et al. 1987; McDonald and Linde 2002), genetic variation for disease resistance is widespread across most if not all host taxa (Lambrechts et al. 2006b; Lazzaro and Little 2008). Understanding to what extent such variation influences disease incidence in the short-term, and mediates its evolution in the long-term is one the major focuses of biology today (Grenfell et al. 2004). Below I discuss how a coevolutionary framework can inform on this issue. I then highlight how a complete understanding of the disease triangle, including host, pathogen and the effects of environmental variation on their interactions, will increase our understanding of host-pathogen coevolution.

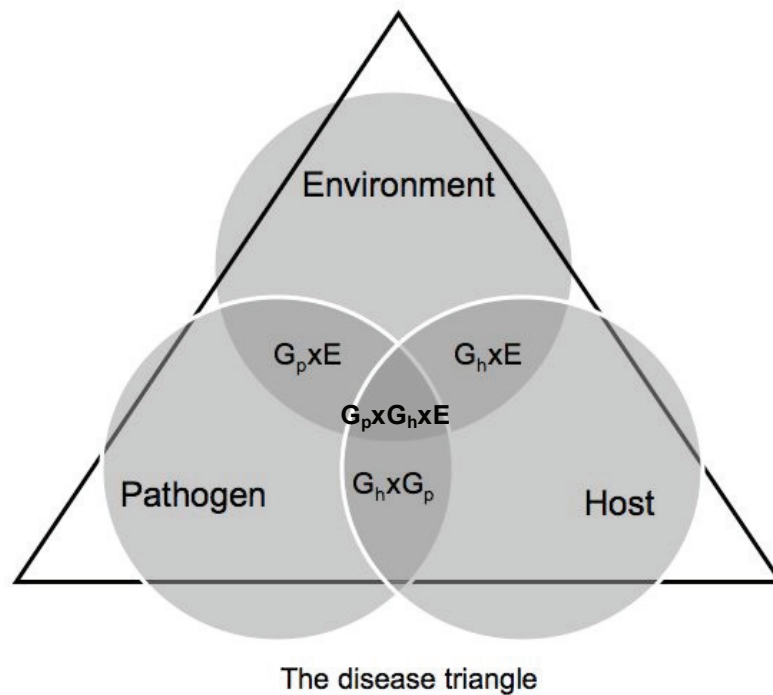


Figure 1.1. The disease triangle. The schematic shows the possible contributions to observed variation in the incidence and severity of infectious disease. Pathogen effects are important but their effects will depend on the environment, possibly in a genotype-specific manner ($G_p \times E$). Host variation in the susceptibility to disease will contribute to the variation in infection, but will also depend on how different host genotypes respond to infection under different environments ($G_h \times E$). The presence of genetic variation in both host and parasite populations allows for genotype-specific patterns of infections ($G_p \times G_h$), fostering coevolutionary dynamics. This suggests that disease is inevitably the outcome of both genotypic interactions between hosts and parasites, and how the environment modulates these interactions ($G_p \times G_h \times E$). Studying the evolution of infectious disease therefore requires that the relative contribution of each of the factors be measured.

1.2 Coevolutionary interactions between hosts and parasites

Coevolution between interacting species occurs when selection operating on one species affects the evolution of the other species (Thompson 1994). Parasites and their hosts present a particular case of coevolution, and the topic has received much attention due to the potentially large detrimental effects of parasites on host fecundity and survival (e.g. Stirmadel and Ebert 1997), which may determine host population cycles and genetic makeup (reviewed Woolhouse et al. 2002). Additionally, parasites exist as large, genetically variable populations, with tremendous evolutionary potential (McDonald and Linde 2002; Woolhouse et al. 2002), possibly hindering the efficacy of some disease control programs (e.g. Gandon et al. 2001; Gandon and Day 2008). A greater understanding of these issues can be gained from the knowledge generated by work on the occurrence of coevolutionary dynamics in natural populations (Lively and Dybdahl 2000; Decaestecker et al. 2007; Duffy and Sivars-Becker 2007; Duncan and Little 2007), and testing predictions about the evolution of virulence (Read 1994; Frank 1996; Ebert 2004; Mackinnon and Read 2004).

1.2.1 Evidence of ongoing coevolution

Documenting host-parasite coevolutionary dynamics is not straightforward (Little 2002; Woolhouse et al. 2002). It requires (1) that traits in both interacting species are detrimental to each other's fitness; (2) evidence that changes in the abundance and the genetic composition of both host and parasite populations occur as a direct result of their antagonistic selection; (3) that genetic variation exist in both host and parasite traits that directly mediate the antagonistic relationship (e.g. host resistance and parasite infectivity); (4) that genetic specificity for infection and resistance is present, as this permits ongoing negative frequency-dependent selection (see *Coevolution and polymorphism* below).

Despite these stringent conditions, several studies have found evidence consistent with the occurrence of antagonistic coevolution in wild populations of hosts and parasites. For example, by comparing the resistance of *Daphnia* host genotypes collected before and after an epidemic of the bacterial parasite *Pasteuria ramosa*, Duncan and Little found that after the epidemic susceptible genotypes decreased, and more resistant genotypes increased in frequency, a pattern of resistance consistent with parasite mediated selection (Duncan and Little 2007). Another study compared the resistance of *Daphnia* from lakes with or without epidemics, also finding a similar pattern of parasite-mediated selection (Duffy and Sivars-Becker 2007). Some laboratory systems, particularly bacteria-phage host-parasite systems, have recently provided evidence for the occurrence of coevolutionary dynamics (Brockhurst et al. 2007).

Other studies have looked at the spatial pattern of adaptation between host-parasite pairs. Due to their larger population size and shorter generation time, parasites are expected to adapt quickly to local, common host genotypes (Kaltz and Shykoff 1998). As such, some workers have attempted to examine the occurrence of coevolution in natural populations by testing whether hosts and parasites are locally adapted (Ebert 1994; Morand et al. 1996; Lively and Dybdahl 2000). While local adaptation provides indirect evidence of the potential for coevolution, the detection of local adaptation is contingent on other processes such as the degree of population structuring and of gene flow between demes (Gandon et al. 1996; Gandon 2002; Morgan et al. 2005). Hence, coevolution cannot be ruled out even in the absence of a clear pattern of local adaptation (Kawecki and Ebert 2004). Recently, Decaestecker and colleagues collected resting eggs of *Daphnia* and long-living transmission spores of the same parasite from lake sediment cores. By exposing hosts and parasites collected from different sediment layers, they were able to test patterns of resistance and infectivity to contemporary, past and future combinations and found that hosts and parasites

were temporally locally adapted to each other, an indication of ongoing coevolution (Decaestecker et al. 2007).

1.2.2 Coevolution and polymorphism

Another motivation behind studies of coevolution is that host-parasite systems offer excellent opportunities to study basic evolutionary questions. The mechanisms maintaining genetic polymorphism are a long-standing and central focus of evolutionary biology (Haldane 1932). The problem arises because directional selection (either stabilising or disruptive) is predicted to erode genetic variation, as selection will favour alleles that result in higher fitness, contradicting the observation that genetic variation is widespread in natural populations (Roff 1997; Byers 2005; Laine and Tellier 2008). Several mechanisms have been proposed to maintain genetic variation (reviewed in Roff 1997) including mutation-selection balance (Lande 1975; Turelli 1984), heterozygote advantage (Robertson 1962; Bulmer 1973), or antagonistic pleiotropy (Rose 1982). However, most models developed to test how much variation can be maintained by such mechanisms use a broad range of parameter values, some of which are argued to be unrealistic (Roff 1997). The key is to recognise that the direction of selection need not always be in the same direction, and hence different alleles could be favoured in different environments. This is the case in negative-frequency dependent selection (when alleles confer an advantage only when rare), and also when the environment is heterogeneous, and different alleles are favoured depending on the environments (Byers 2005; Laine and Tellier 2008). Investigating under what conditions polymorphism is maintained in host-parasite systems can therefore inform on which of these mechanisms is likely to operate elsewhere in nature.

Coevolution between hosts and pathogens is predicted to maintain genetic polymorphism in infectivity and host resistance due to negative frequency dependent selection (Anderson and May 1982; Hamilton 1993; Woolhouse et al. 2002). The occurrence of negative frequency-dependent selection in host-parasite systems is only possible if there is genetic specificity for infectivity, called $G_H \times G_P$ interactions (Lively 1989; Carius et al. 2001; Lambrechts et al. 2006b). These interactions mean that no parasite genotype is universally infective on all host genotypes, and no host genotype is completely resistant to all parasite genotypes. In such cases, labelling a parasite as “virulent” or a host as “resistant” is not clear, as it depends on the genetic context of the infection.

It is these genotype-specific patterns of infection that allow coevolution between hosts and parasites to proceed, as parasites adapt to (infect) common host genotypes (Kaltz and Shykoff 1998), and hosts counter-adapt by evolving resistance to these parasite genotypes. Without this specificity, a very infective parasite, or a very resistant host would simply rise to fixation in the population and no coevolution would occur. Therefore, an essential component of studying coevolution is to identify genetic variation in parasite infectivity and host resistance. Empirical work in several systems has shown that infection outcomes can depend strongly on particular combinations of host and parasite genotypes (Lively 1989; Ebert et al. 1998; Kraaijeveld et al. 1998; Carius et al. 2001; Lambrechts et al. 2005; Lazzaro et al. 2006), providing evidence for the prediction that ongoing negative frequency-dependent selection can maintain polymorphism (Hamilton 1993).

1.3 Virulence: an outcome of coevolution

One of the most relevant aspects of studying coevolution between hosts and parasites stems from the very nature of the parasitic relationship: parasites (or pathogens in general) harm

their hosts. This harm (termed virulence) emerges as an unavoidable consequence of parasitism, because parasites necessarily exploit their hosts as a resource. Virulence can be quantified in many ways (mortality, reduced growth or reproduction, weight loss, anaemia), but essentially, all measures of virulence attempt to quantify the parasite-induced harm experienced by the host during infection (reviewed in Ebert and Bull 2008). How much virulence this exploitation should cause has been a long-standing focus of evolutionary biology (Haldane 1949; Read 1994). After all, if parasites need their hosts to survive, should we not expect selection to favour the evolution of benign parasites, that can keep their main resource alive for longer (Read 1994)?

A large body of work has been dedicated to understanding the evolutionary trajectories of virulence (Anderson and May 1982; Bremermann and Pickering 1983; Frank 1996; Andre et al. 2003; Day 2004; Jensen et al. 2006). Underlying most of this work is R_0 , also called the basic reproductive number (Figure 1.2), which is the number of secondary infections caused on average by a single infected individual in a completely susceptible population (Anderson and May 1979; Kermack and McKendrick 1991). Apart from its application in epidemiology to determine the likelihood of pathogen emergence ($R_0 > 1$) or extinction ($R_0 < 1$) (Matthews and Woolhouse 2005), it also underlies most theoretical models that consider virulence evolution as a result of selection maximizing parasite fitness (Anderson and May 1979; Frank 1996). Intuitively, while faster growing parasites will initially have increased fitness, growing too quickly could result host death before the parasite has matured to the stage where transmission is successful, resulting in reduced parasite transmission success. In agreement with these ideas, models assume that virulence evolution is governed by a trade-off between within-host growth (and the harm caused to the host during growth) and between-host transmission, maximising parasite fitness at non-maximal virulence (e.g. Jensen et al. 2006); Figure 1.2). This resulted in the view that virulence would always evolve

to intermediate or benign levels (Bull 1994; Ebert and Bull 2003), but the current view is that any evolutionary trajectory is possible depending on several host, pathogen and environmental factors (Ebert and Bull 2008).

While models based on R_0 maximization are an extremely simplified view of pathogen evolution, they provide useful null-models to test under which conditions virulence will not follow the expected prediction of reduced virulence. For example, one of the assumptions made by many models is that populations exhibit homogeneous mixing, such that the contact rate of parasites and susceptible hosts follows a mass-action law (Hamer 1906; Regoes et al. 2003), where the probability of infection is directly proportional to the number of infectious particles in the population. In reality, such direct proportionality can be modified by several factors, including genetic variation in host resistance (Knell et al. 1996; Dwyer et al. 1997), host physiological changes that occur at high density, or phenotypic plasticity in immune responses (Ben-Ami et al. 2008). Population structure (sub-division into discrete sub-populations or demes) will also cause deviations from mass-action, as the probability of encounter will not be the same for all possible combinations of hosts and parasites. Models of virulence evolution that incorporate spatial structure suggest that, depending on the degree of structuring and migration between population patches, a wide range of virulence levels can evolve (Boots and Sasaki 1999; Boots et al. 2004; Kamo and Boots 2006). In some cases, extremely virulent parasites evolve that lead to the extinction of local populations (Boots and Sasaki 2002). If migration between patches is low such that susceptible hosts are no longer available, this may lead to the extinction of these parasites themselves, resulting in lower population-level virulence (e.g. Boots and Meador 2007).

In chapter 6 I test another assumption of virulence evolution models, that parasite growth and transmission is positively correlated with virulence. In the next section I discuss why

this relationship might not be constant under environmental variation, and the consequences this may have for coevolution and the evolution of virulence.

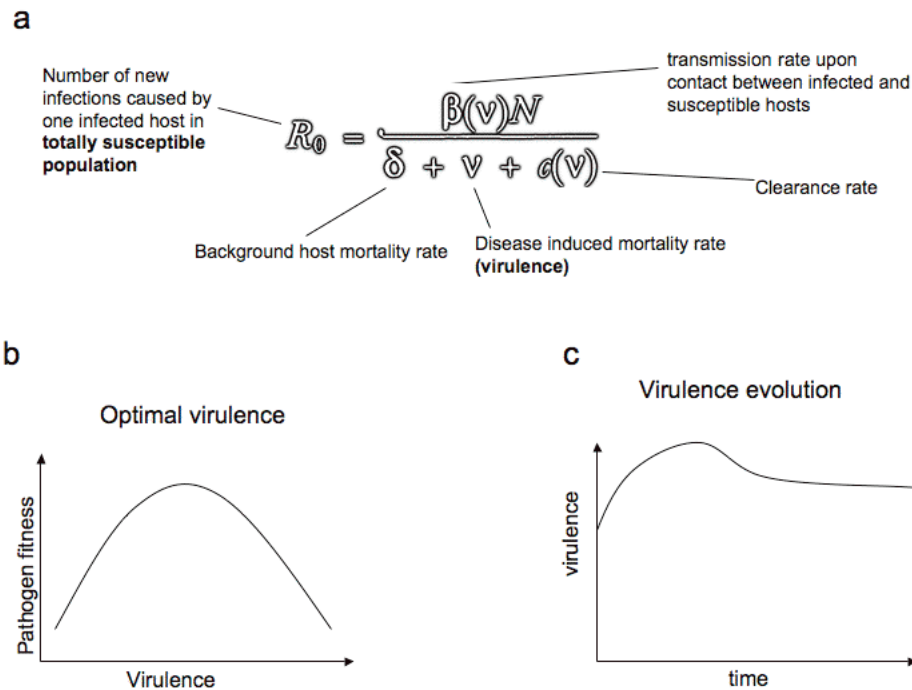


Figure 1.2. The evolution of virulence. (a) Theoretical models of virulence evolution based on R_0 assume that virulence evolves as a consequence of selection on parasite fitness. Parasites with higher growth rates will result in more transmission (β), but increased growth will also result in increased host death (v). If a parasite kills its host too early, it will also reduce its lifetime transmission success, R_0 , because it produces less transmission stages. Therefore, the expectation from R_0 maximization is that pathogen fitness will be maximal for an intermediate level of virulence (b). Selection for the fittest parasites over time (c) should then result in an initial increase in virulence due to very fast growing parasites, but should then reduce over time due to selection for the fittest parasites with intermediate virulence. See text for details and for examples where this outcome might not occur.

1.4 Completing the disease triangle: environmental effects on infection

Most experimental work on parasitism is centred on well-described host-parasite systems, taking advantage of the possibility of performing highly replicated experiments in controlled laboratory settings (e.g. Buckling and Rainey 2002; Mackinnon and Read 2004; Ebert 2008). This is important for disentangling genetic from environmental effects on infection outcomes that are difficult, if not impossible, to separate by studying organisms in their natural environments. However, in addition to the genetic context, a realization is also emerging that infection outcomes are environment-dependent (e.g. Blanford et al. 2003; reviewed in Lazzaro and Little 2008), making disease truly an ‘extended phenotype’ that is an outcome of the interaction between the host, the parasite and their environments (Figure 1.1). Certainly, both the abiotic and the biotic environments vary frequently and sometimes unpredictably in the wild. Therefore understanding how hosts and parasites have evolved and will evolve under common conditions of environmental variation requires that infections be studied under realistic ranges of variation in the environmental variables that are most likely to affect them in their natural settings.

1.4.1 Incorporating environmental variation into studies of host-parasite interactions

As discussed above, the view that the environment is important in determining disease has been prevalent in the plant pathogen community for nearly 50 years, in the form of the disease triangle (McNew 1960; Scholthof 2007; Figure 1.1). Work on infectious diseases in animal and especially in human populations have for the most part ignored environmental variation as a major determinant of disease severity (an exception is found perhaps in epidemiology with some work focussing on the effects of seasonal and climactic variation on disease transmission (e.g. Koelle et al. 2005; Pascual and Dobson 2005). While this may be in part due to the dominating dogma of the “virulent pathogen”, in most cases the relevance

of environmental variation is acknowledged but is simply too difficult to investigate experimentally. First, the genotype of the host can be considered an “environment” in which the parasite must survive, yet apart from a handful of model systems, the genetic details underlying host susceptibility to infection is confined to a few species where genomic data is available and the genetic architecture of resistance has been determined. Genetic markers for parasites, on the other hand, have so far been biased toward pathogenic clinical isolates (Selander et al. 1987), giving a misleading picture of parasite genetic variation. Even when the genetic information is available, testing the determinants of infection in controlled laboratory settings is not possible in the majority of vertebrate animal systems (and certainly not humans). Finally, even when such a system is available, incorporating abiotic environmental variation requires knowledge of the natural ecology of the system, and which variables are most important in determining the outcome of infection, which is challenging. Still, the last decade has seen several studies of parasitism, mostly in invertebrate host-parasite systems, that have incorporated variation mainly in food levels and temperature (reviewed in Lazzaro and Little 2008; Table 1.1).

In the following sections I discuss the possible consequences of this environmental variation on three aspects of host-parasite biology that are addressed in greater detail in the chapters that follow: 1) the effect of climatic variation on the onset, duration and severity of epidemics; 2) the maintenance of genetic polymorphism due genotype-by-environment (GxE) interactions; and 3) the evolution of virulence in variable environments.

Environmental variable	Host-parasite system	Genetic variation	Host traits measured	Parasite traits measured	Reference
Temperature					
10°C, 23°C, 30°C	<i>Paramecium caudatum</i> ; <i>Holospira undulata</i>	3 host clones	Proportion infected;	Parasite load (number of infectious forms)	(Fels and Kaltz 2006)
10°C, 15°C, 20°C, 25°C; Spore dose	<i>Daphnia magna</i> ; <i>Pasteuria ramosa</i>	8/9 host clones	Proportion infected; Proportion reproductive	-	(Mitchell et al. 2005)
Temperature: 25°C, 29°C Host crowding	<i>Drosophila nigrospiracula</i> ; <i>Macropheles subbadius</i>	3 resistance-selected and 3 unselected host lines	Proportion infected; Larval competitive ability; Survival; Female size	-	(Luong and Polak 2007)
Temperature: 18, 25, 28, 31°C	<i>Acyrtosiphon pisum</i> ; <i>Erynia neoaphidis</i>	4 host clones	Proportion infected	-	(Blanford et al. 2003)
16°C, 18°C, 20°C, 22°C; Nutrient concentration	<i>Plantago lanceolata</i> ; <i>Podosphaera plantaginis</i>	8 host 8 parasite genotypes	Proportion infected;	Hyphae formation time; spore production (number and timing);	(Laine 2007a)
Food levels					
High / low food	<i>Paramecium caudatum</i> ; <i>Holospira undulata</i>	5 host clones	Division rate; Survival.	Parasite load (number of transmission stages and average nucleus size)	(Restif and Kaltz 2006)
High and low glucose	<i>Anopheles stephensi</i> ; <i>Plasmodium chabaudi</i>	2 parasite genotypes	Proportion infected; Survival	Oocyst production	(Ferguson and Read 2002)
Food gradient (mg) 0.5 – 3.0	<i>Aedes aegypti</i> ; <i>Vivaria culicis</i>	-	Probability adulthood; Developmental time; Adult dry weight; Survival/longevity	Parasite load (number of infectious forms)	(Bedhomme et al. 2004)
Host's previous food level; Parasite's previous food level	<i>Aedes albopictus</i> ; <i>Ascogregariana taiwanensis</i>	-	Wing length; Developmental time;	Oocyst production	(Tseng 2006)
Glucose gradient	<i>Anopheles stephensi</i> ; <i>Plasmodium yoelli</i>	8 host isofemale lines	Survival	Oocyst production	(Lambrechts et al. 2006a)

Table 1.1. Empirical studies of the effect of abiotic environmental factors on host-parasite interactions.

1.4.2 The effect of climatic variation on the onset, duration and severity of epidemics

Climatic variability is thought to play a key role in determining the temporal and spatial distribution of pathogens (Epstein 2001), particularly for vector and water-borne diseases, due to their sensitivity to changes in seasonal fluctuations in temperature (Lipp et al. 2002; Pascual et al. 2002; Gage et al. 2008; Halstead 2008). As such, linking seasonal climatic variables to fluctuations in disease transmission has been an active field of research (reviewed in Altizer et al. 2006). The need to understand the link between climatic variability and infectious disease is heightened by the latest scientific evidence predicting a global increase in mean temperatures by an average of 4°C over the next century (IPCC 2007). The effect of such an increase in temperature for infectious diseases is not yet clear (Brooks and Hoberg 2007). The general prediction is that an increase in the mean temperature would increase the period of time when the optimal conditions for infection are met, resulting in earlier, longer lasting, and potentially more severe epidemics (Marcogliese 2008). This outcome is contingent on the effect of increased temperature on both host and parasite evolution and their rates of adaptation to changing environments (Visser 2008).

Predicting the long-term effects of climate change is undoubtedly far from simple, as changes in climate are not geographically uniform. So, while climate change is predicted to result in a global increase in mean temperatures, locally it could create conditions for climatic extremes (Easterling et al. 2000). These may cause disruption of seasonal climatic events and destabilize biological systems (Epstein 1995). The effect of seasonal cycle disruption is especially worrying for water-borne diseases, as a strong correlation exists between extreme weather events and outbreaks of enteric diseases, such as cholera (Lipp et al. 2002; Pascual et al. 2002). Other work has shown that climate disruption could result in spatially synchronised disease outbreaks if disruption of seasonal cycles results in changes in

the developmental rate of parasites and, consequently, also changes the temporal dynamics of transmission (Cattadori et al. 2005; Hudson et al. 2006). From the host perspective, changes in seasonal cycles could also cause deviations from optimal levels of immunity in cases where it is largely environment dependent. A recent survey of Red-Spotted Newt populations found evidence that variability in temperature could increase susceptibility to disease by increasing the lag in the production of immune molecules during seasonal transitions, thus prolonging the time needed to acclimatise to new environmental conditions and regain optimal levels of immunity (Raffel et al. 2006).

These examples illustrate how it is important to investigate the effects of climatic variation on infection outcomes in a systematic manner that clearly distinguishes between the genetic and environmental determinants of epidemic onset. The work presented in chapter 3 shows how the use of the *Daphnia*-microparasite system might prove fruitful in studying the role of climate variability on epidemics. Other work in this system has already addressed general epidemiological principles such as the factors affecting transmission (Regoes et al. 2003; Ben-Ami et al. 2008), how host ecology influences the timing of infection (Hall et al. 2007; Pulkkinen 2007; Wolinska et al. 2007), the role of seasonality on infection prevalence (Lass and Ebert 2006), the environmental and ecological causes of epidemic termination (Caceres et al. 2006; Hall et al. 2007), the role of trophic interactions (Hall et al. 2006; Duffy and Hall 2008) and sexual reproduction (Duncan et al. 2006) in reducing the detrimental effects of parasites during epidemics, and how parasite epidemics affect host population genetic structure (Mitchell et al. 2004; Duncan and Little 2007).

1.4.3 Maintenance of polymorphism due to genotype-by-environment (GxE) interactions

The mechanisms maintaining genetic variation in natural populations are a central focus of evolutionary biology, so understanding the maintenance of genetic variation in populations of hosts and their pathogens would naturally attract attention from evolutionary biologists (Haldane 1949; Hamilton 1982). Besides the maintenance of polymorphism due to frequency-dependent coevolutionary dynamics (discussed above, Hamilton 1993), another mechanism potentially maintaining genetic variation is environment-dependent selection, if different genotypes have the highest fitness in different environments. While this is certainly plausible in spatially variable environments where adaptation to local conditions could maintain different genotypes in separate patches, the role of environmental variation over time is less clear. The prediction is that temporally fluctuating environments would simply result in repeated bouts of directional selection, which would erode genetic variation albeit at a slower rate than directional selection in only one direction as occurs in a constant environment (Roff 1997). The maintenance of polymorphism due to temporal fluctuation in the environment would be possible however under negative frequency-dependent selection (Hamilton 1993).

These genotype-by-environment (GxE) interactions can be detected by measuring the fitness of different genotypes across two or more environments and analysing the variance in fitness attributable to genotypic main effects, environmental main effects, or the interaction between genotype and environment (GxE interaction; Stearns 1992; Roff 2002). Graphically, GxE interactions appear as crossing reaction norms (or with divergent slopes), where the slope of the norm indicates the direction and the magnitude of the environmental effect on fitness (Roff 2002; Nussey et al. 2007; Figure 1.3). Significant GxE interactions for fitness literally mean that the rank order of fitness of genotypes changes depending on the environment, so

when detected they provide evidence of the potential for environment-dependent selection to occur in heterogeneous environments (Byers 2005; Laine and Tellier 2008). Possibly the first experimental evidence of GxE interactions was Falconer and Latyszewski's classic experiment using mice selected on the basis of improved growth in two environments (full or restricted rations). It showed that the phenotype (i.e. improved growth) was dependent upon both the genotype (mouse family) and environment (ration size) (Falconer and Latyszewski 1952). Evidence for GxE interactions in host-parasite systems has been increasing, especially over the last decade (Table 1.1). Most of these studies investigate GxE effect on host fitness traits, while few have investigated the effects of environmental variation on the maintenance of parasite polymorphism, despite its relevance to pathogen evolution. In chapter 4, I employ this approach to test whether an environment where the temperature is heterogeneous could maintain polymorphism in parasite populations due to environment-dependent selection.

Host-parasite systems add another level of complexity to the study of GxE interactions because many of the traits that are used to infer host or parasite fitness depend on the genotypes of both host and parasite (Lambrechts et al. 2006b; GxG interactions, see above). This raises two main issues regarding the effect of the environment on host and parasite evolution. First, the question is not necessarily if a GxE interaction is present, but whether $G_H \times G_P \times E$ interactions occur (where G_H and G_P are the host and parasite genotypic effects). If some traits are indeed the product of genotypic specificity, can environmental variation override the genotypic effects and still result in environment-dependent selection? The second point relates to the broader issue of inferring population level fitness from the measurement of few traits that are expected to correlate well with fitness (Lenski and Service 1982; Lande and Arnold 1983), e.g. host fecundity or survival, or parasite infectivity or growth. If such traits are affected differently by genotypic (GxG) and environmental effects

(GxE), which traits are the most appropriate fitness correlates for studies of evolution in a variable environment? I investigate these questions in chapter 5.

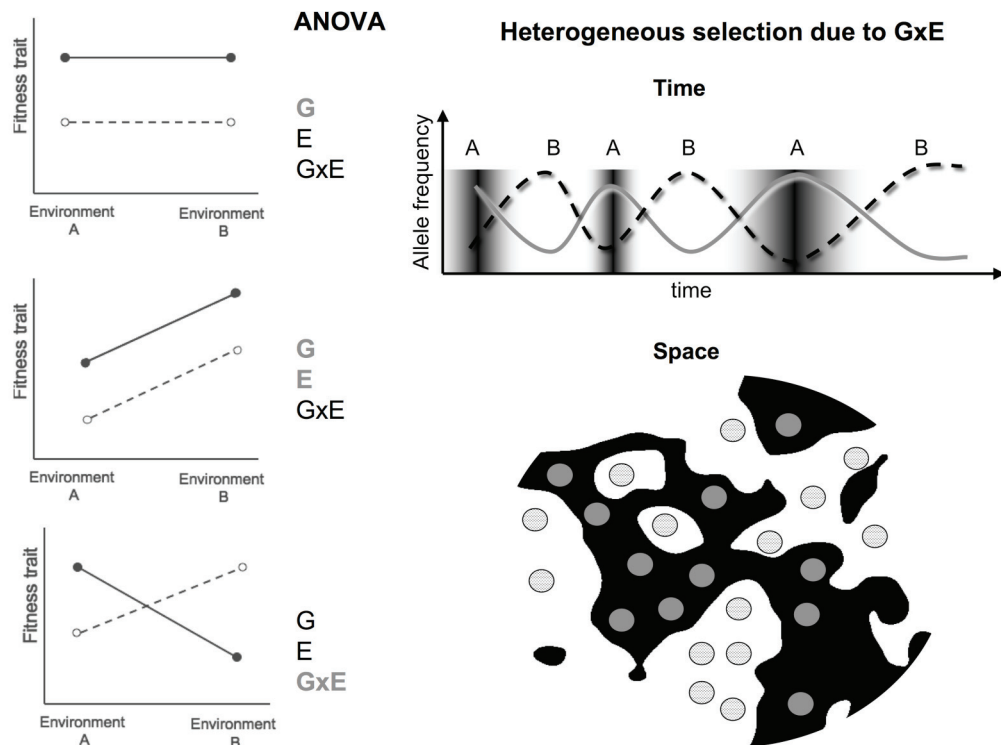


Figure 1.3. Interactions, reaction norms and selection. By measuring the fitness of several genotypes in multiple environments, it is possible to analyse the variance in fitness (e.g. with ANOVA) attributable to genotype effects, environmental effects, and their interaction. Top left: the fitness of two genotypes (grey and white circles) does not change between environment A and B, but differs between genotypes. Statistically, this is revealed by a significant genotype (G) main effect, and selection in these two environments would not alter the frequencies of each genotype over time (under frequency-dependent selection) or space. Middle left: apart from the genotype effect, the fitness of each genotype increases in environment B. However, the magnitude of this increase is the same for both genotypes (the slopes of the reaction norms are the same) and the GxE interaction is not significant. Bottom left: crossing reaction norms indicate that fitness is environment-dependent and a significant GxE interaction is found. Environment-dependent selection is predicted to maintain polymorphism both in time (top right) and in space (bottom right).

1.4.4 Virulence evolution in a variable environment

Maintenance of genetic polymorphism is only one potential outcome of environment-dependent selection on host-parasite systems. From a standard quantitative genetics point of view, the efficiency of selection on host and parasite genotypes will depend on the expressed genetic variance, which is known to be environment-dependent (Falconer and Mackay 1996). Due to this, environmental variation may influence the intensity of coevolution, potentially resulting in coevolutionary “cold spots” or “hot spots” in different environments (Thompson 1994; Thompson 1999), where antagonistic selection between host and parasites is weak or strong, respectively. By changing the strength of antagonistic selection between hosts and parasites, fluctuating environments have the potential to modulate the evolution of one very relevant trait that is a product of coevolution: virulence. If we consider that in natural habitats environmental fluctuation is common (such as in temperature or food availability), our understanding of host-parasite interactions, their coevolution, and the levels of virulence that result from that evolution must incorporate knowledge of how environmental variation impacts the evolution of host and parasite traits.

All theories of virulence evolution are based on the idea that parasites face a trade-off between growth and transmission (Frank and Schmid-Hempel 2008). Implicit in trade-offs (and therefore also in theories of virulence evolution) is that resources are limited and are allocated either towards host growth and reproduction or parasite growth and reproduction. Generally, because traits are physiologically or developmentally linked and resources are finite, it is not possible for all fitness traits to be maximised during evolution; selection increasing one trait value comes at the cost of another (Stearns 1976; Falconer and Mackay 1996; Roff 2002). Due to these costs, traits cannot evolve to any possible value, but rather

exist within a limited phenotypic space whose boundaries are determined by trade-offs (Roff 2002). Therefore, understanding how correlated traits are expressed is central to life-history evolution, as they will determine how phenotypes will evolve.

Relationships between correlated traits (e.g. trade-offs) are often not constant, and the magnitude and even the sign of the relationship between correlated traits can change depending on the environment (Sgrò and Hoffmann 2004). The general implication for phenotypic evolution is that, as genetic correlations change between environments, this could alter the predicted outcome of evolution in constant environments. In the context of host-parasite systems, environment-dependent relationships between host and parasite fitness traits could have important consequences for the evolutionary outcome of parasitism and virulence. For example, under parasitism we expect a strong negative correlation between host and parasite fitness traits. If this correlation is weakened under certain environmental conditions (i.e. if parasitism is reduced), what consequences might this have for detecting coevolutionary dynamics? Will changing these relationships alter trajectories of virulence evolution? In chapter 6 I describe an experiment testing several relationships between host and parasite traits under different environmental treatments. In doing so, I also test the main assumption of virulence evolution models, that the virulence-transmission relationship is positive.

1.5 Thesis outline and aims of each chapter

Chapter 2- Model system. I give a brief overview of the *Daphnia-Pasteuria* host-parasite model system used in the experiments that follow.

Chapter 3 – Lagged minimum temperature drives epidemic prevalence of a crustacean parasite. Based on predictions from laboratory infections under thermal variation, I tested the hypothesis that natural epidemics of this parasite are driven by temperature. I found that elevated minimum temperatures around the expected time of initial exposure correlated strongly with infection prevalence.

Chapter 4 – Temperature dependent costs of parasitism and maintenance of polymorphism under genotype-by-environment interactions. I show that the expression of infections traits in *P. ramosa* depends on temperature in a genotype-specific manner [genotype-by-environment (GxE) interactions]. These GxE interactions are a pre-requisite for the maintenance of polymorphism through environment-dependent selection.

Chapter 5 – Measuring parasite fitness under genetic and thermal variation. I tested if GxG interactions for infectivity could be altered by variation in temperature (GxGxE interactions). Infectivity appeared to be robust to thermal variation, and was also more important in determining parasite fitness relative to the production of transmission stages. This highlights the importance of considering natural infection routes, an aspect that is frequently overlooked in studies of host-parasite systems.

Chapter 6 – Context-dependent virulence-transmission relationships and the evolution of virulence. I tested the fundamental assumption of virulence evolution models, regarding the expected relationship between transmission and virulence, under environmental variation. I found that for realistic ranges of temperature and food the strength and even the direction of these relationships could change. These results suggest that the environment-dependent nature of parasitism may limit predictions about the evolutionary outcomes of virulence based on models that assume constant environments.

Chapter 7 - Testing for temperature-dependent costs of resisting infection in *Daphnia magna*. I measured the cost of resisting infection in terms of fecundity and lifetime survival in hosts exposed to, but not infected by, *P. ramosa*. I found no evidence that variation in temperature increases the cost to the host of resisting infection.

Chapter 8 - General discussion. I discuss the relevance of environment-dependent selection in the wild and highlight areas of research that require greater attention.

CHAPTER 2

THE MODEL SYSTEM

The model system

The work described in this thesis studies the interaction between the planktonic freshwater crustacean *Daphnia magna* and a naturally occurring parasite, the spore-forming bacterium *Pasteuria ramosa*. This model system allows hypotheses about coevolution to be tested, by combining studies in natural populations with controlled laboratory experiments. Below is a brief description of host and parasite biology that is relevant to the chapters that follow. A detailed description of the specific genotypes used in each experiment is given the methods sections within each chapter.

2.1. The host: Daphnia magna

Daphnia spp. are planktonic crustaceans (zooplankton ranging from 1-6 mm as adults). Like most members of the class Branchiopoda, they are filter-feeding inhabitants of temperate freshwater ponds and lakes. Within the Branchiopods, they are classified in the order Cladocera, and possess a two-valved carapace that covers most of the body except for the ventral filtering appendages.

Daphnia reproduction is mostly parthenogenetic, with females usually producing broods of clonal females. Some environmental conditions such as short-day photoperiod, food limitation and crowding (Kleiven et al. 1992), may induce the production of asexual males and haploid eggs (known as ephippia; Figure 2.1). Ephippia can withstand long-periods of environmental stress (e.g. Decaestecker et al. 2007) and if fertilised by males before they are released by females, they will give rise to sexual progeny. In the wild, male and ephippia production usually occur in the spring. In the lab, environmental conditions can be controlled to induce male production if required, or (as in the experiments presented here) to ensure that only clonal females are produced. This allows distinct genotypes to be propagated clonally

from an individual female, which in turn allow high levels of experimental replication within genotype (Ebert 2008).

Daphnia ecology has been the focus of research for more than a century and this knowledge, paired with their amenability to laboratory manipulation make them an ideal model system. A comprehensive review of *Daphnia* physiology and ecology is provided in by Peters, R. H. & deBernardi (1987). Recently, Ebert (2005) reviewed the ecology, epidemiology and evolution of parasitism in *Daphnia*.

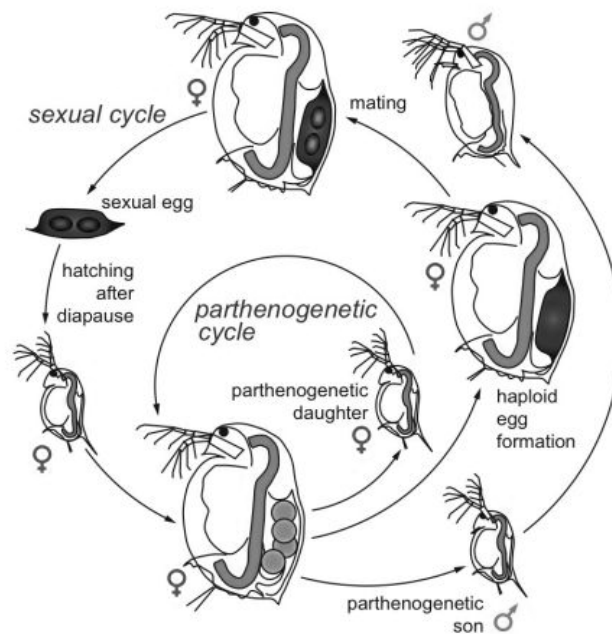


Figure 2.1. *Daphnia* life cycle. Clonal females are produced during the asexual cycle. Some environmental cues might result in the production of males or haploid eggs. Fertilization of these eggs by males results in resting sexual eggs (ephippia) that will hatch after diapause. Diagram from Ebert (2005).

2.2 The parasite: *Pasteuria ramosa*

Daphnia are known to be susceptible to a variety of bacterial, microsporidian, and fungal parasites, (reviewed in Ebert 2005 and Green 1974). All the experiments in this thesis were carried out with the naturally occurring bacterial parasite *Pasteuria ramosa* Metchnikoff 1888. *P. ramosa* is a gram-positive bacterium, closely related to the nematode parasite *P. penetrans*. (Ebert et al. 1996) In natural populations of *D. magna*, *P. ramosa* infection prevalence reaches extremely high levels, especially during the spring and summer months (Mitchell et al. 2004; Duncan and Little 2007).

Daphnia are filter feeders and become infected with *P. ramosa* by filtering transmission spores present in the water. Transmission is exclusively horizontal by transmission spores that are only released from dead hosts. Within the host, *P. ramosa* goes through developmental process that ends in the formation of transmission stage spores (Figure 2.2). These transmission spores are extremely resistant to environmental stress and spores from pond sediments have been shown to be infective during the winter when hosts enter diapause and even from sediment cores a few decades old (Decaestecker et al. 2004; Decaestecker et al. 2007).

Juveniles infected in the first days after hatching will sometimes produce one or even two clutches before reproduction ceases, although some individuals produce no clutches when infected. Apart from sterilization, infected female *Daphnia* grow larger and acquire a darker, red colouration. Uninfected *Daphnia* can be maintained for more than 100 days in lab conditions, while infection causes premature death, ranging from 20 to 60 days post-infection. *P. ramosa* is an obligate *Daphnia* parasite and attempts to culture it have so far been unsuccessful. In experimental conditions, spore solutions are obtained by macerating infected *Daphnia*. These usually harbour 5 to 10 million transmission spores per host.

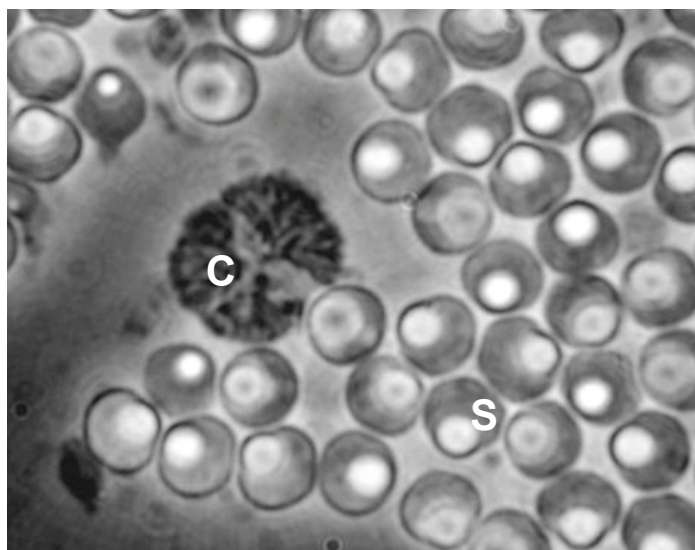


Figure 2.2. *Pasteuria ramosa*. The first visible stage of an infection is the cauliflower stage (C). These develop, branch out and grow into individual spores (S) that fill the host in the final stages of infection and serve as transmission stages for the parasite. Spores vary in size from approximately 3-6 μ m in diameter. Figure from Ebert. 2005

CHAPTER 3

LAGGED MINIMUM TEMPERATURE DRIVES EPIDEMIC PREVALENCE OF A CRUSTACEAN PARASITE

3.1 Summary

Environmental variables such as temperature are likely drivers of the onset, duration and severity of epidemics. In this study, we tracked the infection status of a population of the freshwater, planktonic crustacean *Daphnia magna* during a single season in 2006. As in previous years, this population experienced a summer epidemic of *Pasteuria ramosa*, an obligate, bacterial parasite. Previous laboratory work suggests that several infection-related traits in this system are sensitive to temperature. Here, we aimed to explicitly test if temperature could explain the observed field patterns of parasite prevalence. Laboratory measures of *P. ramosa* infection showed that infections are detectable 15-20 days after exposure to the parasite, and we thus assumed that hosts deemed infected in our fieldwork were initially exposed to the parasite 15-20 days prior to each sampling date. Consistent with these predictions, we found an association between infection prevalence and the temperature approximately 18 days before each sampling date. Specifically, there was a positive correlation with lagged minimum temperature and a negative correlation with the lagged daily thermal range for this period. This presents a scenario where a combination of high minimum temperatures and low daily variability in temperature results in longer periods of parasite infectiousness and/or growth, and thus increased infection prevalence 18 days later. We discuss these results and their implications for temperature-mediated epidemics.

Keywords: epidemic, prevalence, correlation, time-lag, temperature, crustacean, *Daphnia magna*, *Pasteuria ramosa*.

3.2 Introduction

Many infectious diseases show regular seasonal variation in their incidence (Dowell 2001). However, identifying the seasonal drivers of disease is notoriously challenging due to the complexity of specific biological interactions between hosts and parasites, and to the variability of the abiotic environment (Pascual and Dobson 2005; Altizer et al. 2006). Some studies attempt to explain the observed patterns of infection based on seasonally varying environmental factors that might facilitate disease transmission, such as temperature (Checkley et al. 2000), humidity (Chew et al. 1998), or rainfall (Naumova et al. 2005). However, in these cases it is usually difficult to infer a causal relationship between the climatic variable studied and the incidence of infection, because the direct effect of climate on the expression of infection traits is not often testable (Pascual and Dobson 2005).

One approach to overcome the shortcomings of purely correlational studies is to use naturally occurring host-parasite systems that are amenable to direct laboratory manipulation. This makes it possible to directly test, through independent observations in controlled laboratory settings, hypotheses about disease transmission in wild populations. Here we use such a well-established system, the freshwater crustacean *Daphnia magna* and its naturally occurring, bacterial parasite *Pasteuria ramosa* (Ebert 2008). We followed a pond population located near Leitholm in the Scottish Borders, UK. Summer epidemics of *P. ramosa* have been observed previously in this population (Mitchell *et al.* 2004; Duncan & Little 2007), suggesting that temperature is a potential driver of infection. However, whether this is due to a direct effect of temperature on parasite biology, the effect of temperature on the availability of susceptible hosts, or the effect of some other ecological factor, is not clear.

Previous work on this and other *D. magna*-*P. ramosa* populations has found that the likelihood of infection, parasite growth and transmission are generally lower when temperatures are below 20°C (Mitchell et al. 2005; Vale et al. 2008b), suggesting that temperature affects parasite biology directly. Until now, the direct effect of temperature on the onset of *P. ramosa* epidemics in wild populations had received little attention. Thus, we monitored the infection status of a population of *D. magna* during 2006 and investigated the relationship between infection prevalence, host density, and air temperature. Symptoms of *P. ramosa* infections are apparent by visual inspection after a period of parasite within-host growth, usually 15-20 days post-exposure (Ebert *et al.* 1996; Ebert 2005). We hypothesised that if temperature was driving the epidemic, increased prevalence would be observed when temperatures 15-20 days prior to sampling fell within a range conducive to parasite infection and growth. Therefore, we investigated relationships between the prevalence of *P. ramosa* infections and the minimum and maximum temperatures, as well as the daily thermal range (DTR) at a range of periods prior to each sampling date.

3.3 Material and Methods

Study system

Daphnia magna (Crustacea: Cladocera) are planktonic crustaceans that inhabit freshwater lakes and ponds (Peters and De Bernardi 1987). *Pasteuria ramosa* are spore-forming bacteria that cause sterilization and premature death in *D. magna* (Ebert et al. 1996). Transmission of *P. ramosa* is exclusively horizontal, achieved by spores that are released from dead hosts and picked up by *Daphnia* during filtration feeding (Ebert et al. 1996). Within the host, *P. ramosa* spores germinate and develop, culminating in the formation of transmission stage spores (Ebert et al. 1996). In experimental infections at 20°C, when

infection is carried out on 1 day old juveniles, this developmental process takes between 15 and 20 days before signs of infection are obvious (*Daphnia* stop producing eggs, grow larger and red, and bacterial growth is apparent in the haemolymph) (Ebert 2005).

Population sampling

We sampled a population in the Kaimes pond near Leitholm, in the Scottish Borders (2°20.43' W 55°42.15' N). Biweekly samples were taken from May to December 2006. Sampling methods were similar to those described previously (Mitchell *et al.* 2004; Duncan *et al.* 2006). Briefly, three locations within the pond were chosen at random for sampling and these were maintained throughout the sampling period. Sampling was achieved by using a net to sweep each location horizontally just below the water surface, keeping the size of the sweep consistent (approximately 1 metre). Samples were immediately counted, and categorised according to their sex (male/female), developmental stage (adult/juvenile), reproductive state (barren, parthenogenetic, ehippial), and infection status (infected, not infected). We determined the mean density of *D. magna* per litre of water by estimating the volume of water sampled with each sweep of the net. The mean proportion of infected adult *Daphnia magna* in the three locations was taken as a measure of the mean prevalence of *Pasteuria ramosa* infections in the population at each time point.

Meteorological data

We obtained meteorological data from the UK Meteorological Office for Edinburgh Gogarbank, the closest meteorological station to the study population (approximately 80 km / 50 miles from the field site). These data include the daily maximum and minimum temperatures, which were used to calculate the daily thermal range (DTR, the difference

between maximum and minimum daily temperatures). To determine if air temperature was a good estimate of water temperature, the water temperature in the pond was recorded in the following year by measuring the temperature at each of the sampling locations with a thermometer, and the relationship between water temperature and air temperature was tested with correlation.

Data analysis

All analyses were performed using the statistical software package JMP 7 (SAS Institute Inc.). We first tested for a general association between infection prevalence and temperature variables using Pearson correlation. Due to the time-lag between exposure to the parasite and the detection of infection symptoms in the host, we determined the daily maximum and minimum temperature and DTR for each individual day 10-20 days prior to each sampling date. As it is not possible to determine the exact day of infection, nor is it likely that all infected individuals sampled were infected on the same day, we repeated the analysis using a 5-day mean of minimum and maximum temperatures, and DTR, for 3 different periods: 10-15 days, 12-17 days, and 15-20 days prior to sampling. Using this univariable pre-analysis, we pre-selected predictors of infection prevalence. We then included the temperature variables with the highest correlation coefficients in a linear model, together with host density at each sampling date as explanatory variables of infection prevalence (prevalence~temperature x host.density). Because both prevalence and temperature were recorded temporally, it is possible that the data was temporally correlated, which would violate the assumption of independence made in our linear models. We tested the autocorrelation of the residuals of these models using the Durbin-Watson test (Durbin and Watson 1950). The resulting test statistic, d , is a value between 0 and 4; small values (< 1) indicate that successive residual terms are on average close to each other and suggest a

positive autocorrelation, and large values (>3) suggest a negative correlation. In the statistical package JMP 7 (SAS Institute Inc.), the p-value tests the null hypothesis that there is no autocorrelation at significance level 0.05.

3.4 Results

Host density, life-stage composition, and infection prevalence

During the sampling period, host density appeared to fluctuate little (mean=3.5, SD= 4.56), aside from a sharp increase in August/September (average of 19 adult *Daphnia* per litre), followed by a sharp decline (Figure 3.1). Predictably, this increase was reflected by a sharp increase in the number of juvenile *Daphnia* and also barren females during the same period (Figure 3.1). Otherwise, the life-stage composition of the population remained relatively constant throughout the sampling period. Male *Daphnia* and *Daphnia* with sexual resting eggs were rarely detected during the sampling period.

The mean infection prevalence throughout the sampling period was 0.47 (SD= ± 0.27). A notable increase in *P. ramosa* infection was first detected in June, rising sharply to a peak prevalence of 0.82 in July. This peak in infection prevalence was accompanied by a decrease in host density, which then reached almost undetectable levels at the start of August. Prevalence of *P. ramosa* infections was always above 0.20, and two more peaks in infection prevalence were observed in September (mean prevalence 0.68) and November (mean prevalence 0.75) (Figure 3.1). Host density decreased and infection prevalence became undetectable after December 2006.

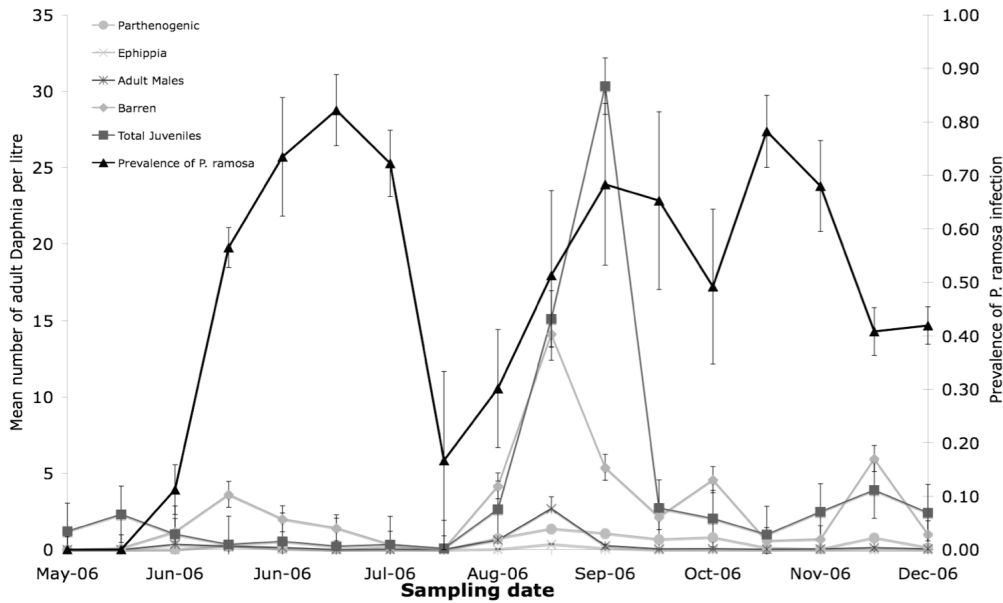


Figure 3.1. Density of *Daphnia magna* in each of the categories recorded (per litre of water), and infection prevalence of *P. ramosa* (▲). Categories are parthenogenetic females (●); females with ehippia (×); adult males (*); barren females (◆); total juveniles (■). All values are the mean \pm SE of 3 sampled spots.

Infection prevalence and temperature

Across the sampling season, mean air temperature ranged between 5°C (December) and 17°C (June/July). The daily thermal range (DTR) across the same period varied greatly; the lowest DTR was observed during May (2.4°C between daily maximum and minimum temperature), and the highest DTR was observed during April (17°C between daily maximum and minimum temperature) (Figure 3.2). We found a positive correlation between water and air temperature ($R^2=0.60$, $F_{1,13}=16.76$, $p=0.0015$), suggesting that variation in air temperature is a reasonable proxy for variation in water temperature.

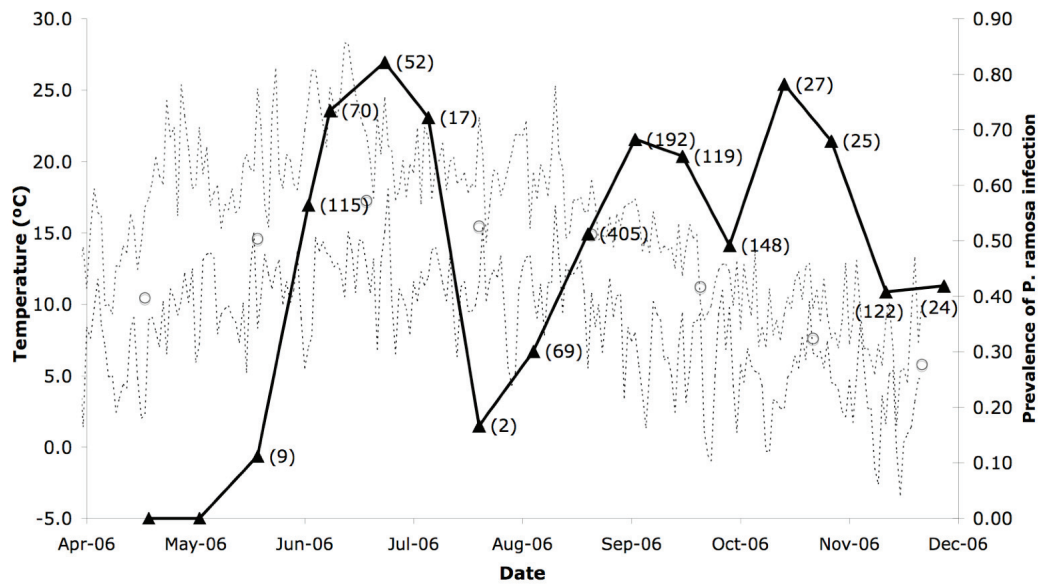


Figure 3.2. The monthly mean temperatures are shown (open circles). The dotted lines above and below the mean temperature are the maximum and minimum daily temperatures, respectively (daily thermal range). The prevalence of *P. ramosa* infection is shown by the black triangles. Numbers of infected adults recorded are shown in parentheses for non-zero prevalence. Data refers to air temperature recorded at the Edinburgh Gogarbank weather station and were provided by the UK Meteorological Office.

We found the strongest correlations between prevalence and the DTR and minimum temperatures experienced on or around 18 days prior to each sampling date. The strongest of these was a negative correlation between prevalence and the daily thermal range experienced 18 days prior to each sampling date, followed by positive correlations between prevalence and the minimum temperature experienced 18 days to each sampling date (Table 3.1).

Temp.	Coeff.	p
DTR 18	-0.5829	0.0141
Min Temp 18	0.539	0.0256
15-20 Min	0.4795	0.0515
Min 19	0.4462	0.0726
Min 17	0.3989	0.1127

Table 3.1. Pairwise Pearson correlations between prevalence and several temperature variables (n=17) (see data analysis for details). We show the correlations with the lowest p-values. Based on these, we defined the temperature variables to include in linear models that tested the effect of temperature and host density on parasite prevalence (see Table 3.2). Temp – temperature variable; Coeff. - Pearson correlation coefficient.

Based on these correlations, we tested the effect of temperature and host density on the prevalence of *P. ramosa* infections. The ANOVA shows that there is no statistically significant effect of temperature or host density on prevalence when no lag period is considered. However, for a lag of 18 days before each sampling date, we found statistically significant effects of minimum temperature and a host density x minimum temperature interaction (Table 3.2). Indeed, prevalence appears to follow lagged minimum temperature closely, except for a period between August and September (Figure 3.3), which coincides with the sharp increase in juvenile density (Figure 3.1), and is the possible driver of the temperature x host density interaction we detected in our analysis (Table 3.2). We also find a significant main effect of the DTR 18 days before each sampling date but whereas the minimum temperature model is significant ($R^2 = 0.41$, $p=0.018$, Table 3.2) the DTR model is not, despite the significant main effect ($R^2 = 0.26$, $p=0.077$, Table 3.2). In addition, the DTR model appears to show some evidence of positively autocorrelated residuals (Durbin-Watson $d=1.06$, $p=0.015$), while the minimum temperature model does not (Durbin-Watson $d=1.59$, $p=0.126$), satisfying the assumptions of the analysis.

Time-lag	Temperature variable	Term	Adj R ²	d.f.	Coeff.	Type I SS	F	p
No lag	Minimum temperature	Whole model	0.05	3,16				0.5560
		Host density			-0.053	0.0207	0.2670	0.6141
		Min			0.045	0.1238	1.5973	0.2285
		Host density x Min			0.009	0.0236	0.3047	0.5903
	Daily thermal range (DTR)	Whole model	0.02	3,16				0.9634
		Host density			0.371	0.0207	0.2336	0.6369
		DTR			0.002	0.0003	0.0033	0.9549
		Host density x DTR			0.007	0.0033	0.0376	0.8492
15-20 day lag	Minimum temperature	Whole model	0.44	3,16				0.049
		Host density			0.216	0.021	0.4097	0.5332
		Min (15-20)			0.035	0.250	4.9567	0.0443
		Host density x Min (15-20)			-0.003	0.248	4.9136	0.0451
18 day lag	Minimum temperature	Whole model	0.41	3,16				0.018
		Host density			0.018	0.021	0.480	0.501
		Min 18			0.024	0.321	7.441	0.017
		Host density x Min 18			-0.008	0.273	6.332	0.026
	Daily thermal range (DTR)	Whole model	0.26	3,16				0.077
		Host density			0.016	0.021	0.381	0.548
		DTR 18			-0.044	0.384	7.067	0.019
		Host density x DTR 18			0.001	0.065	1.190	0.295

Table 3.2. Linear models analysing the effect of host density and temperature on the prevalence of *P. ramosa* during the 2006 epidemic. Host density is the density of adult *Daphnia* per litre of water recorded on the day of sampling. The temperature variables tested are the minimum temperature and daily thermal range of temperature for 3 time-lag periods; no lag (recorded on the day of sampling), 18 day lag (recorded on a single day 18 days prior to each sampling date), and 15-20 lag (the 5-day mean of temperatures recorded between days 15 and 20 prior to each sampling date). These were chosen based on the strongest correlations with prevalence (see Table 3.1). Adj R², Adjusted R-squared; d.f., degrees of freedom (model, total); Coeff., parameter coefficient for model effect; Type I SS, Type I / sequential sum of squares; F, F-test statistic; p, probability.

3.5 Discussion

Variation in temperature is ubiquitous in natural populations and influences many features of plant and animal physiology (Schmidt-Nielsen 1997; Atkin and Tjoelker 2003), affecting the distribution of species (Parmesan 1996), and the phenology of key life-history events (Crick & Sparks 1999; Reed *et al.* 2006). In host-parasite systems, thermal variation has been shown to influence host susceptibility to infection (Blanford *et al.* 2003; Mitchell *et al.* 2005; Fels & Kaltz 2006; Raffel *et al.* 2006), and alter the expression of parasite life-history traits (Fels and Kaltz 2006; Laine 2007a; Vale *et al.* 2008b) and microbial virulence factors (Konkel and Tilly 2000). Thus, it is relevant to investigate how thermal variation in the wild might impact the onset, duration and severity of epidemics. Here we studied a natural population of a freshwater crustacean *Daphnia magna* and a sterilizing bacterial pathogen *Pasteuria ramosa*, and tested the effect of temperature on the prevalence of *P. ramosa* infections in the wild.

The epidemic we observed in 2006 is similar to those reported before in the same population (Mitchell *et al.* 2004; Duncan and Little 2007), except that multiple parasite peaks were observed, whereas in previous epidemics, increased prevalence appeared to follow the increase in host density. Given that epidemic onset usually occurs in late spring or early summer, temperature may play a role in driving an increase in infection prevalence. Previous work on the *Daphnia-Pasteuria* host-parasite system has tested how temperature affects different aspects of host and parasite biology using well-replicated, controlled laboratory infections. For example, an earlier study on host genotypes from this population carried out experimental infections using a general parasite inoculum at 10°C, 15°C, 20 °C, and 25°C and found that infections were more virulent at 20°C and 25°C. Indeed, at the lower temperatures parasite growth was very low or absent, resulting in less virulent infections (Mitchell *et al.* 2005). This suggests that *P. ramosa* is only able to infect and grow within a

given range of temperatures (above 10°C), finding its thermal optimum around 20°C. More recent work has explored the responses of distinct *P. ramosa* genotypes under thermal variation and confirmed the same trend of reduced infectivity and spore growth at 15°C (Vale et al. 2008b). This pattern was further dissected by additional laboratory work indicating that temperature does alter the parasite's ability to establish infection slightly, but curtails within-host growth considerably (D. Allen 2008, unpublished data).

Given these previous results, we had clear predictions regarding the effects of temperature on infection prevalence during natural epidemics of *P. ramosa*. Given that symptoms of *P. ramosa* infection only become apparent approximately 15 days after exposure (Ebert et al. 1996), the infected individuals we collected at each sampling date would have been initially exposed at least 15 days prior. We found a positive association between prevalence and minimum temperature experienced 18 days before each sampling date (Tables 3.1 and 3.2; Figure 3.3). This time lag is consistent with the parasite biology we observe in laboratory conditions regarding its post-infection developmental time. Further, a negative association between prevalence and the daily thermal range (DTR) (Tables 3.1 and 3.2) supports the hypothesis that thermal effects on prevalence are driven by constraints on parasite growth: increased prevalence was observed when a combination of high minimum temperature was accompanied by little fluctuation in temperature, increasing the period of time during which *P. ramosa* is able to infect and grow successfully. This is then reflected approximately 18 days later as increased prevalence (Figure 3.3). The host density x temperature interaction we uncovered further suggests that while a significant proportion of variation in prevalence is explained by lagged temperature, this effect is sensitive to host density. We see this effect by how closely prevalence follows lagged temperature before and after the increase in host density in August and September. Given that during this period we observed an increase in

juvenile density, it is possible that we underestimated infection prevalence due to juvenile infections that are hard to diagnose (Figure 3.3).

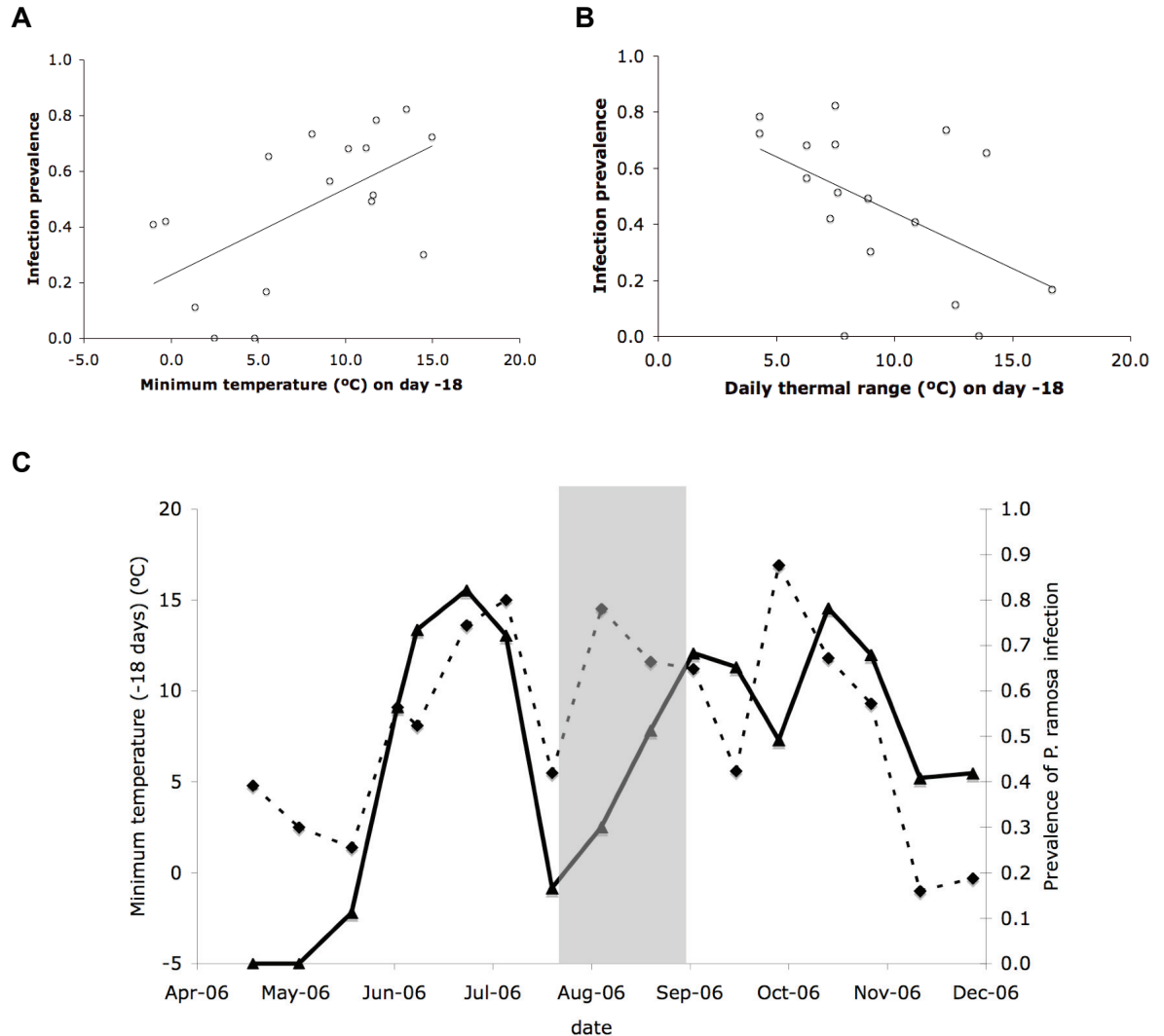


Figure 3.3. Linear bivariate fits of *P. ramosa* infection prevalence and (A) the minimum temperature or (B) the Daily Thermal range – DTR - experienced 18 days prior to each prevalence measurement. C. For a given date the plot shows the *P. ramosa* infection prevalence on that day (full line), and the minimum temperature recorded 18 days prior to that date (dashed line). The grey shading indicates the period when host density increased sharply (see Figure 3.1).

Most studies investigating correlations between climatic variables and disease incidence use time-series modelling approaches that allow forecasting of prevalence based on long-term trends (Allard 1998). These methods have become widely used due to their strong predictive power in disease surveillance (Pelat et al. 2007), but rely heavily on the availability of long-term data, making them inappropriate for the analysis of the data we present. The methodologies are similar in that they test for correlations between explanatory variables and prevalence. However, time-series analyses allow different models to be specified and autocorrelation between variables to be calculated at different time lags, identifying the appropriate time lag in a single analysis (Allard 1998). In this case, our 17 observations fall short of the minimum of fifty, regularly spaced, observations required for time-series.

A strength of our study, however, is that we were able to choose our lag period according to well-founded predictions regarding parasite growth from independent experimental data (Mitchell et al. 2005; Vale et al. 2008b), and our results are consistent with these predictions. In addition, all significant or marginally non-significant correlations were found to coincide with the same period of time lag, on or around the 18 days prior to sampling (Table 3.1). Similar effects of lagged temperature on parasite biology have been reported to drive epidemics in other systems (Kriger and Hero 2007). It would be interesting to test the predictive nature of these results in future epidemics, or compare them with prevalence data from previous years. An epidemic experienced by this population in 2003 (Duncan and Little 2007), showed only a single prevalence peak and so, on its own, offers limited statistical power to test for correlations with weather variables. Gaining a greater understanding of the effect of climatic variability on *P. ramosa* epidemics will require longer surveillance of *D. magna* populations.

In summary, our data illustrate how environmental variables such as temperature that affect parasite biology, can have consequences for the epidemic onset and incidence of infection in natural populations. The combination of laboratory and this field data points strongly to the effects of temperature on parasite growth. This link is important because it provides the empirical support for causation between the environment and disease, lacking in studies that rely solely on correlations. As such, we propose that studying natural epidemics in a model system such as the *Daphnia*-microparasite system can provide a deeper understanding of the link between evolutionary ecology and epidemiology (Galvani 2003; Day and Gandon 2007). Such knowledge is particularly relevant in the context of ongoing climate change (IPCC 2007; Visser 2008) and their possible effects on the ecology and evolution of infectious disease (Epstein 2002; Hudson et al. 2006; Brooks and Hoberg 2007).

CHAPTER 4

TEMPERATURE DEPENDENT COSTS OF PARASITISM AND MAINTENANCE OF POLYMORPHISM UNDER GENOTYPE-BY- ENVIRONMENT INTERACTIONS

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Temperature dependent costs of parasitism and the maintenance of polymorphism under genotype-by-environment interactions *Journal of Evolutionary Biology* 21(5):1418-1427.”

4.1 Summary

The maintenance of genetic variation for infection-related traits is often attributed to coevolution between hosts and parasites, but it can also be maintained by environmental variation if the relative fitness of different genotypes changes with environmental variation. To gain insight into how infection-related traits are sensitive to environmental variation, we exposed a single host genotype of the freshwater crustacean *Daphnia magna* to four parasite isolates (which we assume to represent different genotypes) of its naturally co-occurring parasite *Pasteuria ramosa* at 15°C, 20°C and 25°C. We found that the cost to the host of becoming infected varied with temperature, but the magnitude of this cost did not depend on the parasite isolate. Temperature influenced parasite fitness traits; we found parasite genotype-by-environment (GxE) interactions for parasite transmission stage production, suggesting the potential for temperature variation to maintain genetic variation in this trait. Finally, we tested for temperature-dependent relationships between host and parasite fitness traits that form a key component of models of virulence evolution, and we found them to be stable across temperatures.

Keywords: host-parasite; cost of parasitism; virulence evolution; genetic variation; infectivity; transmission; genotype-by-environment interaction; temperature; *Daphnia magna*; *Pasteuria ramosa*.

4.2 Introduction

Understanding of the mechanisms that maintain genetic variation in natural populations of pathogens is of clear importance for the design of disease control programs because the success of interventions can be undermined by pathogen evolution (Gandon et al. 2001; Galvani 2003; Grenfell et al. 2004; Takala et al. 2007). Frequency dependent host-parasite coevolution can, in theory, maintain substantial genetic variation in infection-related traits (Haldane 1949; Anderson and May 1982; Woolhouse et al. 2002). Where studied, patterns of host and parasite genetic variation have proven compatible with the occurrence of frequency dependent dynamics (Lively 1989; Carius et al. 2001; Wolinska et al. 2006; Decaestecker et al. 2007; Duncan and Little 2007). However, environmental heterogeneity is also potentially a major contributor to the maintenance of genetic polymorphism in fitness traits. When the magnitude of fitness differences between genotypes changes across environments (termed genotype-by-environment (GxE) interaction), this could promote the co-occurrence of different genotypes, particularly if no single genotype outperforms all others across a variable environment (Gillespie and Turelli 1989; Falconer and Mackay 1996; Byers 2005)

Variation in environmental variables like food (Bedhomme et al. 2004) or temperature (Fels and Kaltz 2006) can impact the cost to the host of becoming parasitized. Work in a variety of host-parasite systems have also incorporated parasite or host genetic variation and have found evidence for parasite genotype-by-environment ($G_p \times E$) interactions (Ferguson and Read 2002) or host genotype-by-environment ($G_h \times E$) interactions when environmental variables are manipulated (Blanford et al. 2003; Mitchell and Read 2005; Lambrechts et al. 2006a; Restif and Kaltz 2006). While most of these studies have focused on how these interactions affect host traits such as survival or fecundity, less attention has been drawn to GxE interactions affecting parasite fitness components (Fels and Kaltz 2006; Laine 2007a)

and therefore the effects of these interactions on pathogen genetic variation and evolution are less well understood. In this study, using the freshwater crustacean *Daphnia magna* and its sterilizing bacterial pathogen *Pasteuria ramosa*, we therefore describe how the general cost of parasitism changes with temperature and how individual parasite isolates perform at these temperatures. As *D. magna* tends to live in small, sometimes temporary ponds, thermal variation is likely to be large, and previous studies on the *D. magna*-*P. ramosa* system have shown temperature sensitivity to infection as well as host genotype-by-environment interactions (Mitchell et al. 2005). Here, we performed experimental infections with four parasite isolates (which we consider to be different genotypes) at three temperatures on a single host clone, and measured host traits indicative of fitness costs of becoming infected (mortality and fecundity) and parasite fitness components (infectivity, and the production of transmission stages). We looked for evidence of parasite GxE interactions for these traits that would indicate the potential for the maintenance of genetic variation via environmental variation.

In addition, we tested how temperature affected the relationships between host and parasite fitness components. Testing these relationships was motivated by common models of pathogen evolution which suggest that pathogens will be selected to balance their rate of transmission with their rate of host exploitation, known as the trade-off hypothesis of virulence evolution (Anderson and May 1982; Bremermann and Pickering 1983; Frank 1996; Andre et al. 2003; Day 2004; Jensen et al. 2006). This model assumes particular relationships between host and parasite fitness traits. There is evidence that these relationships might change depending on the genotypes of host and parasite involved (Salvaudon et al. 2005, 2007), but it has not been tested, as far as we are aware, how these relationships might vary with environmental heterogeneity. Clearly, if the expression of these traits changes depending on the specific environmental context, this could confound

predictions about the evolution of virulence. In general, if genetic correlations change between different environments, this could relax the evolutionary constraints imposed by trade-offs (Sgrò and Hoffmann 2004) and contribute to the maintenance of genetic variation in the associated fitness traits (Falconer 1952; Bell and Rebourd 1997; Bell 1997).

4.3 Material and methods

Host and parasite genotypes

Twenty replicates of one host genotype (named GG3) were exposed to four spore types (named Sp1, Sp7, Sp8 and Sp13) at 15°C, 20°C, and 25°C in a fully factorial design. The host genotype and parasite isolates were originally collected from a population near Gaazerfeld, Germany and maintained in the lab in a state of clonal reproduction (host) or frozen (parasite). The parasite isolates (each originally collected from an individual host) have been studied extensively and infections have been shown to differ depending on the combination of host and parasite genotype (Carius et al. 2001). *Daphnia* are filter feeders and become infected with *P. ramosa* by filtering transmission spores present in the water. Infection causes host castration and gigantism, as well as premature death. Within the host, *P. ramosa* goes through a developmental process that culminates in the formation of spores that can be horizontally transmitted when they are released from dead hosts; vertical transmission does not occur (Ebert et al. 1996).

Host acclimation

Before exposing hosts to parasite spores, host maternal lines experienced a period of acclimation to reduce maternal effects, as these have been shown affect infection outcomes in this system (Mitchell and Read 2005). Twenty independent replicates of five GG3

isofemale *Daphnia* were maintained in jars containing 200 ml of artificial medium (Kluttgen et al. 1994), fed 6×10^6 cells per *Daphnia* per day of chemostat grown *Scenedesmus obliquus* algae, and maintained within temperature-controlled incubators with a light:dark cycle of 12:12 hours. Medium was changed with every clutch or every 3 or 4 days regardless of a clutch being present. Although infections were carried out at three different temperatures, all host lines were acclimatised at 20°C in order to synchronise and maximise clutch production. Previous experiments have shown that the temperature of acclimation of the maternal generation does not affect infection outcomes in their offspring (Mitchell et al. 2005). Acclimation lasted at least three generations and all infections were done on second- or third clutch one-day old juvenile females.

Infection and temperature regime

The experiment followed a split-jar design (analogous to a split-brood design), where clutches from an individual replicate jar were split into the different treatments. Each experimental replicate received a single one-day old isofemale, placed in a jar containing 60 ml artificial *Daphnia* medium and sterile sand. Jars with *Daphnia* media were prepared the day before infection and placed in an incubator at the appropriate temperature overnight. This guaranteed that the infection period took place at the desired temperature. Infection was achieved by adding 10,000 spores to each jar. Spore solutions were originally obtained by homogenising infected *Daphnia* in ddH₂O, and these solutions were stored at -20°C until required. *Daphnia* have longer development times at lower temperatures, and Mitchell & Read (2005) have previously shown that the product of temperature and real days (called degree-day) is a reasonable measure of *Daphnia* physiological time. Accordingly, the infection period in all treatments lasted 150 degree-days, i.e., 6 days at 25°C, 7.5 days at 20°C, and 10 days at 15°C (Mitchell et al. 2005; Little et al. 2007). During the infection

period, all replicates were stirred daily and fed low amounts of chemostat grown *Scenedesmus obliquus* algae (1.5×10^6 cells per *Daphnia* per day). All replicates at a particular temperature treatment were grouped within the same incubator, and thus we cannot exclude the possibility that uncontrolled incubator effects have confounded the effects we attribute to temperature. However, we consider it reasonable to assume that "effects of incubators set at a particular temperature" are essentially "temperature effects", and previous experiments that tested for consistency among incubators (Mitchell et al. 2005) support this assumption.

After the infection period, all replicates were transferred to jars with 60 ml clean medium. Food levels were increased and remained in excess of what *Daphnia* can consume daily: (algae cells per *Daphnia* per day) 15°C: 2×10^6 ; 20°C: 3.5×10^6 ; 25°C: 6×10^6 (Mitchell et al. 2005). Females that produced clutches were changed into clean medium, or changed 3 times a week regardless of producing a clutch. The number of offspring produced was counted at every clutch. The experiment lasted 900 degree-days and during this time jars were distributed randomly within trays of 24 jars and the position of the trays was changed regularly to equilibrate any positional effects within the incubators. Hosts that showed signs of infection (no eggs in the brood chamber and red colour) were observed under a dissecting microscope for symptoms consistent with *P. ramosa* infection (sterilization, bacterial growth in the haemolymph). Hosts that died during the experiment were individually frozen in 1.5 ml Eppendorf tubes at -20°C until needed. Counts of *P. ramosa* transmission stages were obtained by homogenizing the dead host with a sterile pestle in 100µl of ddH₂O, and counting two independent samples of this solution in a Neubauer (improved) counting chamber ($0.0025\text{mm}^2 \times 0.100\text{ mm}$ [depth]). The number of transmission spores per *Daphnia* was used as a measure of transmission potential.

Data analysis

All data analysis was done using the statistical software package JMP 7. We focused first on the cost of parasitism to the host in relation to temperature. We determined how the response variables host fecundity (total number of offspring produced) and mortality differed with infection status in hosts exposed to the different parasite genotypes at the three temperatures tested. Square root transformed 'total number of offspring' data were analysed with a generalized linear model assuming normally distributed residuals. Mortality was analysed using proportional hazards and the time scale used was always degree-day to allow comparisons between temperature treatments. Both models were constructed by including "infection status", "temperature" and "parasite genotype" in a full factorial model and then removing the highest order non-significant term in the model until only significant terms were present in the model.

Next we studied how infectivity (the proportion of hosts that became infected) and the number of transmission stage spores produced on the day of death once infection was achieved was affected by temperature, parasite genotype, and the parasite genotype-by-temperature interaction. For infectivity, a binomial distribution of error terms was assumed (Logit function). Parasite transmission was log transformed and a normally distributed error term was used.

Finally, we tested whether the relationships between host and parasite fitness components (production of transmission stage spores, host fecundity and mortality) were modified by temperature. We controlled for the effect of spore genotype by including it as a main effect in a linear model with either the parasite transmission or host fitness components as response variables. We then used the residuals from these models (the total variation not explained by spore genotype) to test if the relationship between parasite and host fitness components

changed with temperature. We used a general linear model with “spore per *Daphnia*” residuals as the response variable, and either the residuals for time to host death (for mortality), or residuals for total number of offspring (for fecundity), and temperature in a full factorial model. The effect of interest for our purposes is a significant interaction between the host fitness component and temperature, suggesting that the amount of variation in the number of spores produced per *Daphnia* can be explained by the host fitness component, but in a temperature-dependent manner.

4.4 Results

A total of 240 female *Daphnia* were individually exposed to infection (4 parasite genotypes x 3 temperature treatments x 20 replicates), of which 26 were lost either due to premature death during the exposure period or due to handling error. Overall, all treatments remained with between 17 and 19 replicates. In all analyses, including replicate as a random variable did not change the outcome, confirming the role of our acclimation period in reducing between replicate variation.

General and parasite-specific costs of parasitism under temperature variation

Daphnia experience sterilization and reduced survival when infected with *P. ramosa* (Ebert et al. 1996). Our experimental design aimed to test whether these costs differed at different temperatures. Fecundity (the total number of offspring produced during the experiment) differed, as expected, between infected and uninfected hosts (Table 4.1; Figure 4.1A). Temperature alone did not have a significant effect on the total number of offspring, but we found a significant interaction between infection status and temperature, showing that being infected will have different costs depending on the temperature at which infections occur (Table 4.1; Figure 4.1A). The significant parasite genotype by temperature interaction

appears to be due to the fact that hosts exposed to Sp1 have particularly low fecundity at 20°C (data not shown), but this is true for both infected and uninfected hosts (hence the three way interaction was not significant). Both a cost of resisting infection and a cost of parasitism could contribute to this pattern. By studying the fecundity of infected and uninfected hosts separately, it is apparent that parasite genotype and temperature have little effect on the fecundity of infected hosts, (Table 4.1, fecundity of infected hosts), but they do effect the fecundity of hosts that remained uninfected following exposure to parasites. The possibility of genotype and temperature-specific costs of resisting infection will be addressed in subsequent experiments (see also Little and Killick 2007).

Mortality analysis also indicated temperature dependent costs of infection. In particular, an infection status by temperature interaction was evident (Table 4.1), being driven by the relatively higher mortality among infected hosts at 25°C (Figure 4.2). However, parasite genotype specific effects on host mortality were not detected (Table 4.1; Figure 4.2).

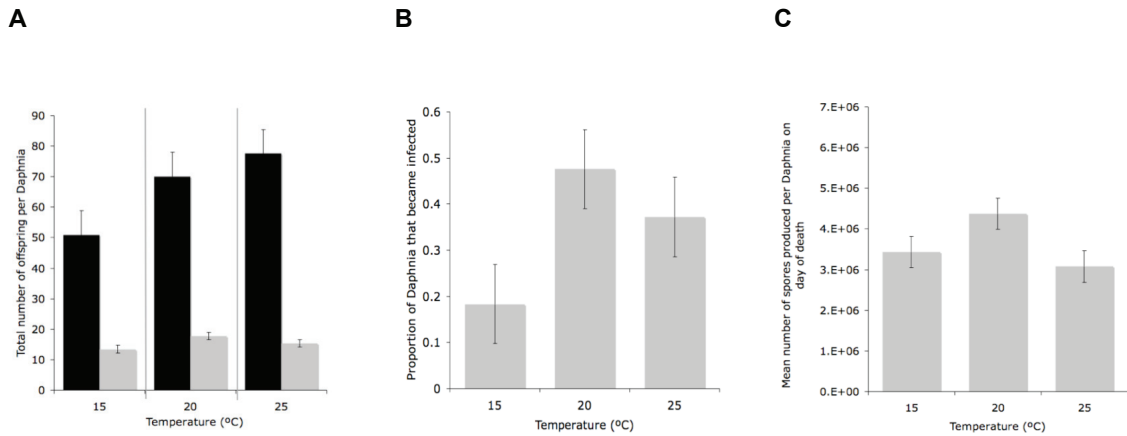


Figure 4.1. The general effects of temperature on infection outcomes. (A) The effect of temperature on the total number of offspring produced. Infected hosts (grey bars); non-infected hosts (black bars). (B) General effects of temperature on infectivity (the proportion of hosts that developed infection) and on (C) the mean number of transmission stage spores produced by each *D. magna* during experimental infection with *P. ramosa* at 15°C, 20°C, and 25°C. See tables 4.1 and 4.2 for statistical details.

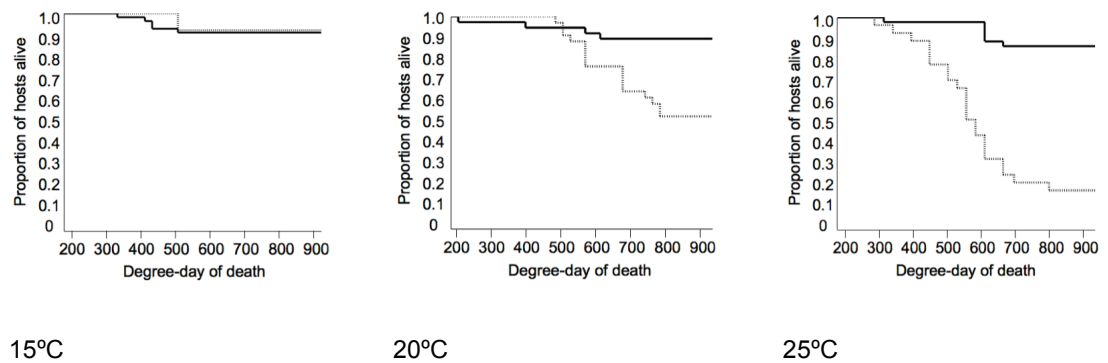


Figure 4.2. Survival curves showing the proportion of *D. magna* exposed to *P. ramosa* during the experiment at 15°C, 20°C, and 25°C. Infected (dotted line); not infected (full line). Timescale is *Daphnia* physiological time (degree-day) to allow comparisons between temperature treatments (see methods for details). See table 4.1 for statistical details.

Fecundity (all data)	DF	L-R χ^2	p
Temperature	2	2.648	0.2660
Infection status	1	171.018	<0.0001
Parasite genotype	3	21.506	<0.0001
Temperature x Infection status	2	15.844	0.0004
Parasite x Temperature	6	18.940	0.0043
Fecundity (infected hosts only)			
Temperature	2	4.678	0.1969
Parasite genotype	3	3.129	0.2091
Parasite x Temperature	6	7.358	0.2890
Fecundity (uninfected hosts only)			
Temperature	2	17.020	0.0007
Parasite genotype	3	39.016	<0.0001
Parasite x Temperature	6	13.280	0.0388
Mortality			
Temperature	2	10.105	0.0064
Infection status	1	4.053	0.0441
Parasite genotype	3	0.341	0.9521
Temperature x Infection status	2	6.511	0.0386
Parasite x Temperature	6	3.390	0.7585

Table 4.1. Summary statistics from generalised linear models testing the cost of parasitism under temperature variation. The effect of temperature, infection status, parasite genotype and their interactions were tested on host fitness traits (fecundity - the total number of offspring - and host mortality). For fecundity, we also present the summary statistics from the separate analysis of hosts that became infected or remained uninfected after exposure. These analyses were identical to the full model, but infection status was not included as an effect. Fecundity analyses were done on square root transformed total number of offspring in a generalised linear model assuming normal residuals. Mortality was analysed using proportional hazards. L-R χ^2 is the likelihood ratio chi-squared test. DF is degrees of freedom.

Parasite genotype and temperature interactions

Variation in infectivity showed significant main effects of both temperature and parasite genotype (Table 4.2). The highest proportion of infected hosts occurred at 20°C (46% on average), followed by 25°C (36%) and 15°C (18%) (Figure 4.1B). Exposure to parasite isolate Sp1 achieved the highest levels of infectivity (60% on average), while isolate Sp8 gave the lowest amount of infection (15% on average) (Figure 4.3A). Differences between parasites were consistent across temperatures, i.e. parasite isolate by temperature interactions were not evident for infectivity (Table 4.2). Among infected hosts, spore production was influenced by temperature main effects (Table 4.2; Figure 4.1C), and showed a parasite by temperature interaction (Table 4.2; Figure 4.3B).

	DF	L-R χ^2	p
Infectivity			
Parasite genotype	3	28.09	<0.0001
Temperature	2	15.98	0.0003
Parasite x Temperature	6	3.13	0.7930
Number of spores per infected <i>Daphnia</i>			
Parasite genotype	3	1.03	0.7950
Temperature	2	12.22	0.0022
Parasite x Temperature	6	16.52	0.0112

Table 4.2. Summary statistics from generalised linear models testing the effects of parasite genotype, temperature, and their interaction on parasite fitness traits (infectivity, and production of transmission spores). L-R χ^2 is the likelihood ratio chi-squared test. DF is degrees of freedom.

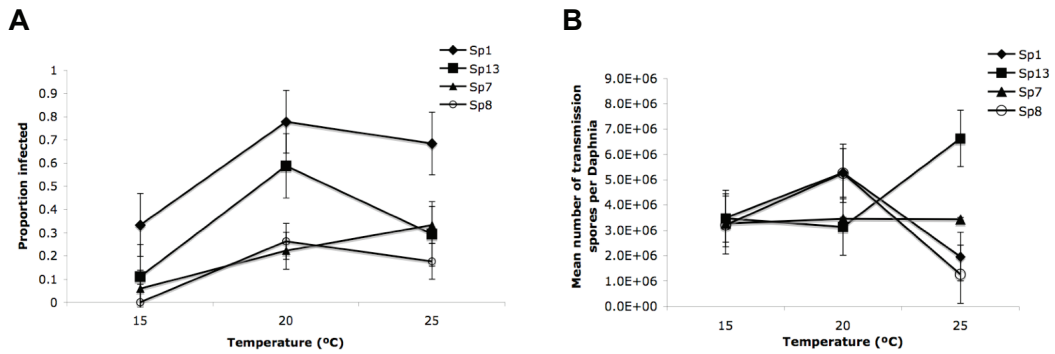


Figure 4.3. Reaction norms for infection-related traits when infected with each spore type. (A) infectivity (the proportion of exposed hosts that developed infection) and (B) the number of transmission spores per infected *Daphnia*. Error bars are standard error of the mean. See table 2 for statistical details.

Does temperature modify relationships between host and parasite fitness traits?

As indicated above, the cost of being parasitized (i.e. virulence) varied with temperature, with higher temperatures leading to greater costs whether measured as mortality or fecundity. Theory on the evolution of virulence assumes particular relationships between parasite transmission and virulence depending on the cost-benefit relationship of host exploitation strategies. Although our experiments were not explicitly designed to study the nature of these relationships, our data can assess if any relationships exist, and if temperature can modify them. We used a general linear model to determine how transmission potential was related to virulence, and if temperature altered that relationship (virulence-by-temperature interaction). No significant relationship between spore production and fecundity was found (Table 4.3; Figure 4.4A), while for host mortality, we found a significant relationship with spore production (Table 4.3; Figure 4.4B). Virulence by temperature interactions were not evident for fecundity or mortality, indicating that the relationship

between virulence and parasite transmission is relatively stable to changes in temperature within the range we tested.

	DF	F ratio	p
Temperature	2	1.172	0.3205
Fecundity(residuals)	1	0.005	0.9424
Temperature x Fecundity(residuals)	2	0.625	0.5407
Temperature	2	0.227	0.7979
Mortality(residuals)	1	13.365	0.0008
Temperature x Mortality(residuals)	2	0.7640	0.4726

Table 4.3. Summary statistics from generalised linear models testing if the relationship between parasite transmission (the number of spores per *Daphnia*) and virulence (measured in terms of host fecundity and mortality) was modified by temperature (virulence-by-temperature interaction). The analysis was performed on residuals after controlling for spore genotype (see data analysis section in methods). DF is degrees of freedom.

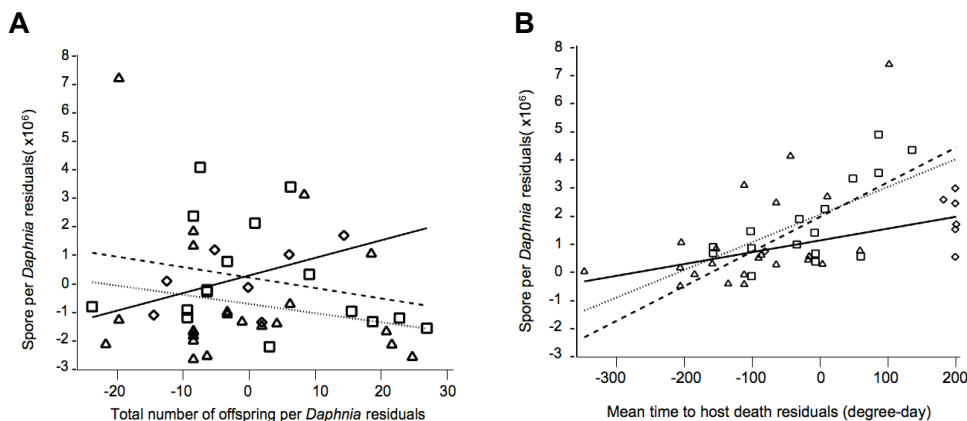


Figure 4.4. Scatterplots of parasite and host life-history traits at 15°C (\diamond - black full line), 20°C (\square - black dashed line), and 25°C (\triangle - dotted line), after correcting for the effect of parasite genotype (residuals shown). (A) Number of spores produced plotted against host fecundity (the total number of offspring), per infected host. (B) Number of spores produced plotted against host mortality (the mean time to host death). Only data for infected hosts that died before the end of the experiment are included. See Table 4.3 for statistical details.

4.5 Discussion

Genetic variation in traits relevant for infection is often attributed to balancing selection resulting from host-parasite coevolution (Haldane 1949; Anderson and May 1982; Woolhouse et al. 2002). However, the maintenance of genetic polymorphism can also occur due to context-dependent selection, as a variable environment can result in changes in the direction and strength of selection on different traits (reviewed in Byers, 2005). Most host-parasite systems experience environmental heterogeneity, and it is therefore highly relevant to enquire about the pervasiveness of genotype-by-environment interactions. The present study showed that the cost of infection to hosts increased with increasing temperature, and that parasite GxE interactions were present for parasite transmission stage production. Other traits (infectivity, host mortality and fecundity) did not show clear evidence of genotype by environment interactions. With reference to the trade-off hypothesis for virulence, we also explored if temperature changed the relationship between host and parasite fitness traits, but our results indicated that, when this relationship existed, it was stable across temperatures.

Thermal optima and the cost of parasitism

Both the proportion of infected hosts and the number of transmission stages produced was higher at 20°C (Figures 4.1B,C). Although we do not know the mechanism by which temperature affects the probability of acquiring infection (whether it affects host resistance directly, induces behavioural changes that affect infectivity indirectly, or reflects a thermal optimum of the parasite), we can speculate on its effects given what we know regarding *Daphnia* physiology and *P. ramosa* infection biology. Transmission of *P. ramosa* occurs horizontally through the release of water-borne spores from dead infected hosts (Ebert et al. 1996), and infection takes place when spores are filtered by *Daphnia* during feeding. If the

likelihood of a host acquiring an infection correlates positively with its filtration rate, we should expect the highest infectivity to occur for maximized filtration rates. Indeed, previous studies on the effect of temperature on *Daphnia* filtration rate have found a maximum close to 20°C (Peters and De Bernardi 1987 and contained references). Additionally, spore production in infected individuals was also highest on average at 20°C (Figure 4.1C), suggesting a possible optimum for parasite growth once successful infection has occurred. However, it is difficult to disentangle this hypothesis from the effect of a larger establishing population due to higher host filtration rate. Estimates of the rate of parasite growth across temperatures would shed light on these issues.

GxE interactions and the maintenance of polymorphism

The number of transmission stages produced per infection is likely to be an important parasite fitness component. Support for this idea comes from numerous studies showing that dose (i.e. the number of parasite spores a host is exposed to) strongly influences the likelihood of achieving a successful infection (Ebert et al. 2000; Little and Ebert 2000; Regoes et al. 2003; Ebert 2004; Little and Killick 2007; Ben-Ami et al. 2008). We found that the production of transmission stages was influenced by temperature, but its effect depended on the parasite genotype involved in the infection, i.e., genotype-by-environment (GxE) interactions were present. For the specific parasite genotypes we tested, there was a switch in the rank order of transmission stage production. For example, infections with isolates Sp1 and Sp8 yielded the most spores at 20°C but produced the least number at 25°C, where parasite isolate Sp13 was most productive (Figure 4.3B). Given that no parasite isolate outperformed all other isolates across all temperatures, this switching can presumably promote the co-occurrence of distinct isolates in environments where temperature fluctuates.

While the occurrence of GxE interactions is generally interpreted as evidence that genetic variation could be maintained due to context-dependent selection (Gillespie and Turelli 1989), direct evidence linking levels of environmental variation and levels genetic diversity are not conclusive (Maynard Smith and Hoekstra 1980; Bell and Rebourd 1997; Byers 2005). Without prior knowledge of the selection history or past levels of genetic variation, the cause-effect relationship is uncertain (Byers 2005). Many of the quantitative models developed to examine the conditions that would favour such a link have also reached mixed conclusions (Levene 1953; Gillespie and Turelli 1989; Sasaki and de Jong 1999), and tend to require strict conditions; specifically, that the contrast in the fitness effects of traits in different environments needs to be considerable in order for genetic variation to be maintained (Maynard Smith and Hoekstra 1980).

Maintenance of polymorphism by environment-dependent selection requires fitness differences between genotypes across environments. The production of transmission stages (a reflection of within-host growth) is an important parasite fitness component as it strongly influences the likelihood of subsequently transmitting to and infecting susceptible hosts. While the GxE interaction we found for this trait across temperatures would suggest that parasite genotypes differ in fitness across temperatures, producing transmission stages is not the only trait relevant for parasite fitness. For example, being able to infect a host is most likely a strong determinant of parasite fitness, as without gaining entry in to the host, no transmission stages are produced. Infectivity is a trait that has been shown in previous work in this system (Carius et al. 2001) to be determined by the specific combinations of host and parasite genotypes (genotype-by-genotype interactions). Therefore, experiments incorporating genetic variation for both host and parasite would provide a more complete picture of how parasite fitness is affected by environmental variation.

Understanding which fitness traits are important and how environmental variation affects them in the wild is necessary to fully comprehend how genetic variation is maintained. Testing for differences in fitness effects in natural populations presents a formidable challenge, as it requires information about genetic variation and how frequencies of specific genotypes change in response to environmental variation. Tracking parasite genotype frequencies and temperature in the wild, as has been achieved for hosts (Little & Ebert 1999, Carvalho 1987), would provide an idea of how parasite fitness varies under thermal variation. This could be complemented with laboratory experiments using the same parasite genotypes under controlled temperature treatments to test their effect on parasite fitness components. However, while there is evidence for *P. ramosa* genetic variation (Carius et al. 2001; Jensen et al. 2006; Little et al. 2008), and some *P. ramosa* genetic markers have been developed (Mouton et al. 2007; Mouton and Ebert 2008) these have not yet revealed substantial *P. ramosa* within-population genetic variation.

The relationship between host and pathogen fitness traits and the evolution of virulence

The evolution of life-history traits depends on the expression of heritable genetic variation (Falconer and Mackay 1996). Several studies of genotype-by-environment interactions have shown that such expression differs with the environment (Falconer 1952; Ebert et al. 1993), with implications for the expression of host and parasite life-history traits (Laine 2007a). However, traits are also correlated with each other, possibly leading to constraints on their evolution imposed by trade-offs, a key component of life-history evolution theory (Stearns 1976; Falconer and Mackay 1996). Such theory is at the basis of the mathematical framework developed to understand virulence evolution that assumes that a) transmission and virulence are coupled, and b) pathogen evolution will proceed by a trade-off between within-host growth (and the consequent fitness cost to the host) and between host

transmission (Anderson and May 1982; Frank 1996). It is possible that the strength and direction of such relationships are environment-specific (Sgrò and Hoffmann 2004). In particular, changing the relationships between host and parasite traits could modify the costs associated with exploiting the host and potentially alter the course of virulence evolution.

We found a positive relationship between parasite transmission (measured as the number of spores produced during an infection) and the survival time of infected individuals, and this relationship was robust to changes in temperature (Table 4.3; Figure 4.4B). Such a relationship is expected, as there is a direct cost to the parasite in killing its host too quickly as it will have less time to produce spores. For obligate killing parasites (where host death is required for transmission to occur), theory suggests that virulence should evolve such that parasites maximize their use of host resources, killing them around the time when host growth decelerates (Ebert and Weisser 1997). Empirical support for such an optimum for virulence has been found in this system, where parasite lifetime transmission was maximal when hosts died between 50-55 days, coinciding with a phase of decelerating growth (Jensen et al. 2006). In comparison to our study, this work was performed at 20°C, hence 50-55 days corresponds to approximately 1000 degree-days. As our experiment only continued until degree-day 900 we are most likely on the left side of the hump-shaped optimum curve, where the relationship between lifetime transmission and host survival is positive.

For another measure of host fitness (fecundity) there was an unexpected lack of relationship with parasite spore production (Table 4.3; Figure 4.4A). Host resources that are not allocated by the host to reproduction are available to the parasite, hence parasites should evolve to castrate hosts as soon as possible to maximize resource availability (Ebert 2004). Therefore, in the absence of any constraint, we should expect a strong negative relationship between parasite fitness and host fecundity as they both compete for the same resources, and this has

indeed been shown in previous *Daphnia-Pasteuria* studies (Ebert *et al.*, 2004). Possibly, in our experiment resources were abundant to the point where parasite growth was maximised while hosts were still able to allocate resources toward reproduction, thereby weakening the expected negative relationship between parasite and host fitness. Although the food levels we used were similar to the ones reported in Ebert *et al.* (2004), it is still possible that food quality differed between the two studies. Although we have no way of testing this possibility, we find no alternative explanation why parasite fitness should show no relationship with host fecundity. Interestingly, the slopes diverge between temperatures and the negative slope for 20°C and 25°C is the expected pattern (Figure 4.4A). Relationships between host and parasite fitness traits have been shown to change depending on the host and parasite genotypes (Salvaudon *et al.* 2005, 2007) but further experimentation is needed to ascertain if these relationships also change with abiotic environmental variables, such as temperature.

In summary, we have presented evidence for temperature dependent costs of parasitism and for the potential maintenance of genetic variation in parasite infection traits through the presence of GxE interactions for spore production. We also explored the possibility that temperature could alter the relationships between host and parasite traits that could direct virulence evolution, but found that when these relationships existed they did not change across temperatures. Although our experimental system certainly does not capture either the full levels of host and parasite genetic variation or the environmental heterogeneity found in the wild, it adds strength to the increasing realization that knowledge of how environmental variation affects infection parameters is essential for our understanding of disease ecology and evolution.

CHAPTER 5

MEASURING PARASITE FITNESS UNDER GENETIC AND THERMAL VARIATION

This chapter has been submitted to *Heredity* as “Vale PF, Little TJ. Measuring parasite fitness under genetic and thermal variation”.

5.1 Summary

Accurate measures of parasite fitness are essential to study host-parasite evolution. Parasite fitness depends on several traits involved in establishing infection, growth and transmission. Individually these traits provide a reasonable approximation of fitness, but they may also be under the shared control of both host and parasite genetics ($G_H \times G_P$ interactions), or be differentially sensitive to environmental variation. Using the natural host-parasite system *Daphnia magna*-*Pasteuria ramosa*, we performed experimental infections that incorporated host and parasite genetic variation at three different temperatures, and compared measures of parasite fitness based only on growth rate, or incorporating the ability to infect. We found that infectivity was most important for parasite fitness, and depended mainly on the combination of host and parasite genotypes. Variation in post-infection parasite growth and killing time depended on the parasite genotype and its interaction with temperature. These results highlight the merits of studies that can incorporate natural infection routes, and emphasize that accurate measures of parasite fitness require knowledge of the genetic control and environmental sensitivity of more than one trait. Additionally, no $G_H \times G_P \times E$ interactions were present, suggesting that the potential for genetic specificities to drive frequency-dependent coevolution in this system is robust to thermal variation.

Keywords: parasite fitness; genotype-by-environment interaction; genotype-by-genotype interaction; temperature; infection; transmission.

5.2 Introduction

Parasite growth has commonly been used as parasite fitness correlate, particularly in empirical work testing evolution of virulence theory (Read and Schrag 1991; Ebert 1998; Jensen et al. 2006). Models of coevolutionary interactions, however, make the important point that the host and parasite genotype will together determine virulence, as well as the probability of becoming infected (Lambrechts et al. 2006b). Empirical work motivated by this coevolutionary perspective has shown that infection outcomes, and ultimately the fitness of both host and parasite, can indeed depend strongly on particular combinations of host and parasite genotypes, called $G_H \times G_P$ interactions (Carius et al. 2001; Lambrechts et al. 2005; Lazzaro et al. 2006).

In addition to the genetic context, infection outcomes may depend on the abiotic environment. Laboratory studies designed to tease apart genetic effects on infection outcomes tend to minimise environmental variation, but such variation is widespread in the wild, and could affect genotypes differently, leading to genotype-by-environment interactions (GxE, reviewed in Lazzaro and Little 2008). For example, genotype-by-food level interactions have been found to have strong effects on infection outcomes in mosquito-*Plasmodium* systems (Ferguson and Read 2002; Lambrechts et al. 2006a), a mosquito-microsporidian system (Bedhomme et al. 2004), and during bacterial infection of a ciliate (Restif and Kaltz 2006). Experimental variation in temperature has uncovered GxE interactions affecting infection outcomes in the pea aphid-fungal system *Acyrtosiphon-Erynia* (Blanford et al. 2003), and the waterflea-bacterial system *Daphnia-Pasteuria* (Mitchell et al. 2005).

These interactions between genotypes or between genotypes and the environment could result in host and parasite genotypes having context-dependent fitness (Lambrechts et al. 2006b; Lazzaro and Little 2008), with implications for the maintenance of genetic variation in the wild. $G_H \times G_P$ interactions, for example, can generate frequency-dependent selection, which prevents the loss of rare alleles and fosters coevolution between host and parasite populations (Hamilton 1993). Alternatively, $G \times E$ interactions can maintain genetic variation when alternate genotypes are favoured in different environments (Byers 2005; Laine and Tellier 2008). $G_H \times G_P \times E$ interactions could favour different combinations of coevolving genotypes in different environments, or generate different rates of coevolution between hosts and parasites in different environments (Thompson 1994; Thompson 1999), further shaping the temporal and spatial distribution of genetic diversity and patterns of local adaptation (Kawecki and Ebert 2004; Laine 2008). In all cases, a correct assessment of whether environmental variability can maintain polymorphism clearly relies on an accurate measurement of fitness. While parasite growth or transmission potential is a commonly used (and reasonable) parasite fitness correlate, a complete assessment of fitness must incorporate all stages of infection, as a parasite must infect, grow, and transmit to new hosts. Each of these traits may be under differential genetic (host or parasite) and environmental control.

Previous studies of the host-parasite system used presently, the crustacean *Daphnia magna* and its specialist bacterium *Pasteuria ramosa*, have found that temperature is an important determinant of infection, and a potential driver of epidemics in wild populations (Mitchell et al. 2005). Experimental studies in this system have found evidence for host genotype \times temperature interactions ($G_H \times E_T$) affecting the probability of infections becoming established (Mitchell et al. 2005), parasite genotype \times temperature interactions ($G_P \times E_T$) affecting parasite growth (Vale et al. 2008b), as well as strong $G_H \times G_P$ interactions for infectivity (Carius et al. 2001). Given that *Daphnia* inhabit temperate ponds where

temperature fluctuates on a seasonal or even daily scale, studying these interactions can inform whether evolutionary or coevolutionary trajectories are sensitive to environmental variation. We now extend the study of interactions with an experiment simultaneously incorporating host genetic variation, parasite genetic variation, and temperature variation. We measured the probability of a host becoming infected, and post-infection host mortality and parasite growth at 15°C, 20°C, and 25°C. In so doing, we examine which infection traits are more sensitive to thermal variation and important in determining parasite fitness. This design also allowed us to assess if previously described genetic specificities (i.e. $G_{HX}G_P$ interactions) can be altered under thermal variation ($G_{HX}G_P \times E_T$ interaction).

5.3 Material and methods

Host and parasite isolates

The experiment used long-term laboratory isolates of hosts and parasites. All host genotypes (GG3, GG4, GG7 and GG13) and parasite isolates (Sp1, Sp7, Sp8, Sp13) were originally collected from a German population near Gaarzerfeld, Germany, in a study showing that infection outcomes depend on the specific combination of host and parasite (Carius et al. 2001). Since this time, host clones have been maintained in the lab in a state of clonal reproduction and parasite isolates have been kept frozen except for occasional experimental use. *Daphnia* are filter feeders and become infected with *P. ramosa* by filtering transmission spores present in the water. Within the host, *P. ramosa* goes through a slow developmental process, culminating in the formation of transmission stage spores, apparent 15-20 days post-infection at 20°C. Transmission is exclusively horizontal, achieved by spores that are released from dead hosts. During the infection process, infected female *D. magna* become sterilized, and ultimately die prematurely (Ebert et al. 1996).

Infections and temperature treatments

Prior to infection, host maternal lines were acclimated for three generations to equilibrate maternal and environmental effects. Twelve replicate jars of each isofemale line (5 *Daphnia* per jar) were contained in 200 ml of artificial medium (Kluttgen et al. 1994), fed 6×10^6 cells per *Daphnia* per day of chemostat grown *Scenedesmus obliquus* algae, and maintained within temperature-controlled incubators with a light:dark cycle of 12:12 hours. Medium was changed with every clutch or every 3 to 4 days regardless of a clutch being present. Although infections were carried out at three different temperatures, all host lines were acclimated at 20°C. Acclimation at the same temperature allows clutch production to be synchronised, reducing among-replicate variation in the time of initial exposure across treatments. Previous experiments have shown that the temperature of acclimation of the maternal generation does not affect susceptibility to infection in their offspring (Mitchell et al. 2005). Subsequently, infections were carried out at 15°C, 20°C and 25°C by splitting replicates among three incubators at these temperatures. In previous experiments where temperature was manipulated, host genotypes showed consistent phenotypes regardless of which incubator they were studied in, indicating that incubators do not vary substantially apart from the temperature at which they are set (Mitchell et al. 2005).

Infections followed a split-jar design (analogous to a split-brood design), where clutches from each replicate jar of each host genotype were split into the different treatments (four parasite genotype exposures at three temperatures). Each experimental replicate consisted of a single, one-day old (< 24h) female, placed in a jar containing 60 ml artificial *Daphnia* medium and sterile sand. These jars were placed in an incubator at the appropriate temperature overnight prior to the beginning of infection to guarantee that the entire

infection period occurred at the desired temperature. All hosts were exposed to 10,000 parasite spores per jar. Spore solutions were originally obtained by homogenising infected *Daphnia*, and these solutions were stored at -20°C until required. Prior to infection, spore solutions were thawed, shaken thoroughly and counted using a Neubauer counting chamber (0.0025mm² x 0.1mm depth). *Daphnia* have longer development times and slower filtration rates at lower temperatures (Peters and De Bernardi 1987) so a measure of *Daphnia* physiological time is useful. Degree-day is a reasonable measure of *Daphnia* physiological time and is simply the product of temperature and real days, with *Daphnia* producing the first clutch after 250-280 degree-days across a range of temperatures (Mitchell et al. 2005). Following previous temperature manipulation studies in this system (Mitchell et al. 2005; Little et al. 2007; Vale et al. 2008b). *Daphnia* were exposed to parasites for 150 degree-days, i.e., 6 days at 25°C, 7.5 days at 20°C, and 10 days at 15°C. During the infection period, all replicates were stirred daily and fed low amounts of chemostat grown *Scenedesmus obliquus* algae (1.5x10⁶ cells per *Daphnia*). Low food increases the *Daphnia* filter feeding rate (Peters and de Bernardi 1987) and stirring increases the likelihood of *Daphnia* encountering transmission spores, both of which increase the chances of infection (Mitchell and Little, unpublished data).

After the infection period, all replicates were transferred to jars with 60 ml clean medium and following the procedure of Mitchell et al. (2005) food levels were increased to 2 x10⁶ at 15°C, 3.5 x10⁶ at 20°C, and 6 x10⁶ at 25°C to be in excess of what the *Daphnia* can consume, but not so high as to induce mortality. From degree-day 300-500 hosts were observed under a dissecting microscope for symptoms consistent with *P. ramosa* infection (sterilization and bacterial growth in the haemolymph). Jars were checked daily for dead hosts and these were removed from the jars as soon as detected, individually placed in 1.5 ml Eppendorf tubes and frozen at -20°C. The experiment lasted 900 degree-days and at this time all remaining

Daphnia were individually frozen. Counts of *P. ramosa* transmission stage spores were obtained by crushing the dead host with a sterile plastic pestle and counting two independent samples of this solution in a Neubauer (improved) counting chamber (0.0025mm² x 0.1mm depth). The number of transmission spores was used as a measure of transmission potential. Offspring production was not recorded, as this experiment focused on parasite fitness traits. Throughout the experiment, jars were distributed randomly within trays of 24 jars and the position of the trays was changed regularly to equilibrate any positional effects within the incubators.

Data analysis

Analyses were performed using the statistical software packages JMP 7 (SAS Institute Inc.) and R (R 2005). We tested fixed effects of host genotype, parasite genotype and temperature on infectivity (the proportion of hosts infected), host mortality (mean time to host death) and parasite transmission potential as estimated by counting transmission spores. For infectivity, we used a generalised linear model with a binomial error structure and a logit function. Host survival (the time to host death) was analysed for infected individuals only, using proportional hazards. The time scale used was always degree-day to allow comparisons among temperature treatments, and all individuals that were alive by the end of the experiment were entered as censored data. Spore counts were square-root transformed to obtain normally distributed data and analysed using ANOVA.

The number of spores produced per infected host was analysed, firstly, by counting the spores produced by an individual infection, irrespective of whether that individual died during the experimental period or survived until the end. Variation in this measure can thus be attributed either to different rates of parasite growth or to differential host survival rates,

but by incorporating both factors this represents the lifetime transmission potential of an infection (see Jensen *et al.* 2006). Secondly, we controlled for variation due to differential survival by analysing the number of spores produced per degree-day a host remained alive (i.e. spores / host / degree-day). This measure represents, roughly, parasite growth rate.

The above analyses of spore counts were restricted to infected individuals. However, we also considered that failure to infect is extremely detrimental for parasite fitness. We therefore studied the lifetime transmission potential of each parasite genotype on all hosts, regardless of infection status (hosts that did not become infected contribute zero spores and thus parasite genotypes produce, on average, a number of spores that is weighted by their infectivity on all hosts). Given that many host-parasite combinations yielded unsuccessful infections, this resulted in a high number of data points being zero. To correct for the resulting overdispersion of the data, we used generalized linear models with both a Poisson and a quasi-Poisson error structure in R (Crawley 2007). However, we found the results from these to be essentially identical to the linear model, and we therefore present only the latter for consistency.

While the significance of each term was determined based on the reduced model, we also report the proportion of variance explained by each of the terms in the full model. For infectivity, the deviance of each term was divided by the total deviance of the binomial model mentioned above. For spore production data, variance proportions were calculated for each term as their sequential sum of squares (SS) divided by the total SS in the model. For survival, to enable straightforward calculation of the effect sizes of each term, we analysed 'day of death' using ANOVA and calculated variance as for spore production. The reduced model in both ANOVA and formal survival analysis (proportional hazards) did not yield different qualitative results.

5.4 Results

We exposed a total of 576 individual *Daphnia* to infection (12 replicates of 4 host genotypes exposed to 4 parasite genotypes at 3 temperatures), of which 38 replicates were lost during the experiment, mainly due to death during the infection period. Due to the small size of hosts and early stage of infection it was not possible to determine why these replicates died, so we removed them from all subsequent analyses. Most of the treatments remained with a total of 12 replicates with the lowest number of replicates being 9 for three of the 25°C treatments.

Across all combinations of host and parasite genotypes, infectivity was highest at 20°C (33%), and lower at 15°C (20%) and at 25°C (16%) (Figure 5.1). The reduced model (Table 5.1) shows that the proportion of hosts that became infected can be explained mostly by main effects of temperature and host genotype. These main effects explained over 25% of the variance. There was a significant interaction between host genotype and parasite genotype explaining nearly 13% of the variance, and also a significant interaction between host genotype and temperature, but this effect explained less than 3% of the variance. (Table 5.1). The three-way interaction was not significant in the full model ($\chi^2_{18}=8.271$, $p=0.974$).

15°C						20°C						25°C					
	GG3	GG4	GG7	GG13	Mean		GG3	GG4	GG7	GG13	Mean		GG3	GG4	GG7	GG13	Mean
Sp1	0.75	0.82	0.00	0.00	0.39	Sp1	1.00	1.00	0.08	0.00	0.52	Sp1	0.70	0.82	0.00	0.00	0.38
Sp13	0.27	0.00	0.00	0.25	0.13	Sp13	0.75	0.00	0.09	0.25	0.27	Sp13	0.09	0.10	0.00	0.00	0.05
Sp7	0.25	0.25	0.00	0.00	0.13	Sp7	0.67	0.17	0.20	0.00	0.26	Sp7	0.42	0.18	0.09	0.00	0.17
Sp8	0.17	0.00	0.00	0.08	0.06	Sp8	0.75	0.08	0.00	0.20	0.26	Sp8	0.00	0.08	0.00	0.00	0.02
Mean	0.36	0.27	0.00	0.08	0.18	Mean	0.79	0.31	0.09	0.11	0.33	Mean	0.30	0.30	0.02	0.00	0.16

Figure 5.1. Infectivity across temperatures. The matrices show the proportion infected for each combination of host (columns) and parasite (rows) genotypes. Numbers are the proportion of 12 individual replicates that became infected. Combinations of host and parasite genotypes with higher infectivity are darker compared to combinations with little or no infectivity. See Table 5.1 for statistical details.

Temperature influenced survival of infected hosts, explaining 23% of the variation in the time to death (Table 5.1; Figure 5.2A). The direction and magnitude of this effect depended on which parasite genotype was involved in the infection. This parasite genotype by temperature interaction explained 8% of the variation for the time to host death (Table 5.1; Figure 5.2B). In some cases, we observed a complete switch in the rank order of parasite genotypes between temperatures (Figure 5.2B). Notably, none of the hosts infected with parasite genotype Sp8 died during the experiment at 20°C, but this parasite genotype is the most virulent at 25°C, killing all hosts it infected by degree-day 600 (24 days).

Infectivity	Source	DF	L-R χ^2	p	% Variance full model
	Host	3	104.9	<0.0001	20.69
	Parasite	3	2.147	0.5425	10.79
	Temperature	2	28.21	<0.0001	4.91
	Host x Parasite	9	62.28	<0.0001	12.58
	Host x Temperature	6	13.56	0.0350	2.82
	Parasite x Temperature	6	8.68	0.1924	1.61
	Host x Temperature x Parasite	-	-	NS	1.54
	Error	490			45.06
	Total	537			100
Survival among infected hosts					
	Host	3	2.20	0.5328	3.87
	Parasite	3	4.93	0.1772	4.61
	Temperature	2	13.69	0.0011	23.14
	Host x Parasite	-	-	NS	5.15
	Host x Temperature	-	-	NS	2.13
	Parasite x Temperature	6	14.30	0.0265	8.10
	Host x Parasite x Temperature	-	-	NS	0.93
	Error	93			52.07
	Total	120			100

Table 5.1. Summary of statistical analyses testing the effects of temperature, host and parasite genotypes on infectivity and host survival. Infectivity was modeled using a generalized linear model (GLM), assuming a binomial error structure. Survival was analysed with proportional hazards analysis on infected individuals only. We present the test statistics for all the terms in the reduced model. % Variance explained by each term refers to the full model. DF are degrees of freedom for the reduced model. L-R χ^2 is the likelihood ratio Chi-square. Terms are significant for $p > 0.05$. NS, not significant. See methods section for statistical details.

The lifetime transmission potential of parasites could be explained by temperature, and also by an interaction between parasite genotype and temperature (nearly 30% of the variance, combined; Table 5.2). Host genotype had no effect on lifetime transmission potential, suggesting that within-host growth is only controlled by the parasite genotype and is conditioned by the temperature it experiences (Figure 5.3A). Removing host genotype as a main effect did not improve the overall model fit. After controlling for differential survival (by studying spore/ *Daphnia*/ degree-day, which is a measure of parasite growth rate) we found that the parasite x temperature interaction was no longer significant, and variation in spore production could be explained to a large extent by significant main effects of temperature (39% variance explained) and parasite genotype (3.2% variance explained, Table 5.2). Incorporating information on the infectivity of each parasite genotype also dissipated the parasite genotype by temperature interaction (Table 5.2; Figure 5.3B).

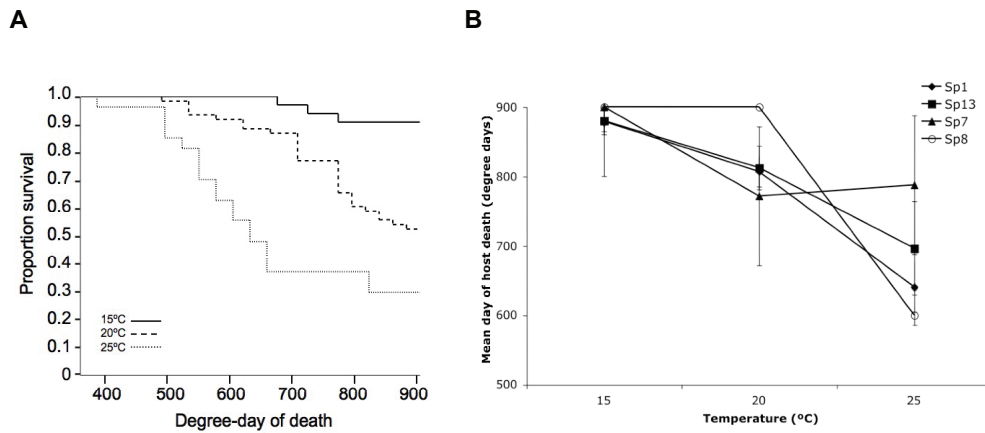


Figure 5.2. Host survival under thermal variation. (A) Survival curves for infected hosts at 15°C (full black line), 20°C (dashed line) and 25°C (dotted line). (B) Reaction norms for the mean time to host death in infected hosts. Each data point is the mean trait value for each parasite genotype. Error bars are standard error of the mean. See Table 5.1 for statistical details.

Lifetime transmission potential	Reduced model			Full model	
	DF	F Ratio	F	DF	%var
Source					
Host	3	0.8825	0.453	3	1.61
Parasite	3	1.9871	0.120	3	3.62
Temperature	2	18.1799	<0.0001	2	22.10
Host x Parasite	-	-	NS	6	2.42
Host x Temperature	-	-	NS	4	1.23
Parasite x Temperature	6	2.258	0.043	6	7.75
Host x Parasite x Temperature	-	-	NS	3	1.93
Model				27	40.66
Error				93	59.34
Total				120	100
Parasite growth rate					
Host	3	1.0747	0.363	3	1.60
Parasite	3	2.1579	0.097	3	3.20
Temperature	2	39.3594	<0.0001	2	38.95
Host x Parasite	-	-	NS	6	1.44
Host x Temperature	-	-	NS	4	0.88
Parasite x Temperature	6	1.2771	0.274	6	3.53
Host x Parasite x Temperature	-	-	NS	3	1.06
Model				27	50.66
Error				93	49.34
Total				120	100
Spores on all hosts (inf. + n.inf)					
Host	3	53.211	<0.0001	3	15.63
Parasite	3	30.3754	<0.0001	3	8.92
Temperature	2	30.1396	<0.0001	2	5.90
Host x Parasite	9	15.9416	<0.0001	9	14.05
Host x Temperature	6	9.0014	<0.0001	6	5.29
Parasite x Temperature	-	-	NS	6	0.44
Host x Parasite x Temperature	-	-	NS	18	2.36
Model				47	52.60
Error				490	47.40
Total				537	100

Table 5.2. Summary of statistical analyses testing the effects of temperature, host and parasite genotypes on different measures of parasite growth. We present the test statistics for all the terms in the reduced model. % Variance explained by each term refers to the full model. DF are degrees of freedom for the reduced model. F is the F-ratio test statistic. Terms are significant for $p > 0.05$; NS, not significant. See methods section for statistical details.

5.5 Discussion

A growing body of empirical work, mostly on plant and invertebrate host-pathogen systems, has shown that infection is dependent on the host and parasite genotypes involved ($G_{HX}G_P$) (Thompson and Burdon 1992; Carius et al. 2001; Lambrechts et al. 2006b; Salvaudon et al. 2007), and on the environmental context (E) in which host and parasite genotypes interact ($G_{HX}E$, $G_{PX}E$ and $G_{HX}G_{PX}E$, (Mitchell et al. 2005; Laine 2007a; Lazzaro and Little 2008). Here we tested whether simultaneous variation in temperature (E_T), and host and parasite genetic background, could modify infection outcomes when the crustacean *Daphnia magna* is exposed to the bacterium *Pasteuria ramosa*. Our experiment included four genotypes of the host and four genotypes of the parasite that were previously established to show substantial differences in infection-related traits as well as genetic specificity for infectivity ($G_{HX}G_P$ interactions, (Carius et al. 2001). This genetic specificity was confirmed, but we also uncovered a set of genotype-by-environment ($G \times E$) interactions. A host genotype-by-environment interaction ($G_{HX}E_T$) was present for the probability of becoming infected, but not for traits that were measured later in the infection process. By contrast, parasite genotype-by-environment interactions ($G_{PX}E_T$) were not evident for the probability of becoming infected, but were important for traits (transmission potential and the mortality of infected hosts) that were relevant once infections were established. Three-way, $G_{HX}G_{PX}E$, interactions were not evident.

Infectivity

The $G_{HX}E_T$ for infectivity is not attributable to changes in the relative rank order of host and parasite genotypes across temperatures, but is instead associated with changes in the magnitude of differences in infectivity between temperature treatments for different host genotypes (Figure 5.1). While both scenarios will influence the strength of selection, the

long-term maintenance of polymorphism via GxE is only possible when the contrast in fitness effects is such that genotypes switch their rank order across environmental gradients (Maynard Smith and Hoekstra 1980). Moreover, in our experiment, the total variation in infectivity due to variation in temperature or $G_{H \times E_T}$ was small (less than 5% or 3% respectively), which indicates infectivity is a trait robust to thermal variation, as was recently reported in a plant-pathogen system (Laine 2007b, 2008). In a previous study of thermal variation on infection rates in a different population of the *Daphnia-Pasteuria* system, crossing reaction norms for infectivity were detected across temperature treatments (Mitchell et al. 2005). The strength of $G_{H \times E_T}$ could differ between populations, but additionally, the earlier study incorporated more host genotypes (16 host genotypes across 2 experiments) than the present one, which might increase the power to detect variation explained by $G_{H \times E}$ effects. With respect to the mechanisms that might underlie $G_{H \times E_T}$, infection occurs by ingestion of spores during filtration feeding, and thus $G_{H \times E_T}$ could reflect genotype-specific differences in filtration rate at different temperatures (Hall et al. 2007).

Host mortality

Host mortality varied considerably across temperature treatments, and the direction and magnitude of this change depended on the parasite genotype ($G_{P \times E_T}$, Table 5.1, Figure 5.2B). $G_{P \times E_T}$ for mortality of infected hosts will affect the parasite directly, as transmission in this system is exclusively achieved through the release of transmission spores at host death (Ebert et al. 1996). For example, at 20°C, parasite genotype Sp8 did not kill any of the hosts it infected during 900 degree-days (45 days) (Figure 5.2B) and produced the least number of spores compared to the other genotypes tested. However, at 25°C this situation was reversed; not only did this parasite genotype kill all the hosts it infected, it did so faster than any other parasite genotype (Figure 5.3A). Results from both demographic and

epidemiological models show that selection favours early over late births, so in an epidemic there can be a benefit to early transmission and short generation time even if it lowers the pathogen's lifetime reproduction (Bull and Ebert 2008). This highlights how more virulent strains might be afforded an evolutionary advantage under some environmental conditions, in this case, increased temperature. Indeed, given that *P. ramosa* epidemics tend to coincide with increases in temperature in the wild (Mitchell *et al.* 2004; Duncan & Little 2007), such a harmful parasite genotype would be the most likely to gain prevalence if an increase in mean temperature were to occur. However as we discuss below, an accurate assessment of parasite fitness must take into account additional fitness correlates.

Parasite growth and measures of parasite fitness

The number of transmission stages produced is an important parasite fitness component. Once infection was achieved we found that the genotype of the host had no effect on how infections progressed. Rather, our results show that parasite spore production was determined by temperature, and the magnitude and direction of this effect depended on the parasite genotype ($G_{P \times E_T}$, Table 5.2, Figure 5.3A). This genotype-by-temperature interaction follows previous reports on the effect of temperature on parasite growth and transmission potential in this (Vale *et al.* 2008b) and other systems (Blanford *et al.* 2003; Laine 2007a). We attempted to dissect the causes of variation in the number of spores produced by controlling for differential survival among hosts and thus estimating parasite growth rate. We did not find a $G_{P \times E_T}$ for parasite growth rate (Table 5.2), suggesting that variation in survival substantially contributed to the $G_{P \times E_T}$ for lifetime transmission potential.

The $G_{P \times E_T}$ interaction we observed for lifetime transmission potential indicates the potential for environment-dependent selection to maintain polymorphism in the parasite population

(Byers 2005; Laine and Tellier 2008). However, producing transmission stages also depends on infecting the host, and our analysis of infectivity revealed a somewhat different set of interactions (in particular $G_H \times E_T$) than did our analysis of transmission stage production (where was $G_P \times E_T$ more important). Therefore, we were interested in determining if the $G_P \times E_T$ we observed for differences in transmission stage spores remained when information about transmission stage production was combined with infectivity. To achieve this, we considered the average spore production of each parasite genotype on all hosts, regardless of their infection status. This allows for a more complete measure of parasite fitness that incorporates both its probability of infecting a host and growth following infection: hosts that did not become infected contribute zero spores. The $G_P \times E_T$ that was significant for lifetime transmission potential among infected hosts (Table 5.2; Figure 5.3A) was no longer evident once this information about parasite infectivity was incorporated into parasite fitness (Table 5.2; Figure 5.3B). Notably, parasite genotype Sp1 is by far the genotype with highest (estimated) fitness at all temperatures (Figure 5.3B), even though it was not the genotype producing the highest number of spores (Figure 5.3A) during infections. This is a reflection of it having the highest mean infectivity across all host genotypes (Figure 5.1) and highlights the importance of infectivity for parasite fitness. These data suggest that variation in the number of transmission stages produced per infection between different parasite genotypes is not large enough to overcome the large differences in their ability to infect. The ability to infect is the strongest determinant of parasite fitness.

This result highlights the importance of considering variation that arises through the natural route of infection. When the natural route of infection is not possible, some studies artificially inject parasites directly into their hosts and measure parasite growth rate only (this is common, for example, in *Drosophila* and in vertebrate models). In the present study, ignoring variation in infectivity would have given a very different picture of parasite fitness. Another study measuring parasite local adaptation has also emphasized how using different measures of parasite fitness can lead to different conclusions about trajectories of parasite evolution (Laine 2008). In some sense, by simultaneously taking infectivity and spore production into account, our measure of parasite fitness is closer to the fitness measures obtained in bacteria-phage systems, where measuring phage population growth rate takes into account both the ability to infect and growth within the host cell (Abedon 2008).

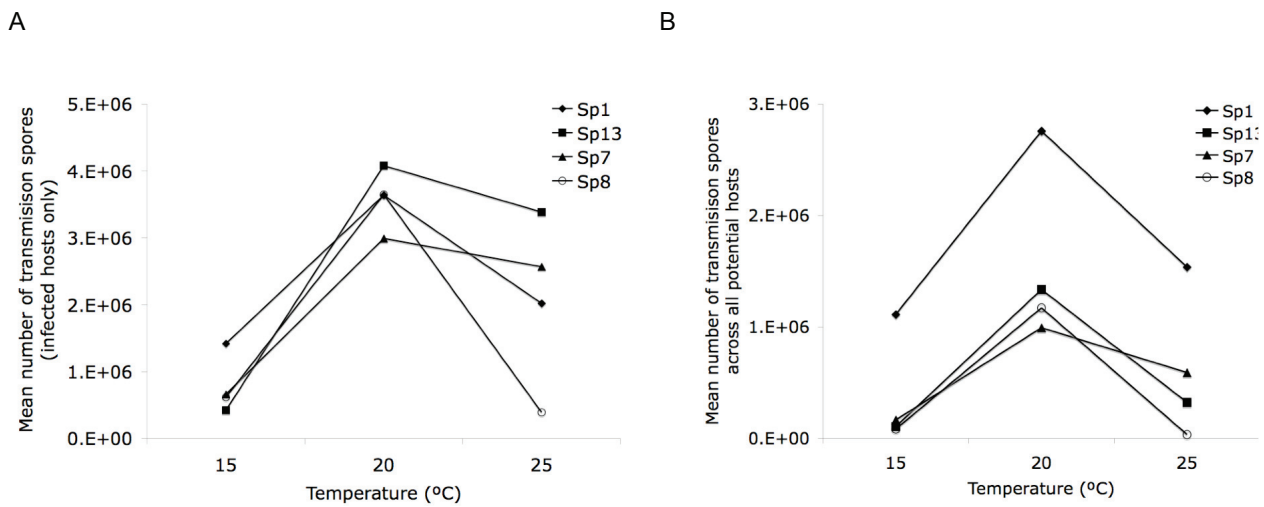


Figure 5.3. Measures of parasite fitness considering spore production among infected hosts only or incorporating the ability of parasite genotypes to infect. (A) The average number of spores produced during the experiment across all infected hosts is shown for three temperatures (parasite lifetime transmission potential). (B) The number of transmission spores produced across all hosts, regardless of infection status incorporates information on the infective ability of each parasite genotype. Where infection was not successful, spore production was zero, and hence this measure of parasite fitness provides a measure of the productivity of infection for each parasite genotype, where the number of spores is weighted by the relative infectivity of each parasite genotype across all host genotypes. Error bars are omitted for clarity. See Table 5.1 for statistical details.

This does not necessarily mean that the observed $G_{p \times E_T}$ for spore production is inconsequential, however. Previous studies have shown that the number of *P. ramosa* spores a host is exposed to (i.e. dose) strongly influences the likelihood of achieving a successful infection (Ebert et al. 2000; Ben-Ami et al. 2008). Thus, the detection of parasite $G_{p \times E_T}$ interactions for both transmission stage production and time to host death (the timing of transmission in this system), could essentially change the “dose” of infection, raising the

possibility that subsequent cycles of infection will result in different patterns of infectivity. Testing this hypothesis experimentally, however, may not be straightforward. For example, Ben-Ami and colleagues (Ben-Ami et al. 2008) recently tested the relationship between parasite dose and infectivity for 14 host-parasite combinations (seven host genotypes and two parasite genotypes) in a constant environment. Five of these combinations showed a density dependent relationship between parasite dose and infection levels, and for these combinations the relationship was found to fit well with a model that assumed the existence of non-inherited phenotypic differences in host susceptibility (phenotypic plasticity). For this reason, predictions about how $G_p \times E$ interactions for transmission spore production will affect infectivity levels in subsequent infection cycles occurring in a variable environment are not intuitively obvious, and will depend on the particular combinations of host and parasite genotypes involved, and on the extent to which hosts are plastic in their responses to infection.

How relevant are $G \times G \times E$ interactions?

One outcome of this study was that a significant $G_H \times G_P \times E_T$ was not detected for any of the traits we measured. Studies on other systems have found strong $G_H \times G_P \times E$ interactions (Tetard-Jones et al. 2007), while other workers (Heath and Tiffin 2007; Laine 2007a), found results similar to the present one: considerable $G \times E$ interactions for individual traits, but only weak or insignificant three-way interactions. Thus the general importance of $G \times G \times E$ remains uncertain; they may only be relevant for particular systems.

One clearly important source of environmental variation concerns differences between the field and laboratory. Work on a snail-schistosome system (Theron et al. 2008), for example, found that patterns of host-parasite compatibility ($G_H \times G_P$) depended substantially on whether

individuals were recently field-collected or were longer-term laboratory cultures. In a recent investigation of parasite local adaptation (Laine 2007b), two experiments found discordant results when measuring parasite fitness either in a field-transplant experiment or in a laboratory cross-infection experiment. Such patterns have led some authors to propose that both laboratory-based and field-transplant experiments should be the norm for local adaptation studies (Nuismer and Gandon 2008), though this certainly will not always be possible. Despite these concerns, the lack of a significant three-way interaction in our experiments has an important implication. $G_H \times G_P$ indicate the potential for frequency dependent coevolutionary interactions, and our results suggest that the signal of coevolutionary dynamics in the wild should shine through the noise of thermal variation.

CHAPTER 6

CONTEXT-DEPENDENT VIRULENCE- TRANSMISSION RELATIONSHIPS AND THE EVOLUTION OF VIRULENCE

6.1 Summary

The evolution of virulence (parasite induced harm) is of central importance to the success of some disease control programs. Knowledge about how this harm might evolve can be gained from studies of how antagonistic host and parasite traits coevolve. Models consider that virulence evolution is constrained by a trade-off that parasites face between within-host growth and between-host transmission. A central assumption is that parasite growth is positively correlated with virulence, and generally, the form of the relationship will critically shape the longer-term evolution of virulence. It has rarely been tested, however, if the nature of the parasite growth and virulence relationship could be context dependent, specifically, whether environmental variation might markedly change the form of the relationship. Yet this seems plausible, given recent work showing pervasive genotype-by-environment interactions in host-parasite systems. We tested the expected positive relationship between growth and virulence under environmental variation. We exposed 8 genotypes of the freshwater crustacean *Daphnia magna* to its bacterial parasite *Pasteuria ramosa* at 15°C, 20°C, and 25°C under limiting or abundant food, and measured host fecundity, mortality and time to castration, and parasite lifetime transmission potential. We demonstrate that under these conditions, the strength and even the direction of parasite growth - virulence relationship can change direction. These results illustrate that the environment-dependent nature of parasitism may limit predictions about the evolutionary outcomes of virulence based on models that assume constant environments.

Keywords – host-parasite coevolution, evolution of virulence, virulence-transmission relationship, environmental variation, trade-off, temperature, food, *Daphnia magna*, *Pasteuria ramosa*.

6.2 Introduction

Virulence can be defined as the harm or reduction in fitness incurred by a host during infection (e.g. mortality, anaemia, a loss in fecundity). Virulence emerges as an unavoidable consequence of parasitism, as parasites exploit their hosts as a resource. How much harm this exploitation should cause has been a long-standing focus of evolutionary biology (Haldane 1949; Read 1994). Intuitively, while faster growing parasites will initially have increased fitness, growing too quickly will be counterproductive if it causes premature host death, resulting in reduced parasite transmission success. In agreement with these ideas, models predict that virulence evolution is governed by a trade-off between within-host growth (and the consequent fitness cost to the host that this growth entails) and between-host transmission, maximising parasite fitness at intermediate virulence (Anderson and May 1982; Frank 1996; Jensen et al. 2006). The main assumption of these models is that parasite growth and transmission are positively correlated with virulence. This assumption has led some to propose that virulence evolution can be curtailed by intervention practices that select for slow parasite growth (Williams and Nesse 1991; Ewald 1994). The validity of this expectation may be questionable in the absence of clear empirical support for the expected relationship between parasite growth and virulence relationship (Ebert and Bull 2003). Recent evidence suggests that disease control measures targeting parasite growth might select for faster growing, more virulent variants if control measures are not completely effective (Gandon and Day 2008; Mackinnon et al. 2008; Read and Mackinnon 2008). Clearly, the success of such interventions on virulence evolution relies on a greater understanding of how correlated host and parasite traits are, and the conditions in which the assumed relationships are found (Elliot 2003; Gandon and Day 2003).

The assumption of a positive correlation between growth rate, transmission and virulence is supported by empirical evidence in some host-parasite systems (Ebert and Weisser 1997; Lipsitch and Levin 1997; Mackinnon and Read 1999; Thrall and Burdon 2003; Mackinnon and Read 2004; de Roode et al. 2008). However, some have failed to find the expected positive correlation (Weiss 2002; Salvaudon et al. 2007) and others have even found evidence consistent with negative correlations between transmission and virulence (Sacristan et al. 2005; Little et al. 2008). All of this work has focused on host-parasite systems in controlled laboratory settings, but it is becoming increasingly clear that infection outcomes are environment-dependent (reviewed in Lazzaro and Little 2008). In recent years, work from several host-parasite systems has demonstrated that the expression of host and parasite traits depends not only on the genotypes that are tested (Carius et al. 2001; Mackinnon et al. 2002; Lambrechts et al. 2006b), but also on genotype-specific responses to different environments (GxE interactions) (Ferguson and Read 2002; Mitchell et al. 2005; Lambrechts et al. 2006a; Laine 2007a; Vale et al. 2008b). Additionally, most virulence studies focus on host fitness correlates (anaemia, reduction in weight, or fecundity) as a measure of virulence. These may be a reasonable approximation of parasite-induced harm, but may not represent fitness; our understanding of virulence evolution will be enhanced if the relationship between parasite transmission and host reductions in fitness can be studied (Day 2002; Ebert and Bull 2003, 2008).

Environmental variation could potentially alter trajectories of phenotypic evolution (Falconer 1952; Falconer and Latyszewski 1952). Indeed, the form and strength of the relationship between correlated traits is often not constant but can be modulated by the environment, as shown by numerous examples of environment-dependent trade-offs (Sandland and Minchella 2003; Sgrò and Hoffmann 2004; Mckean et al. 2008). The general implication for phenotypic evolution is that, as genetic correlations change between

environments, this could relax the constraints imposed by trade-offs, changing the predicted outcome of evolution in constant environments. In the context of host-parasite systems, environment-dependent relationships between host and parasite fitness could vary the strength of parasite-mediated selection and change the evolutionary outcome of virulence. There is evidence that these relationships might change depending on the genotypes of host and parasite involved (Mackinnon and Read 2003; Salvaudon et al. 2005, 2007), but how these relationships vary with heterogeneity in the abiotic environment has not received much attention.

Here we test if the main assumption in models of virulence evolution (a positive relationship between parasite transmission and virulence) holds under different environmental conditions. We exposed eight genotypes of *D. magna* to *P. ramosa* at 15°C, 20°C, and 25°C at either limiting or abundant food and measured the correlation between parasite transmission and three proxies of host fitness trait that relate to virulence: fecundity under infection, the time to castration, and the time to host death.

6.3 Material and Methods

Study system

Daphnia magna (Crustacea: Cladocera) is a cyclically parthenogenetic, planktonic crustacean that inhabits freshwater ponds. Each host genotype used was originally propagated from a single individual and maintained in a state of clonal reproduction since they were isolated. *Pasteuria ramosa* is an obligate bacterial parasite of *D. magna* (Ebert et al. 1996). Infection occurs when long-living transmission spores in the water column or pond sediment are ingested during filter feeding. Successful infection initiates spore germination, followed by a period of within-host growth, which culminates in the formation of

transmission spores. Infection causes host sterilization and premature host death (females may be completely castrated or may sometimes produce one or two clutches before they are irreversibly castrated). Symptoms of *P. ramosa* infection are visible by naked eye 10-20 days post-infection (empty-brood chamber, red colour, and apparent bacterial growth in the haemolymph). Transmission is exclusively horizontal and is only achieved by the release of transmission spores from cadavers of infected hosts (Ebert 2005). This experiment used host and parasite isolates from two populations. Hosts GG3, GG4, GG7, and GG13 were collected from a population in Gaarzerfeld, Germany (GG) and have been previously characterized (Carius et al. 2001). Host genotypes KA5, KA24, KA47, and KA51 were collected from the Kaimes (KA) population near Leitholm, Scottish Borders in 2007.

Temperature and food variation

Hosts were exposed to sympatric parasite spores under high or low food treatments, at three temperatures, in a fully-factorial design. All infected hosts were followed until their death in order to gain precise measures of parasite lifetime transmission. For each host genotype, twelve replicate 'maternal line' jars (5 female *Daphnia* per jar) were maintained for two generations under constant environmental conditions (20°C, 12:12 light:dark, regular feeding and water changes) to equilibrate environmental effects. Experimental infections were performed on 3rd generation maternal line female *Daphnia*, taken from the second or third clutch of the second-generation maternal line. The experiment followed a split-jar design, where clutches from each replicate maternal line jar were split into the different treatments: three temperature treatments (15°C, 20°C, and 25°C) and two food regimes that consisted of daily feeding with chemostat-grown *Chlorella vulgaris* microalgae (High food - $Abs_{\lambda=665}=1.5$, approximately 1.5-2 ml per jar; Low food $Abs_{\lambda=665}=0.3$, approximately 0.3-0.5 ml per jar). Female *Daphnia* were placed individually in jars containing sterile sand and 60

ml of artificial *Daphnia* medium. These jars were placed in trays of 24 jars each and split between incubators at the temperatures described above.

Infections were carried out on 5-day old female *Daphnia*, by adding a fixed number of spores to each jar. For each population, a separate spore solution was prepared by mixing several “single strain” spore solutions in equal proportions. The GG spore solution was made by combining four “single strain” solutions in equal volumes (see parasite solutions in Vale *et al.* 2008b). The KA spore solution was also made from four parasite genotypes, each isolated from the KA host genotypes used in the present experiment (KA5, KA24, KA47, and KA51). Each “single strain” solution was originally obtained by crushing a single infected host and propagating the resulting parasite spores on hosts of the same genotype. These were then maintained frozen at -20°C. Hosts from the GG population received 30,000 spores per jar, while hosts from the KA population required 150,000 spores per jar. Based on our previous experience studying these populations, these spore doses would achieve comparable infection levels in the two sets of hosts (see doses in Carius *et al.* 2001 and Mitchell *et al.* 2005). Absolute exposure time differed with the temperature treatment. *Daphnia* show an increased metabolic rate with increasing temperature, and as infection occurs during filtration feeding, it is important to account for these differences during infection by exposing hosts for the same period of physiological time. Previous studies have found degree-days (the product of real days and temperature) to be a reasonable measure of *Daphnia* physiological time, with females producing their first clutch between 250-285 degree-days across a range of temperatures (Mitchell *et al.* 2005). In the current experiment, hosts in all treatments were exposed to parasite spores for 100 degree-days. During this period all jars were stirred daily and fed either high or low food according to their allocated treatment.

After the exposure period, hosts were transferred to 60 ml jars containing clean medium and no parasite spores or sand. Individuals were monitored daily for infection and mortality. Infections became apparent after approximately 500 degree-days in all temperature treatments. Medium was refreshed with every clutch, or every 4 days if no clutches were produced (in the case of infected individuals). Offspring production was recorded for all individuals until degree-day 700 (or until reproduction ceased due to infection). After 700 degree-days, we considered that individuals that continued to reproduce and showed no other symptom of infection to be uninfected. As the focus of the experiment was on infected individuals, replicates judged to be uninfected were removed from the experiment. Feeding occurred daily according to the high or low food treatment described above. The positions of the jars within trays, and of trays within incubators were changed with every feeding to minimize positional effects. Infected individuals that died were individually frozen in 1.5 ml Eppendorf tubes at -20°C. We counted the number of spores produced on the day of death of each host by adding 500µl of ddH₂O to each Eppendorf tube and homogenising each *Daphnia* using a motorised plastic pestle. Spores were quantified using a Cell Counter (Casy, Model TT). 100µl of the homogenate was added to 10 ml of Casyton isoton solution (10⁻² dilution), and this solution was read in the cell counter with the appropriate dilution factor. Previously, we calibrated the cell counter by comparing serial diluted samples of microscope counts using a haemocytometer, and cell counter counts of all particles within the size range of *P. ramosa* transmission spores (2.9 – 6.5 µm); we found this method to be repeatable and accurate.

Data analysis

Data analysis was performed using the statistical software package JMP 7 (SAS Institute Inc.). As response variables, we measured three host traits related to or influenced by

virulence: (1) the number of offspring produced by infected hosts (2) the time to host castration and (3) the time to host death. To test whether food levels or temperature modified the relationship between transmission potential (as measured via counts of transmission spores) and virulence, we used ANCOVA, with temperature (15°C, 20°C, 25°), food (low, high) and host genotype as categorical factors, and the number of parasite transmission spores as a continuous covariate. For each host trait analysed, environmental effects on the relationship with virulence are detected by a significant interaction between spore production and temperature or food. The number of offspring produced in infected hosts was Log transformed for normality. Time to host castration and time to host death were analysed using proportional hazards. All analyses started with a fully factorial model containing all explanatory variables; model reduction was carried out based on the likelihood ratio chi-squared statistic (Sokal and Rohlf 1995).

It is important to note that one of the assumptions of ANCOVA is that the covariate is measured without error (Sokal and Rohlf 1995; Quinn and Keough 2002). In this case (and for biological examples in general) the covariate is a quantity measured during the experiment and will have some associated error. While this will result in an underestimation of the true regression slope (Quinn and Keough 2002), measurement error should be uncorrelated with the response (and the true value of the covariate), and should underestimate the regression slopes equally across treatment groups. This should still allow for a meaningful interpretation of the interactions between the covariate (number of spores) and the environmental treatments (food, temperature), which is our main aim. ANCOVA also requires homogeneity of the regression slopes between groups in order to compare their means. Because we are explicitly testing if the slopes of these regressions change between groups (if spore x food or spore x temperature interactions are present), we cannot interpret the main effects in the linear model when interactions are present (i.e. when slopes differ).

Again, because our aim is simply to test for interactions between treatments, violating this condition is not problematic for our interpretation (Quinn and Keough 2002).

Cross-environment genetic correlations

In addition to the overall effect of the food and temperature treatments, we were also interested in the individual correlation for each treatment combination. For all relationships we mention above, we performed linear regression on the means of each genotype for each combination of food and temperature treatments. This is essentially a genetic correlation between host and parasite traits that we can compare across environmental treatments. This method allows us to test some specific predictions about transmission-virulence relationships. For example, given that *P. ramosa* only transmits once the host is killed (Ebert et al. 1996), our measurement of parasite growth (spores produced at host death) is an estimate of lifetime transmission potential (LTP), which is close to parasite fitness. However, LTP can be attributed either to different rates of parasite growth or to differential host survival rates. Previous work has described a hump-shaped relationship between parasite LTP and time to host death (Jensen et al. 2006), but this was observed with allopatric host parasite combinations, said to bring out hidden additive genetic variance. In our case, given that we used sympatric host-parasite combinations, it is less likely that we would observe such a hump, because parasites that produce less transmission spores than average and also take longer to kill and transmit will have been selected against. It is more likely that we observe a positive relationship between LTP and the time to host death. For fecundity, we do not expect a hump, but rather a negative relationship between host offspring production and parasite LTP (Ebert et al. 2004). Based on these predictions, we can test whether the environmental treatments we imposed modify these relationships.

6.4 Results

We considered the correlation between parasite transmission (measured by the production of transmission spores) and three host traits directly relevant to measures of virulence: offspring production, time to castration and mortality. A change in the slopes of these correlations with different environmental treatments would indicate that the relationship between transmission and virulence can be modified by that environmental variable. This is shown in our ANCOVA by a significant interaction between spore production and food or temperature, indicating that the relationship between the response variable (virulence) and spore production is modified by the environmental treatment. The relationship between spore production and all three traits were modified by food (Food x spore production interaction, Table 6.1). The relationships between spore production and host castration and mortality were also modified by temperature (Temperature x spore production interaction, Table 6.1).

Under the assumption of a positive relationship between transmission and virulence, increased parasite growth should result in higher parasite fitness and lower host fitness. As such, an equivalent way of considering these relationships is analysing the expected negative relationship between host and parasite fitness. This distinction makes it easier to interpret the relationships shown in Figure 6.1, where we have plotted proxies of host fitness which presumably have a negative relationship with virulence (more offspring, longer time to castration and mortality) and parasite fitness (lifetime transmission potential) on the x-axis. As such, we see that the expected negative correlation between host and parasite fitness is not always found. Significant negative slopes were only found in the low food treatment for offspring production at 25°C, and castration and mortality at 15°C (Table 6.2). In the high food treatment, almost all the correlations showed positive slopes, significant at 15°C for

offspring production and at 25°C for mortality. The remainder of the temperature food combinations showed a non-significant correlation between virulence (or host fitness) and parasite growth. Given the expectation of a relationship between the two, these non-significant correlations are informative and suggest that the harm induced during parasite growth is reduced under some environmental treatments.

Host offspring production	df	χ^2	P
Host genotype	7	18.83	0.0087
Temperature	2	57.89	<0.0001
Food	1	114.83	<0.0001
Spore production	1	2.45	0.1176
Food*spore production	1	5.80	0.0160
Time to host castration			
Host genotype	7	34.57	<0.0001
Temperature	2	67.39	<0.0001
Food	1	10.01	0.0016
Spore production	1	1.58	0.2090
Temperature*spore production	2	8.53	0.0141
Food*spore production	1	5.09	0.0240
Temperature*food	2	4.89	0.0866
Time to host mortality			
Host genotype	7	51.38	<0.0001
Temperature	2	143.59	<0.0001
Food	1	5.40	0.0202
Spore production	1	8.75	0.0031
Host genotype*temperature	14	28.32	0.0129
Temperature*food	2	13.46	0.0012
Temperature*spore production	2	10.68	0.0048
Food*spore production	1	4.22	0.0399

Table 6.1. Environmental effects on the relationship between parasite growth and virulence.

Summarised statistical analyses testing the effect of host genotype, food and temperature on the relationship between host traits indicative of virulence (fecundity relative to uninfected hosts, time to castration, and mortality) and parasite growth / transmission (spore production on the day of host death). For each host trait analysed, environmental effects on the relationship with virulence are detected by a significant interaction between spore production and temperature or food (the relationship between the host and parasite trait is modified by the environmental variable). (See Figure 6.1) Df, degrees of freedom; χ^2 , likelihood ratio chi-squared test. See *data analysis* section for statistical details.

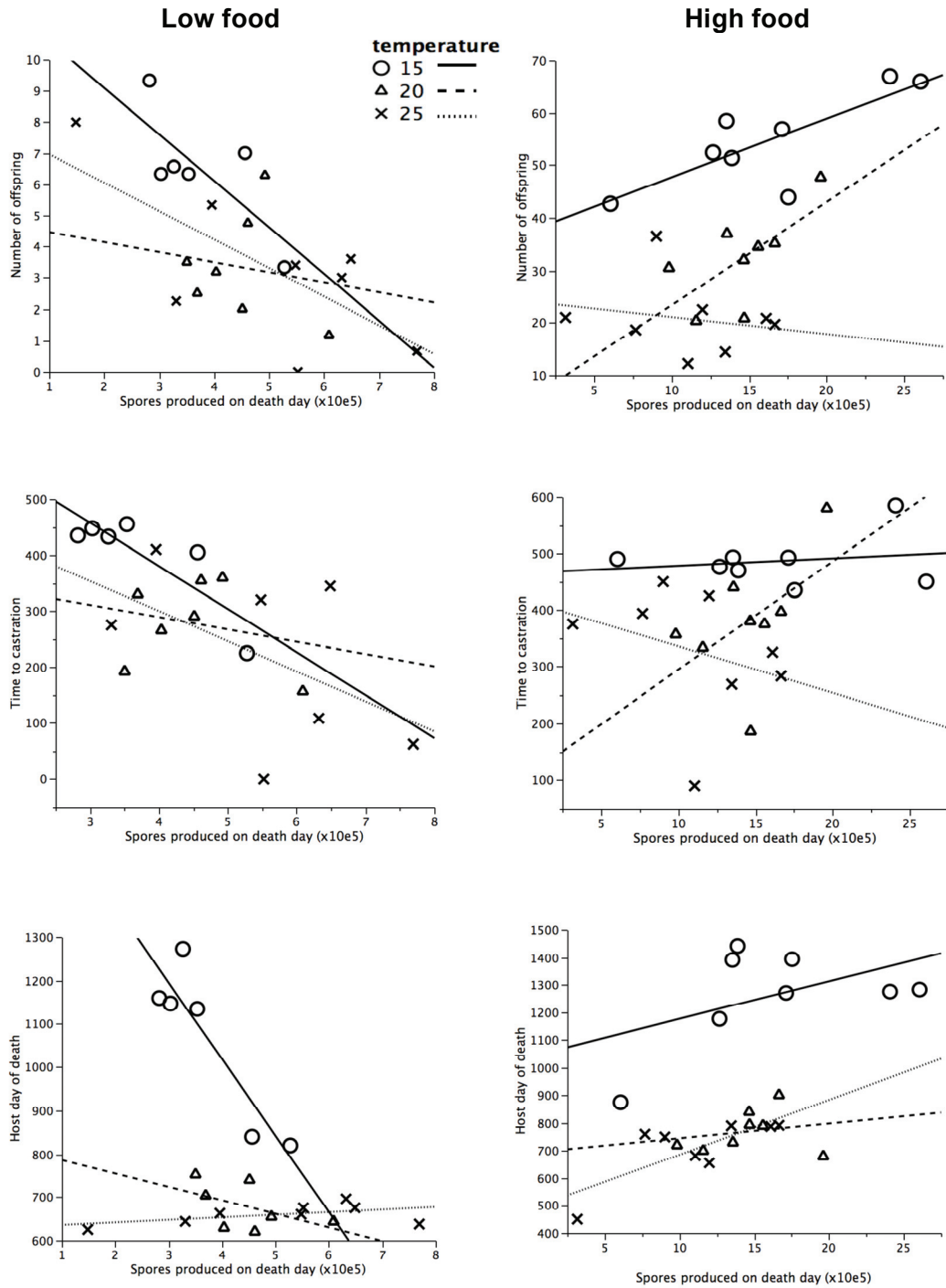


Figure 6.1. Genetic correlations between virulence and parasite growth. Each data point is the mean of each host genotype (2-11 replicates) infected under low (left panel) or high (right panel) food treatments. 15°C- Circles, full line; triangles, dashed line – 20°C; crosses, dotted line – 25°C). Host day of death and time to castration are shown in degree-days. See Tables 6.1 and 6.2 for details.

	Low food		High food	
Host offspring production	Adj. R²	Coeff. (sig.)	Adj. R²	Coeff. (sig.)
15°C	0.46	-1.49 (x)	0.57	1.12 (*)
20°C	0	-0.32 (ns)	0.35	1.96 (x)
25°C	0.43	-0.91 (*)	0	-0.33 (ns)
Host castration				
15°C	0.65	-76.96 (*)	0	1.30 (ns)
20°C	0	-21.86 (ns)	0.16	19.12 (ns)
25°C	0.11	-53.81 (ns)	0	-8.19 (ns)
Host mortality				
15°C	0.76	-176.41 (**)	0.12	13.67 (ns)
20°C	0.11	-31.24 (ns)	0	5.27 (ns)
25°C	0.14	5.89 (ns)	0.52	19.78 (*)

Table 6.2. Goodness of fit and regression coefficients from a linear regression model fit to the means of each genotype for all food and temperature combinations, for the relationship between parasite growth (spore production) and virulence-related traits (see Figure 5.1). Adj. R², adjusted R squared (negative values are reported as zero); Coeff. (sig.) regression coefficient (significance); ns – $p > 0.10$; x – $p < 0.10$; * – $p < 0.05$; ** – $p < 0.01$.

6.5 Discussion

The evolution of virulence can be understood in terms of natural selection on parasite growth rate: fast growing parasite genotypes may be favoured over more prudent ones as long as they do not kill their hosts before achieving transmission (Anderson and May 1982; Frank 1996). A central assumption underlying this work is that parasite growth relates positively to virulence (Read and Schrag 1991; Ebert 1998; Jensen et al. 2006). One likely cause of this is resource competition (resources are limited and are allocated either towards host growth and reproduction or towards parasite growth and transmission), and this has been demonstrated in the *P. ramosa*-*D. magna* system, where parasite induced sterilisation appears to redirect resources to the benefit of the parasite (Ebert et al. 2000, 2004). We have

now shown that under realistic ranges of temperature and food, the strength and direction of the relationship between parasite growth and virulence can substantially depend on environmental conditions (Figure 5.1).

Notable among these changes was how the relationship between parasite growth and virulence can be modified by food availability (Table 6.1, Figure 5.1). While greater parasite growth generally limited host survival under low food, when resources were abundant, parasite growth and host mortality showed a positive relationship (Table 2, Figure 5.1). Presumably, under high food, resource competition was not occurring between host and parasite, and when hosts were doing well (living longer), parasites benefited as well. From a parasite perspective, well-fed hosts are good resources, and the longer a host remains alive the greater the number of transmission spores the parasite will produce (Ebert et al. 2004). For host fecundity, we observed, again, that under limiting resources, increased parasite growth detracted from host fitness, but that under abundant resources it is possible for both host and parasite to simultaneously prosper. This possibility depends on temperature; the correlations between parasite growth and time to host castration and mortality were also modified by temperature, shown by the significant temperature x spore production interaction (Table 6.1). At 20°C, host and parasite fitness showed a negative correlation, but at 15°C host and parasite fitness showed a positive relationship, in particular for time to castration.

Notably, host genotype did not alter the nature of any of the relationships we studied (no host genotype x spore production interactions). This contrasts with other work from a plant-pathogen system showing that the relationship between host and parasite fitness can be modified by the host and parasite genotypes (Salvaudon et al. 2005, 2007). It is possible that there are fundamental biological differences in the way host and parasite fitness traits are

expressed in both systems, making their relationships more or less malleable to environmental perturbation. It is also plausible that these genotype-specific effects are more easily detected when pure parasite strains are studied. Our infection protocol used a general mix of parasite genotypes, so it is not possible to know precisely which parasite genotype infected which host genotype. No other studies that we are aware of have studied these relationships under genetic and environmental variation, so more data are needed before a general picture can emerge.

Implications of environment-dependent selection on host-parasite coevolution

Environment-dependent relationships between parasite growth and virulence could have important consequences for the evolutionary outcome of parasitism and virulence. The cost of parasitism, inferred directly from the negative relationship between host and parasite fitness, could be very high in some environments (steep negative slope), but greatly reduced in others. The strength of the parasitic relationship relates directly to the rate of coevolution (Thompson 1999), and the potential for antagonistic coevolution to maintain polymorphism (Hamilton 1980, 1982; Duncan et al. 2006). We should emphasize, however, that positive correlations (Table 6.2, Figure 5.1) do not equate with mutualism because our analysis only included hosts that developed infection. Even in these cases there is always a cost of being infected relative to uninfected hosts (Mitchell et al. 2005; Vale et al. 2008b).

That host and parasite traits are in greater conflict under low food (i.e. resource competition is acting) is not unlike trade-offs between life-history traits of single species, which are more readily detected under resource-limited environments. For example very resistant host genotypes may show reduced fecundity or competitive ability, but only under low food treatments (Sandland and Minchella 2003; Sgrò and Hoffmann 2004; Mckean et al. 2008). In

this case, the ‘trade-off’ we study is different in that it is between host and parasite fitness traits, though it is also brought about by competition for limited resources. Our results are also consistent with predictions about coevolution over productivity gradients, where different rates of coevolution in predator-prey systems are predicted to be higher when the productivity of the prey is higher (Hochberg and van Baalen 1998). Recent empirical data from a bacteria-phage system confirm these predictions (D. C. Lopez-Pascua and Buckling 2008).

Such context-dependent rates of coevolution are predicted by the ‘geographic mosaic’ theory of coevolution (Thompson 1994), which predicts the existence of either “cold spots” or “hot spots” of coevolution (where antagonistic selection is weak or strong, respectively) over a variable geographic range (Thompson 1999). One study has demonstrated that a plant-insect interaction, studied in two populations, either remained mutualistic over time or varied between antagonism, commensalism, and mutualism due to differences in the ecological contexts (Thompson and Fernandez 2006). We highlight that this may also be the case over temporally variable environments (Nuismer et al. 2003). Experimental data show that rates of coevolution between a bacterium and its phage parasitoid vary over space and time when migrating populations differ across productivity gradients (Forde et al. 2004). Given natural levels of variation in food availability and temperature in *Daphnia* populations (Steiner 2004), our results indicate that similar changes in the nature of the interaction between this host and parasite may occur.

In summary, we have shown that the positive relationship between parasite transmission and virulence (and the resulting negative relationship between host and parasite fitness) that is central to models of virulence evolution is not constant and may be contingent on abiotic

environmental factors, such as fluctuating food and temperature. These results suggest that expectations about virulence evolution based on assumptions of these relationships in constant environment may not hold. More data on the nature of such correlations in different environmental treatments is needed in order to incorporate this information into models of virulence evolution. Broadly, if we consider that in natural habitats fluctuation in temperature or food availability is common, our understanding of host-parasite interactions, their coevolution, and the levels of virulence that result from that evolution must incorporate knowledge of how environmental variation impacts the evolution of host and parasite traits.

CHAPTER 7

TESTING FOR TEMPERATURE-DEPENDENT COSTS OF RESISTING INFECTION IN THE CRUSTACEAN *DAPHNIA MAGNA*

7.1 Summary

Parasites are a ubiquitous cause of host morbidity and mortality, and ongoing coevolution with their hosts has resulted in the evolution of a variety of host mechanisms that permit resistance to parasites. These mechanisms can be physiologically costly however, if they result in lower investment in other host life-history traits such as reproduction or lead to reduced survival. Laboratory studies of infection tend to focus on the genetic determinants of host resistance, keeping environmental variation is to a minimum, but environmental variation is common in the wild. In this study, we test whether the cost of mounting an immune response is temperature-dependent. First we measured parasite growth for two isolates of the castrating bacterial parasite *Pasteuria ramosa* at 15°C, 20°C, and 25°C. We then exposed the crustacean *Daphnia magna* to these isolates with a single dose, a double dose, or not at all at 15°C, 20°C, and 25°C and measured early fecundity and long-term survival. We found strong effects of temperature on overall fecundity and survival, but no clear evidence for a cost of resisting infection for any of the traits measured. Overall, these results suggest that costs of resisting infection are either small or non-existent across a range of realistic thermal environments.

Key-words: cost of resistance, environment-dependent costs, temperature, *Daphnia magna*, *Pasteuria ramosa*, environmental immunology.

7.2 Introduction

Parasites are ubiquitous in nature, and due to the morbidity and mortality they cause their hosts, impose on them a potentially strong selective force (Haldane 1949; Anderson 1971; Woolhouse et al. 2002). During coevolution with their parasites, hosts have evolved a wide range of resistance mechanisms that actively reduce the detrimental effects of infection. These range from parasite avoidance behaviours (Hart 1992; de Valdez 2007; Hutchings et al. 2007), to non-specific innate immune responses (Ausubel 2005), and immune mechanisms that exhibit specificity and memory (Mitchison 1990; Kurtz 2005). Despite their clear benefit in the presence of parasites, launching these immune mechanisms can be costly if resources that would otherwise be allocated to growth or reproduction are instead used to fight off infection (Stearns 1976; Sheldon and Verhulst 1996). Physiological costs of launching an immune response can arise if the energetic requirements of immune responses or their immunopathological effects result in a loss in performance (Moret and Schmid-Hempel 2000; Graham et al. 2005; Sadd and Siva-Jothy 2006; Colditz 2008). These costs can be assessed by comparing the reduction in performance (growth, survival, or fecundity) in groups of hosts that are not exposed to parasites, with hosts that are exposed but do not become infected due to fighting off infection. Evidence for such costs of resistance has been found in both invertebrate (Moret and Schmid-Hempel 2000; Sadd and Siva-Jothy 2006; Little and Killick 2007; Mckean et al. 2008) and vertebrate hosts (Bonneaud et al. 2003; Colditz 2008).

Most laboratory studies of infection minimise environmental variation in order to better reveal the genetic basis of resistance. However, recent studies have found pervasive effects of environmental variation (such as variation in nutrition or temperature) on the expression of host and parasite traits (Ferguson and Read 2002; Bedhomme et al. 2004; Mitchell et al.

2005; Lambrechts et al. 2006a; Restif and Kaltz 2006; Laine 2007a; Vale et al. 2008b).

Given this context-dependent expression of traits relevant to infection, it is likely that the physiological cost of mounting an immune response could also vary with the environment in which infection occurs (Sandland and Minchella 2003). One recent study measured the physiological costs of launching an immune response in *Drosophila melanogaster* under different nutritional environments, and found that costs of resisting infection were only present (or were augmented) in food-limited environments (McKean et al. 2008). Given that food levels are likely to vary considerably in the natural environment, these context-dependent costs could result in changes in the strength and direction of selection on resistance if they vary between genotypes, offering a possible explanation for the maintenance of variation in genes associated with resisting infection (Sandland and Minchella 2003).

Here we test if the physiological cost of resisting infection varies with temperature when *Daphnia magna* is exposed to *Pasteuria ramosa*, a sterilizing, bacterial pathogen. *Daphnia* spp. are cyclically parthenogenetic planktonic crustaceans found in most temperate freshwater ponds and lakes. As such, their habitats experience considerable seasonal and daily fluctuations in temperature (Chapter 3). These fluctuations in temperature have important consequences for the timing and duration of epidemics. For example, the prevalence of *P. ramosa* has been reported to increase during the summer months, reaching 100% in some years (Mitchell et al. 2004; Duncan and Little 2007). Conversely, this parasite shows limited infectivity and growth below 15°C (Mitchell et al. 2005; Vale et al. 2008b). To test for temperature dependent costs of resisting infection, we conducted two experiments. In a preliminary experiment we measured parasite growth at 15°C, 20°C, and 25°C, as costs of resisting infection are likely to be augmented when hosts are faced with faster growing parasites. In the main experiment, we exposed individual *Daphnia* either once

or twice to *P. ramosa* at 15°C, 20°C, and 25°C and measured early fecundity and long-term survival, relative to unexposed control individuals.

7.3 Materials and Methods

Host and parasite isolates

Daphnia spp. are cyclically parthenogenetic crustaceans found in most temperate freshwater ponds and lakes. The clone used in these experiments (GG4) was originally collected from a pond population near Gaarzerfeld in Northern Germany in 1997 (Carius et al. 2001).

Pasteuria ramosa is a naturally occurring bacterial parasite. Infection occurs when transmission stage spores are taken up by *Daphnia* during filtration feeding. Infection causes castration (infected hosts sometimes release one or two clutches) and gigantism, eventually resulting in premature host death. Transmission is exclusively horizontal and occurs at host death when spores are released from the infected cadavers (Ebert et al. 1996). The parasite isolates used in these experiments (Sp1 and Sp7, called genotypes 1 and 7 in Carius et al. 2001) were originally isolated from individual hosts and have since been maintained as frozen solutions.

Experiment 1: Measuring parasite growth

Before assessing the cost of resistance, we conducted an experiment designed to measure the within-host growth dynamics of the two parasite genotypes used (Sp1 and Sp7). One host genotype (GG4) was exposed to these parasite genotypes at 15°C, 20°C, and 25°C in a full factorial design. While previous work has shown that these genotypes differ in their infectivity on this host clone (Carius et al. 2001), within-host growth has not been measured directly. Quantification of within-host growth necessarily requires successful infection, so

hosts received 200,000 spores per *Daphnia*, more than ten times the usual dose necessary for successful infection in these host-parasite genotype combinations (Carius et al. 2001). It is not possible to sample parasites from the same host during an infection, so host replicates exposed to *P. ramosa* were sacrificed at several time-points post-infection. Forty-eight replicates for each of the 6 treatments (3 temperatures x 2 parasite genotypes, total 288 jars) were exposed to the parasite and maintained in identical conditions during the infection and observation periods. The infection period lasted 200 degree-days (the product of the real day and temperature, (Mitchell et al. 2005)), during which all replicate *Daphnia* were maintained in jars with 60 ml of artificial pond medium, lined with sterile sand and stirred daily to maximise infection. After 200 degree-days, 3 replicates were sacrificed for each of the 6 treatments every 50 (25°C treatment) or 60 (15°C and 20°C treatments) degree-days. This lasted until degree-day 800 (total of 9-10 sampling dates) usually enough time for infections to develop and in some cases even for host death to occur. When sacrificed, *Daphnia* were placed individually in 1.5 ml Eppendorf tubes and kept at -20°C until spores were counted. We obtained counts of *P. ramosa* transmission spores by crushing the dead host with a sterile pestle and counting three independent samples of this solution in a Neubauer (improved) counting chamber.

Experiment 2: Immune challenge and temperature treatments

Hosts were exposed to each spore isolate either once (single exposure) or twice (double exposure), or to a control solution containing either deionised water or a macerated healthy daphnia, at 15°C, 20°C, or 25°C (Figure 7.1). Prior to exposure, 30 replicate “maternal line” jars (five *Daphnia* per jar) were maintained for three generations in a climate control chamber at 20°C, 12:12 hour light:dark cycle, fed equal amounts of chemostat grown *Chlorella* sp. microalgae, and were changed into clean medium regularly to equilibrate any

maternal effects on the hosts used in the infection. For the infection, the second clutch from the third-generation of each maternal line replicate jar was split into the different treatments. Each infection replicate (Sp1 or Sp7, control, single, or double exposure, 15°C, 20°C, or 25°C), received a single female (< 24 hours old) placed in a jar containing 60 ml of artificial *Daphnia* medium and a teaspoon of sterile sand. All infection jars were prepared in advance and kept in climate control chambers at the desired temperature overnight. There were 30 replicates per treatment, except for control treatments, which had 24 replicates per temperature, giving a total of 432 experimental jars. All infection replicates received 2500 *P. ramosa* spores on the first day and double exposure treatments received an additional 2500 spores on degree-day 120 (Figure 7.1). The spore solutions were obtained by homogenising infected *Daphnia* in deionised water. Hence, control replicates received the equivalent volume of either a solution of crushed healthy *Daphnia* or deionised water (12 replicates each, per temperature). This allowed us to be sure that any effect on host life-histories was due to a response to the spores and not simply a general response to crushed *Daphnia*, as might be expected as part of a predator avoidance response (Pijanowska and Kowalczewski 1997; Laforsch et al. 2006). The infection period lasted 200-degree days, during which time all replicates (including controls) were stirred daily and fed low amounts of food ($Abs_{\lambda=665}=0.3$). The combination of low food and sand results in hosts grazing on the bottom of the jar, increasing the chance of encountering spores and reducing variation in infection levels.

After the infection period, all replicate *Daphnia* were transferred to jars containing 60 ml artificial *Daphnia* medium, food levels were increased and they were maintained at the desired temperature (15°C, 20°C, or 25°C) in climate control chambers (12:12 light:dark cycle). The number of offspring from each clutch was recorded until degree-day 700, and the medium changed with every clutch (or every 3 days if host reproduction ceased due to

infection). Infection was assessed between degree-day 400-500. By this time infected individuals were red in colour, larger and had ceased reproducing. Hosts that died were collected, dried, and individually frozen at -20°C in Eppendorf tubes. Host mortality was recorded until degree-day 1200, and the day of death was used to assess differences in survival between treatments. During the experiment, all replicate jars were randomly assigned to trays (24 jars per tray) and the position of jars within trays and of trays within incubators was changed regularly to equilibrate possible positional effects.

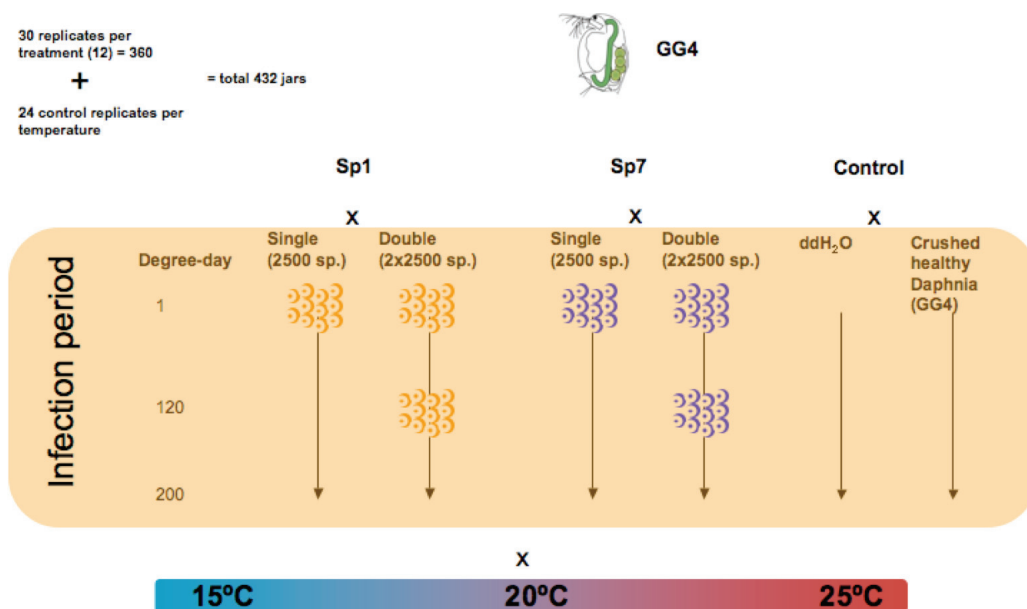


Figure 7.1. Experimental design for immune challenge experiment. Up to thirty individual replicate host *Daphnia* (clone GG4) were split among the 12 infection dose and temperature treatments (two parasite genotypes, single or double exposure, at 15°C , 20°C and 25°C). The host was exposed to either parasite isolates Sp1, Sp7 or two control solutions (water or a solution of crushed healthy *Daphnia*). Hosts in the single dose treatment received 2500 spores on day 1; double dose treatment received a second parasite exposure (an additional 2500 spores) on degree-day 120. The infection period lasted a total of 200 degree-days.

Data analysis

All data was analysed using the statistical software JMP 7 (SAS Institute Inc.). In experiment 1, we analysed the differences in parasite growth rate due to parasite genotype (nominal), temperature (nominal), and the degree-day sacrificed (continuous) in a fully-factorial model using ANCOVA. The response variable “number of spores” was Box-Cox transformed for normality. In experiment 2, we first tested if the proportion of hosts that became infected was influenced by the parasite isolate they were exposed to, the dose of exposure (single or double), or the temperature at which exposure occurred. Infectivity was analysed only on hosts that were exposed to parasites using a generalized linear model (GLM) with a binomial error structure (Logit link function). We next tested for differences in fecundity and survival between hosts that were exposed to parasites but did not develop infection, relative to unexposed controls. Only hosts that remained uninfected (exposed and not exposed) were included in these analyses. Fecundity was analysed using a linear model on the total number of offspring produced recorded during the experiment. Survival (the time to host death) was analysed using proportional hazards; hosts that did not die by degree-day 1200 were entered as censored data. Degree-day was used as the time scale to allow comparisons between temperature treatments. This is simply the product of the real day by the temperature, and has been used as an approximate measure of *Daphnia* physiological time (Mitchell et al. 2005). All analyses were performed by fitting the full model and removing non-significant terms to obtain the best, reduced model.

7.4 Results

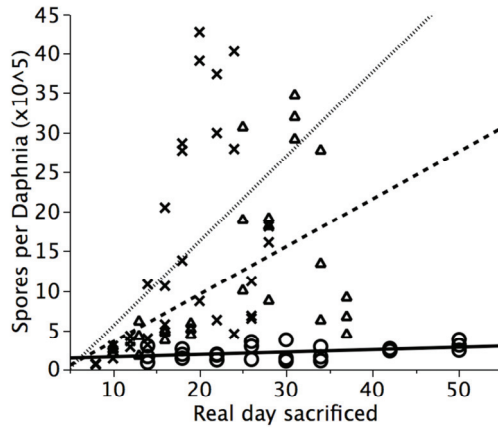
Experiment 1: Measuring parasite growth

The number of spores increased significantly throughout the experiment (Figure 7.2), but did so differently depending on temperature and parasite genotype (Parasite x temperature interaction, Degree-day x temperature interaction and Degree-day x parasite genotype interaction, Table 7.1). For both parasite genotypes, growth was reduced at 15°C (mean 4.77 (\pm 0.48) $\times 10^5$ spores per *Daphnia*) compared to the other temperatures (20°C: mean 8.5(\pm 0.46) $\times 10^5$; 25°C: mean 9.1(\pm 0.41) $\times 10^5$ spores per *Daphnia*) (Figure 7.2).

Source	DF	Seq SS	F	p
Parasite genotype	1	53.14	5.04	0.0263
Temperature	2	553.90	26.26	<0.0001
Degree-day sacrificed	1	564.57	53.52	<0.0001
Parasite genotype x Temperature	2	175.58	8.32	0.0004
Parasite genotype x Degree-day sacrificed	1	65.25	6.19	0.0140
Temperature x Degree-day sacrificed	2	143.96	6.82	0.0015

Table 7.1. Summary statistics from an ANCOVA testing the effects of temperature and parasite genotype on parasite growth over time (measured in degree-days). DF, degrees of freedom; Seq SS, Type I / sequential sum of squares; F, F-ratio test statistic. See Figure 7.2.

Sp1



Sp7

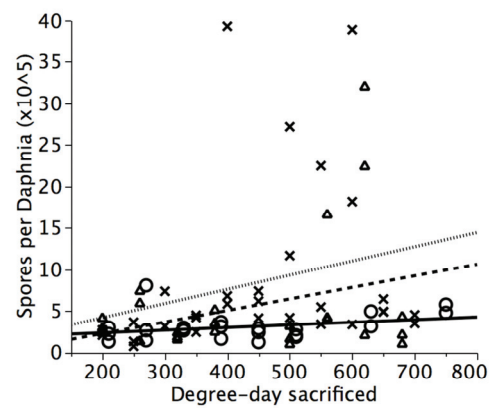
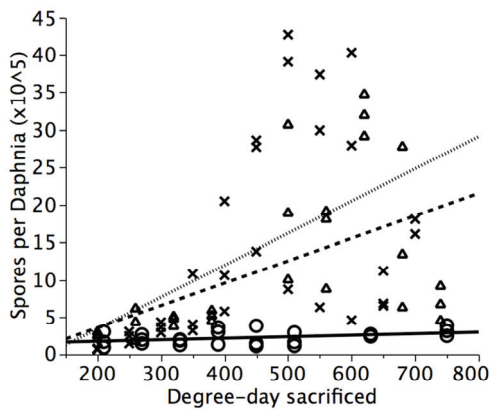
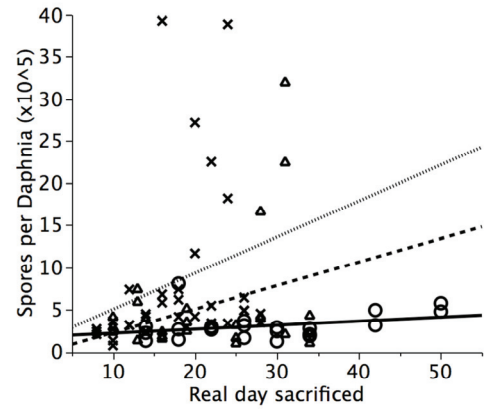


Figure 7.2. Parasite growth over time plotted as the number of spores present on the day an individual *Daphnia* was sacrificed. Each data point is a sacrificed individual. Hosts were exposed to parasite genotypes Sp1 (left panel) and Sp7 (right panel) at 15°C (full line), 20°C (dashed line) or 25°C (dotted line). Time of sacrifice is shown in real days (top) and degree-days (bottom).

Details of linear fits for degree days are as follows (R^2 ; equation): Sp1, 15°C (0.16; $y = 1.3038208 + 0.0020401x$); Sp1, 20°C (0.28; $y = -2.480836 + 0.0300526x$); Sp1, 25°C (0.26; $y = -0.193091 + 0.0428473x$); Sp7, 15°C (0.10; $y = 1.7784069 + 0.0031661x$); Sp7, 20°C (0.10; $y = -0.468417 + 0.0139083x$); Sp7, 25°C (0.07; $y = 0.8134007 + 0.0170688x$).

Experiment 2: Immune challenge and temperature treatments

We exposed a total of 360 individual *Daphnia* to either single or double doses of two parasite isolates (plus an additional 72 unexposed controls) at 15°C, 20°C, and 25°C (Figure 7.1). Of the total 432 experimental jars, some were lost during the infection period, either due to chance or to experimental error. All exposed treatments remained with at least 24 replicates out of a possible 30. Control replicates remained with at least 10 out of a possible 12 replicates. No significant difference was detected between our two control treatments (ddH₂O and healthy crushed *Daphnia*) for either fecundity ($F_{1,65}=0.316$, $p=0.576$) or survival ($\chi^2=0.212$, $df=1$, $n=66$, $p=0.645$), therefore we combined these into one ‘control’ treatment, resulting in 20-23 replicates per temperature.

Infectivity differed noticeably between parasite isolates. While exposure to parasite isolate Sp1 yielded 30-50% infection in all temperature treatments, infections in hosts exposed to parasite isolate Sp7 were almost totally unsuccessful (there was a single infected host at 15°C, data not shown). For hosts exposed to parasite isolate Sp1, we found a significant effect of temperature on the proportion of hosts that became infected (Table 7.2). Double exposures resulted in higher infectivity at 20°C and 25°C ($\chi^2=4.36$, $df=1$, $p=0.0368$), but not at 15°C. Despite this, we did not find any significant effect of the dose of exposure or a dose-by-temperature interaction (Table 7.2).

Source	D F	Infectivity		Fecundity			Survival	
		χ^2	p	DF	F	p	χ^2	p
Sp1								
Dose	1	0.3851	0.5349	2	1.41	0.2469	2.647	0.2662
Temperature	2	7.4905	0.0236	2	89.11	<.0001	23.86	<.0001
Dose x Temperature	2	4.5495	0.1028	4	0.30	0.8780	9.409	0.0516
Sp7								
Dose				2	0.76	0.4684	0.038	0.9811
Temperature				2	137.39	<.0001	69.36	<.0001
Dose x Temperature				4	1.05	0.3824	2.67	0.6147

Table 7.2. Summary statistics of analyses testing infectivity and the cost of resisting infection when hosts were exposed either to parasite isolate Sp1 (top) or Sp7 (bottom). Note that analysis of infectivity was not possible in hosts exposed to Sp7 due to no infection. Infectivity was analysed in a GLM with binomial error structure. Fecundity was analysed in a linear model and Survival was analysed using proportional hazards. DF, degrees of freedom; χ^2 , chi-squared test statistic, F, F-ratio test statistic. See figures 7.3 and 7.4.

We aimed to measure the cost of resisting infection as the reduction in either fecundity or survival in hosts that were exposed to parasites but did not develop infection, relative to unexposed controls. Due to reduced infection in hosts exposed to parasite isolate Sp7 we cannot be certain that any immune eliciting occurred in these hosts. Therefore all subsequent analyses were performed separately for each parasite genotype. For hosts exposed to either parasite genotype (Sp1 or Sp7), we found significant main effects of temperature on fecundity and survival (Table 7.2), but no effect of dose or a dose by temperature interaction for either trait (Table 7.2; Figures 7.3 and 7.4). However, survival analysis of hosts exposed to parasite isolate Sp1 revealed a marginally non-significant dose-by-temperature interaction ($p=0.0516$; Table 7.2). Notably, at 25°C hosts that received a double exposure showed increased survival compared to hosts that were not exposed or that were only exposed once. It is also worth noting that the survival pattern among hosts exposed to parasite isolate Sp1 at

20°C shows the expected trend with non-exposed hosts showing the highest proportion survival and hosts exposed to a double dose of Sp1 showing the lowest survival (Figure 7.4).

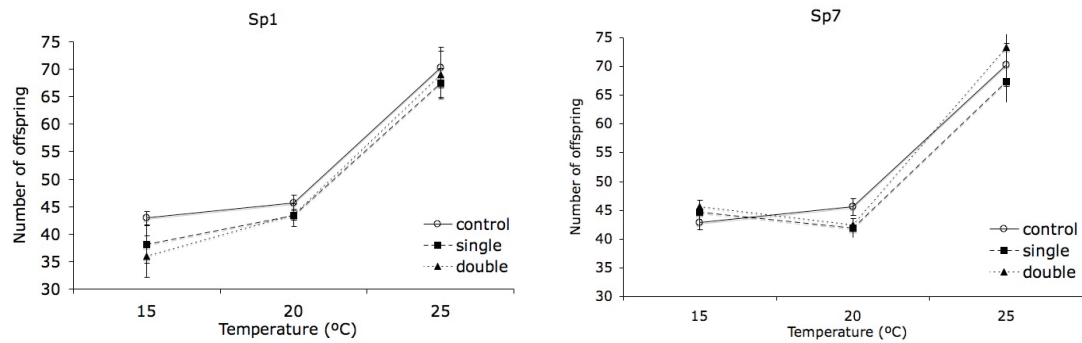
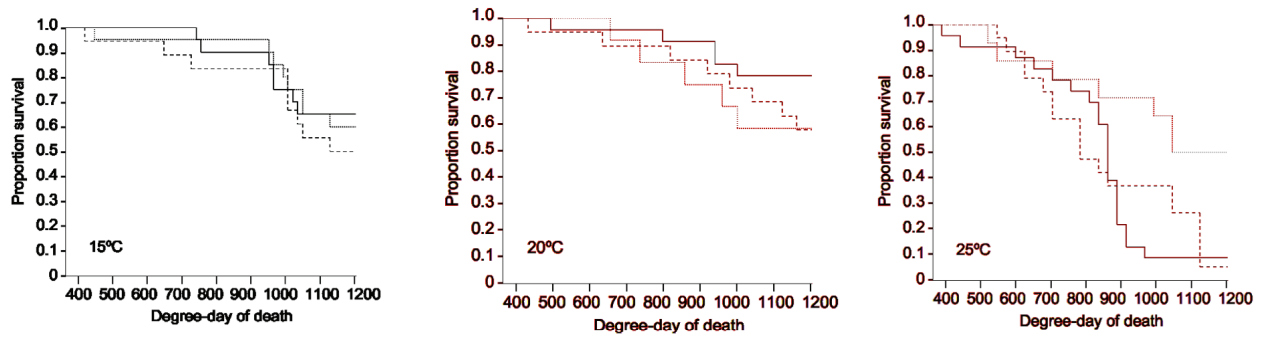


Figure 7.3. Average number of offspring produced per *Daphnia* until degree-day 700 at 15°C, 20°C, and 25°C. Graphs show hosts that were not exposed to parasites (full black line) or that were exposed to either a single dose (dashed line) or to a double dose (dotted line) but did not develop infection. Error bars are standard error of the mean.

Exposed to parasite isolate Sp1



Exposed to parasite isolate Sp7

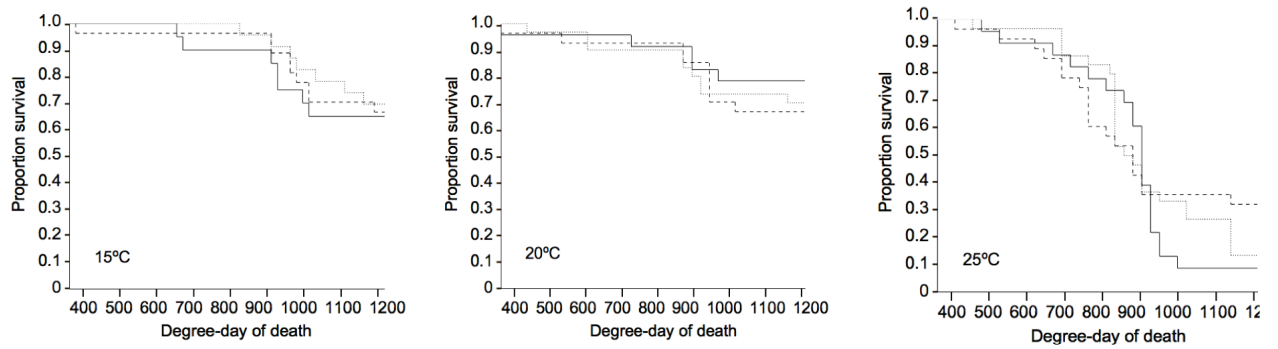


Figure 7.4. The proportion of hosts alive recorded until degree-day 1200 at 15°C, 20°C, and 25°C.

Graphs show hosts that were not exposed to parasites (full black line) or that were exposed to either a single dose (dashed line) or to a double dose (dotted line) but did not develop infection.

7.5 Discussion

This study aimed to measure the physiological cost of resisting infection under thermal variation when the crustacean *Daphnia magna* was exposed to the bacterial parasite *Pasteuria ramosa* at 15°C, 20°C and 25°C. We chose combinations of host-parasite genotypes that have been shown in the past to yield either high (parasite Sp1) or moderate (parasite Sp7) infection levels at the temperatures we tested (Carius et al. 2001; Little and Killick 2007; Vale et al. 2008b). In addition, we tested for temperature dependent differences in growth rate that might help explain any putative cost we might find (Figure 7.2, Table 7.1). Given the reduced parasite growth at 15°C (Figure 7.2), we predicted costs to be absent at this temperature and present at 20°C and 25°C where parasite growth increased. We measured the cost of resisting infection as the reduction in either fecundity or survival in hosts that were exposed to parasites but did not develop infection, relative to unexposed controls. Any significant differences between these two treatments would reflect the cost of having successfully resisted infection, but we found no significant differences between exposed and unexposed hosts for these traits.

Studying resistance necessarily requires hosts to be exposed but to remain infection free, and our infection protocol applied doses either singly or doubly in order to remain with enough uninfected replicates for subsequent analyses. This was successful with parasite isolate Sp1, as infection levels were as expected, but hosts exposed to parasite Sp7 failed to become infected at all. Due to this, we could not be certain that any immune eliciting occurred in these hosts.

We chose early fecundity and long-term survival as host fitness traits for measuring these costs. While fecundity is usually an appropriate measure of host fitness, it is also the main

trait affected by *P. ramosa* infection. Hence patterns of fecundity may reflect the effect of a low-level infection that remained undetected or was controlled by the host, or result from fecundity compensation strategies that are known to occur when *Daphnia* are exposed to parasites (Ebert 2004). Therefore costs measured using fecundity are potentially hard to interpret and may be confounded with the direct effect of parasite exposure (Little and Killick 2007).

Patterns of host survival, however, are more easily interpreted. While parasite treatment did not result in significant differences in survival overall, our analysis did reveal a marginally non-significant dose-by-temperature interaction when hosts were exposed to parasite isolate Sp1 (Table 7.1). At 25°C, hosts died at a faster rate on average relative to exposure at 15°C or 20°C (Figure 7.4), confirming previous results in this system when temperature has been manipulated that the cost of being infected is higher at this temperature (Vale et al. 2008b). Notably, when hosts were exposed to parasite isolate Sp1 at 25°C, less than 10% of non-exposed or single-exposed hosts remained alive after 1200 degree-days (48 days), but more than 50% of double exposed hosts survived within the same period (Figure 7.4, right panel; Table 7.1). This higher survival was unexpected given that control hosts followed the previously reported pattern of increased mortality at this temperature (Vale et al. 2008b). Studies of *Daphnia* maintained at stressful temperatures have shown increased expression of certain classes of heat-shock proteins (Hsp) (Bond et al. 1993; Bond and Bradley 1995, 1997). There is also evidence that the expression of a specific class of Hsp, Hsp60, is positively correlated with the prevalence of epibiont parasites, suggesting that parasitism might induce the expression of these molecules in *D. magna* (Pauwels et al. 2007). Given that we observe increased survival in double exposed hosts at 25°C that did not develop infection, it is possible that increased exposure to *P. ramosa* might have resulted in the expression of a class of Hsp that increased host survival at this high temperature. To our

knowledge, no comprehensive study testing the effects of *P. ramosa* exposure on the expression of Hsp has been carried out, but pursuing this avenue of research could provide some interesting information about the mechanistic nature of environment-dependent parasite-mediated selection in the wild.

In the 20°C treatment, the pattern of survival in hosts exposed to parasite isolate Sp1 was the expected for a cost of resisting infection, with double-exposed hosts dying at a marginally faster rate compared to single-exposed or non-exposed hosts, although these differences between exposure (dose) treatments were not significant (Table 7.1). The same pattern of host survival was observed in a previous study, using the same combination of host genotype and parasite isolate used here, at 20°C (Little and Killick 2007). However, in that experiment the differences between parasite exposure treatments were significant. Several reasons could explain why the current experiment did not reproduce this result clearly. Different food sources were used in the two experiments and it is possible that food quantity or quality varied sufficiently to reduce the cost in the current experiment. Indeed, other work has demonstrated how physiological costs of resistance are usually revealed under stressful or resource-limited environments (Sandland and Minchella 2003; Mckean et al. 2008).

The experimental design also differed slightly between the two studies. While this experiment used one host per 60ml jar to allow recording of individual fecundity and survival, Little and Killick (2007) included six hosts per 200 ml jar. Even if food levels were in excess of what *Daphnia* could consume daily in that study, it is possible that density-dependent effects could augment the cost of resisting infection due to competition between individuals. Situations of high host density would fall into the broad category of “stressful environment” thought to bring out such costs (Sandland and Minchella 2003). One study has shown how the cost *acquiring* an infection is greater in high-density scenarios due to the

reduced competitive ability of infected hosts (Bedhomme et al. 2005). In general, the role of density-dependent effects on costs of resistance requires further attention.

Another relevant difference between these two studies pertains to the infection protocol. Both studies exposed hosts either singly or doubly, but the timing of these exposures differed. While in this study all hosts were initially exposed at day 1 and then received an additional exposure in the double exposure treatment, the infection protocol in Little and Killick (2007) exposed double-exposure hosts on days 1 and 6, and single exposure hosts only on day 6. Therefore, in that experiment the effect of dose of exposure is possibly confounded with the effect of the day of initial exposure. Indeed, it is possible that the higher mortality they observed in double exposed hosts occurred because these hosts were initially exposed at a much younger age. The experiment presented here controlled for this by initially exposing all hosts at the same age.

Taken together, these results suggest that costs of resisting infection are, at best, small to the point of being undetectable under a realistic range of thermal variation. Certainly, they were not revealed at 25°C, which we consider to be a stressful environment for *Daphnia*, supported by the higher death rate of unexposed hosts at this temperature.

Chapter 8

GENERAL DISCUSSION

General discussion

It has long been recognised that the expression of quantitative traits will differ depending on the environment (Falconer 1952). If traits affecting fitness are expressed differently in different environments, this could lead to changes in the direction and strength of selection (termed genotype-by-environment [GxE] interactions), which are hypothesised to affect rates of coevolution (Thompson 1994; Thompson 1999) and promote the co-occurrence of different genotypes through heterogeneous selection (Gillespie and Turelli 1989; Byers 2005; Laine and Tellier 2008).

The work described in this thesis explored the effects of variation in temperature and food levels on infection outcomes using the model host-parasite system *Daphnia-Pasteuria*. The experiments I conducted illustrate that variation in these abiotic factors can have marked effects on the general performance of hosts and parasites during infection, with important implications in the wild as shown in chapter 3, where the temperature at the time of exposure was found to correlate with prevalence during an epidemic. Chapter 4 showed that the general infectivity and growth of *P. ramosa* is optimal within a specific thermal range, affecting the general cost of infection at different temperatures.

I also investigated whether these effects were genotype-specific, with special emphasis on parasite genetic variation and fitness. Several studies in a variety of host-parasite systems have reported GxE effects on host fitness (Lazzaro and Little 2008), but fewer had explored the effect of environmental variability on parasite fitness. In chapter 4 I showed that GxE interactions for parasite fitness traits do indeed occur in this system and could potentially maintain variation in parasite populations. *Potentially*, because GxE requires that *fitness* be environment dependent, not only the expression of a single trait. While parasite growth and

transmission potential are reasonable and commonly used fitness correlates, in chapter 5 I showed that measures of parasite fitness change considerably when the ability to infect is taken into account, to the extent where GxE interactions for measures of parasite transmission were dissipated. Whether this affects the potential for the maintenance of polymorphism in heterogeneous environments is unclear. Certainly, GxE effects on parasite transmission will influence the probability of infection in subsequent infection cycles in the wild in a way that is not immediately obvious from the common-garden experiments presented here. Still, these results add to a growing body of work in host-parasite systems that demonstrate unequivocally that the expression of host and parasite traits and the outcome of infection is context-dependent (Lazzaro and Little 2008).

Such context-dependence has obvious implications for the expression of disease in common ecological settings, but equally for the rate at which coevolution between hosts and parasites proceeds. As discussed in chapter 6, one of the possible consequences of this may be that the evolution of virulence becomes unpredictable when environmental heterogeneity is present. Each of the preceding chapters concluded with a detailed discussion of these topics. Below I highlight four areas where more work is required in order to fully understand the extent of environmental effects on host-parasite interactions and their evolution: (1) studies of environmental variation in natural populations, (2) using experimental evolution to study adaptation under environmental variation (3) incorporating environmental variation into models of coevolution and virulence evolution, and (4) developing host-parasite systems to test theoretical predictions about evolutionary epidemiology under environmental variation.

8.1 Environmental variation in natural populations: does the “E” matter?

Common garden infection experiments, such as the ones described in this thesis, allow us to learn more about how environmental treatments influence infection outcomes. However, while this approach may help to validate specific predictions from theoretical models or generate new hypotheses, it remains restricted to particular model systems and simple experimental communities. Despite the increasing number of examples from laboratory host-parasite systems indicating the occurrence of environment-dependent infection outcomes, the importance of GxE interactions in changing the strength and direction of selection in the field remains obscure. Simply identifying that traits are expressed differently in different environments only tells us that GxE interactions can occur, but without quantifying the effect sizes of these interactions in natural populations we can say little about how or if they will change evolutionary outcomes. Indeed, if the contrast in fitness effects due to environmental variation is not large, this would reduce the relevance of such interactions in affecting the coevolutionary process, and question their importance in the maintenance of genetic variation (Maynard Smith and Hoekstra 1980; Gillespie and Turelli 1989; Byers 2005).

Perhaps the most challenging question is then to ask how robust genotype interactions are against environmental variation in the wild: does the “E” in GxE (or GxGxE) really matter (Vale et al. 2008a)? Clearly, common garden experiments alone are no substitute for studies of GxGxE in natural populations. Some such studies have tried to correlate levels of genetic variation along environmental clines in wild populations, usually finding that the pattern of GxE interaction is consistent with local adaptation (Byers 2005), and could thereby maintain environment-dependent polymorphism. However, without knowledge of the ancestral state of these populations, it is difficult to establish if such phenotypic variation is caused by

environment-dependent selection or if it preceded the environmental clines studied (Byers 2005).

So it appears that studies of natural populations on their own cannot clearly decipher the relevance of GxGxE any more than common-garden experiments can on their own. Some authors have defended the view that the complexity and sheer scale of these questions – spanning molecular, physiological, and ecological levels of biological organization and operating over evolutionary timescales – make their answers only attainable within the framework of large, collaborative, multidisciplinary projects that extend beyond the capabilities of a single research lab (Thompson 1999). Recent efforts to combine field and lab studies of the same host-parasite system (Laine 2007b) along with a recently proposed theoretical framework to partition the contribution of the genetic and environmental causes of local adaptation from such experimental studies (Nuismer and Gandon 2008), are a clear step in that direction.

8.2 Understanding pathogen adaptation under environmental variation

The importance of interactions between host and parasite genotypes and with their environment is not only important for our understanding of evolution, but could also have applied consequences for disease management. If infection outcomes are context-dependent, anti-parasitic intervention strategies could be thwarted if the environmental variation encountered in the wild is not taken into account. For example, being able to predict the fate of particular genes or genotypes (e.g. introduced resistance genes) in variable environments is of extreme importance for disease control programs (Lambrechts et al. 2008). Other studies have shown how the rate of environmental change can have clear consequences for the evolution of antibiotic resistance. In this case, sudden changes in the concentration of

antibiotic resulted in fewer, but fitter bacterial genotypes, while gradual changes resulted in the maintenance of more genotype variability but with intermediate fitness (Perron et al. 2008).

Perhaps, before considering host-parasite coevolution under environmental variation, it might be prove more fruitful to first focus on the question of how adaptation proceeds under environmental variation in individual populations. Despite a long-established theoretical framework of adaptation (Fisher 1930), it is only recently that predictions from this theory have been experimentally tested (Orr 2005; Rokyta et al. 2005; Kassen and Bataillon 2006; Martin et al. 2007). Yet, we are still far from predicting how populations will adapt under common conditions of environmental variation (Visser 2008). Studies with simple forms of variation (periodic fluctuation in temperature or food) suggest that the mode and tempo of environmental heterogeneity determine the amount of genetic variation maintained, and the outcome of adaptation (Kassen and Bell 1998; Collins et al. 2007). The effect on adaptation of more complex and realistic settings is still poorly understood and lacks experimental testing.

This knowledge is vital to our understanding of how pathogen evolution and adaptation proceed under environmental variation. Pathogens exist as large, genetically variable populations, with tremendous evolutionary potential. This facilitates adaptation to changing environments over short timescales, hindering the efforts of disease control programs (Gandon et al. 2001). In addition, the most important environmental factor for a pathogen – its host – is a living, evolving organism, adding another level of environmental complexity. For these reasons, understanding pathogen adaptation under environmental variation is at the centre of evolutionary epidemiology, a field that aims to understand how population

dynamics of hosts and parasites affect their evolutionary dynamics (Day 2004; Day and Gandon 2007).

8.3 Incorporating environmental variation into models of coevolution and virulence evolution

One of the main challenges of evolutionary epidemiology is to understand how the environmental factors that determine the epidemiological dynamics can feed back into the evolutionary dynamics of disease (Day and Gandon 2007; Gandon and Day 2009). A theoretical framework for evolutionary epidemiology is currently being developed (Day 2004; Day and Gandon 2006, 2007) based on Fisher's Fundamental Theorem of adaptation (FFT) (Fisher 1930). Following Price's reinterpretation of FFT (Price 1972; Frank 1995), the dynamics of pathogen adaptation can be partitioned into the combined effect of natural selection, recurrent mutation, and environmental change (Day and Gandon 2006). Because this framework focuses on the evolution of fitness rather than using life-history traits as fitness correlates, and by explicitly describing the feedback between epidemiological and evolutionary dynamics, it provides a direct measure of how populations are expected to evolve under environmental variation.

In addition to models of coevolutionary dynamics, very few models of virulence evolution have incorporated environmental variation. The experiment described in chapter 6 is the first known empirical test of the transmission-virulence relationship under environmental variation, and provides a unique opportunity to understand the evolution of virulence under environmental variation. In the future, I aim to combine this empirical data with already established mathematical models of virulence evolution to generate specific predictions about how virulence will evolve under environmental variation.

8.4 Host-parasite model systems to study evolutionary epidemiology

The theoretical framework for evolutionary epidemiology described above is only beginning to generate predictions about how host-parasite coevolution is expected to proceed under environmental variation. It will be particularly useful to ask questions about the interplay between the ecological and the evolutionary dynamics of pathogen populations when they are not at equilibrium, such as in situations of disease emergence and periodic epidemics (Bull and Ebert 2008). It is this information that yields the most promise for virulence management programs (Gandon and Day 2003). As this framework is expanded and applied to more scenarios of disease evolution (e.g. host and parasite genetic variation, co-infection), a slew of theoretical predictions will require experimental validation.

The work presented in chapter 3 highlights how the use of *Daphnia* and similar model systems might prove useful in studying the role of abiotic factors on epidemics. Of course, there are not many systems that combine the ease of monitoring natural populations and the possibility of controlled infection experiments (Nuismer and Gandon 2008). However, several plant (Laine 2007b) and animal (Schmid-Hempel 2001) systems possess the desired characteristics for studying epidemics in controlled laboratory or semi-natural environments.

Microbial systems are especially desirable for this purpose, as in addition to the aforementioned attributes, they offer the exciting possibility of performing experimental evolution over short time scales (Jessup et al. 2004). For example, several bloom-forming algae and their viruses are regularly monitored in their natural habitats and some have been isolated and propagated in the lab (Nagasaki 2008). Many of these populations experience seasonal viral epidemics that result in algal population crashes (Tomaru et al. 2008; Yoshida

et al. 2008). Experimental infections using algal and viral isolates from these populations have found intra-species host specificity and distinct virulence profiles for each viral strain (Tomaru et al. 2008). This knowledge has so far been applied to controlling toxic algal blooms, but such a system offers tremendous potential for studying general evolutionary and epidemiological questions. Experimental evolution on algae and virus isolates on the other hand, could be used to address questions about the evolution of virulence and the feedback between evolution and epidemiology. Studying natural epidemics in such model systems would provide an invaluable test bed for predictions about how evolutionary processes might influence epidemiological dynamics, offering a deeper understanding of the link between evolutionary ecology and epidemiology (Galvani 2003; Day and Gandon 2007).

8.5 Concluding remarks

The work described in this thesis highlights how infection outcomes are sensitive to variation in temperature and food levels. This was apparent in the general effect of temperature on host and parasite performance, and in genotype-specific effects of these abiotic variables, suggesting the potential for maintenance of polymorphism via environment dependent selection. These effects could influence the strength of coevolution between hosts and parasites and possibly alter the evolutionary trajectories of virulence. A comprehensive assessment of the frequency and magnitude of these effects will require more data from natural populations, incorporating environmental variation into models of host-parasite coevolution and virulence evolution, and developing model systems that combine the study of natural populations with experimental evolution in the lab. Only through such a comprehensive and multidisciplinary approach can true insight be gained into the evolution of infectious disease under environmental variation.

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Appendix I – Publication list

Accepted for publication

Vale PF, Little TJ. Measuring parasite fitness under genetic and thermal variation (accepted for publication in *Heredity*)

Published

Vale, P. F., M. Stjernman, and T. J. Little. 2008. Temperature dependent costs of parasitism and the maintenance of polymorphism under genotype-by-environment interactions *Journal of Evolutionary Biology* 21(5):1418-1427.

I also contributed to two other articles that are not part of my thesis that were published in peer-reviewed journals:

Vale, PF, Salvaudon, L, Kaltz, O, Fellous, S (2008) The role of the environment in the evolutionary ecology of host-parasite interactions. *Infection, Genetics and Evolution* 8(3):302-305.

Little TJ, Birch J, Vale P and Tseng M (2007) Parasite transgenerational effects on infection. *Evolutionary Ecology Research* 9(3):459-469.

Temperature-dependent costs of parasitism and maintenance of polymorphism under genotype-by-environment interactions

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Pasteuria ramosa;
 temperature;
 transmission;
 virulence evolution.

Abstract

The maintenance of genetic variation for infection-related traits is often attributed to coevolution between hosts and parasites, but it can also be maintained by environmental variation if the relative fitness of different genotypes changes with environmental variation. To gain insight into how infection-related traits are sensitive to environmental variation, we exposed a single host genotype of the freshwater crustacean *Daphnia magna* to four parasite isolates (which we assume to represent different genotypes) of its naturally co-occurring parasite *Pasteuria ramosa* at 15, 20 and 25 °C. We found that the cost to the host of becoming infected varied with temperature, but the magnitude of this cost did not depend on the parasite isolate. Temperature influenced parasite fitness traits; we found parasite genotype-by-environment ($G \times E$) interactions for parasite transmission stage production, suggesting the potential for temperature variation to maintain genetic variation in this trait. Finally, we tested for temperature-dependent relationships between host and parasite fitness traits that form a key component of models of virulence evolution, and we found them to be stable across temperatures.

Introduction

Understanding of the mechanisms that maintain genetic variation in natural populations of pathogens is of clear importance for the design of disease control programmes because the success of interventions can be undermined by pathogen evolution (Gandon *et al.*, 2001; Galvani, 2003; Grenfell *et al.*, 2004; Takala *et al.*, 2007). Frequency-dependent host–parasite coevolution can, in theory, maintain substantial genetic variation in infection-related traits (Haldane, 1949; Anderson & May, 1982; Woolhouse *et al.*, 2002). Where studied, patterns of host and parasite genetic variation have proven compatible with the occurrence of frequency-dependent dynamics (Lively, 1989; Carius *et al.*, 2001; Wolinska *et al.*, 2006; Decaestecker *et al.*, 2007; Duncan & Little, 2007). However, environmental heterogeneity is also potentially a major contributor to the maintenance of genetic polymorphism in fitness traits. When the magnitude of

fitness differences between genotypes changes across environments [termed genotype-by-environment ($G \times E$) interaction], this could promote the co-occurrence of different genotypes, particularly if no single genotype outperforms all others across a variable environment (Gillespie & Turelli, 1989; Falconer & Mackay, 1996; Byers, 2005).

Variation in environmental variables, such as food (Bedhomme *et al.*, 2004) or temperature (Fels & Kaltz, 2006), can impact the cost to the host of becoming parasitized. Work in a variety of host–parasite systems have also incorporated parasite or host genetic variation and have found evidence for parasite genotype-by-environment ($G_p \times E$) interactions (Ferguson & Read, 2002) or host genotype-by-environment ($G_h \times E$) interactions when environmental variables are manipulated (Blanford *et al.*, 2003; Mitchell *et al.*, 2005; Lambrechts *et al.*, 2006; Restif & Kaltz, 2006). Whereas most of these studies have focused on how these interactions affect host traits such as survival or fecundity, less attention has been paid to $G \times E$ interactions affecting parasite fitness components (Fels & Kaltz, 2006; Laine, 2007) and therefore the effects of these interactions on pathogen genetic variation and evolution are less well understood.

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In this study, using the freshwater crustacean *Daphnia magna* and its sterilizing bacterial pathogen *Pasteuria ramosa*, we therefore describe how the general cost of parasitism changes with temperature and how individual parasite isolates perform at these temperatures. As *D. magna* tends to live in small, sometimes temporary ponds, thermal variation is likely to be large, and previous studies on the *D. magna*–*P. ramosa* system have shown temperature sensitivity to infection as well as host genotype-by-environment interactions (Mitchell *et al.*, 2005). Here, we performed experimental infections with four parasite isolates (which we consider to be different genotypes) at three temperatures on a single host clone, and measured host traits indicative of fitness costs of becoming infected (mortality and fecundity) and parasite fitness components (infectivity and the production of transmission stages). We looked for evidence of parasite $G \times E$ interactions for these traits that would indicate the potential for the maintenance of genetic variation via environmental variation.

In addition, we tested how temperature affected the relationships between host and parasite fitness components. Testing these relationships was motivated by common models of pathogen evolution which suggest that pathogens will be selected to balance their rate of transmission with their rate of host exploitation, known as the trade-off hypothesis of virulence evolution (Anderson & May, 1982; Bremermann & Pickering, 1983; Frank, 1996; André *et al.*, 2003; Day, 2004; Jensen *et al.*, 2006). This model assumes particular relationships between host and parasite fitness traits. There is evidence that these relationships might change depending on the genotypes of host and parasite involved (Salvaudon *et al.*, 2005, 2007), but it has not been tested, as far as we are aware, how these relationships might vary with environmental heterogeneity. Clearly, if the expression of these traits changes depending on the specific environmental context, this could confound predictions about the evolution of virulence. In general, if genetic correlations change between different environments, this could relax the evolutionary constraints imposed by trade-offs (Sgro & Hoffmann, 2004) and contribute to the maintenance of genetic variation in the associated fitness traits (Falconer, 1952; Bell, 1997; Bell & Rebound, 1997).

Materials and methods

Host and parasite genotypes

Twenty replicates of one host genotype (named GG3) were exposed to four spore types (named Sp1, Sp7, Sp8 and Sp13) at 15, 20, and 25 °C in a fully factorial design. The host genotype and parasite isolates were originally collected from a population near Gaazerfeld, Germany, and maintained in the laboratory in a state of clonal reproduction (host) or frozen (parasite). The parasite isolates (each originally collected from an individual

host) have been studied extensively and infections have been shown to differ depending on the combination of host and parasite genotypes (Carius *et al.*, 2001). *Daphnia* are filter feeders and become infected with *P. ramosa* by filtering transmission spores present in the water. Infection causes host castration and gigantism, as well as premature death. Within the host, *P. ramosa* goes through a developmental process that culminates in the formation of spores that can be horizontally transmitted when they are released from dead hosts; vertical transmission does not occur (Ebert *et al.*, 1996).

Host acclimation

Before exposing hosts to parasite spores, host maternal lines experienced a period of acclimation to reduce maternal effects, as these have been shown to affect infection outcomes in this system (Mitchell & Read, 2005). Twenty independent replicates of five GG3 isofemale *Daphnia* were maintained in jars containing 200 mL of artificial medium (Kluttgen *et al.*, 1994), fed 6×10^6 cells per *Daphnia* per day of chemostat grown *Scenedesmus obliquus* algae, and maintained within temperature-controlled incubators with a light : dark cycle of 12 : 12 hours. Medium was changed with every clutch or every 3 or 4 days regardless of a clutch being present. Although infections were carried out at three different temperatures, all host lines were acclimatized at 20 °C to synchronize and maximize clutch production. Previous experiments have shown that the temperature of acclimation of the maternal generation does not affect infection outcomes in their offspring (Mitchell & Read, 2005). Acclimation lasted at least three generations and all infections were performed on second- or third clutch 1-day old juvenile females.

Infection and temperature regime

The experiment followed a split-jar design (analogous to a split-brood design), where clutches from an individual replicate jar were split into the different treatments. Each experimental replicate received a single 1-day old isofemale, placed in a jar containing 60 mL of artificial *Daphnia* medium and sterile sand. Jars with *Daphnia* media were prepared the day before infection and placed in an incubator at the appropriate temperature overnight. This guaranteed that the infection period took place at the desired temperature. Infection was achieved by adding 10 000 spores to each jar. Spore solutions were originally obtained by homogenizing infected *Daphnia* in ddH₂O, and these solutions were stored at –20 °C until required. *Daphnia* have longer development times at lower temperatures, and Mitchell & Read (2005) have previously shown that the product of temperature and real days (called degree-day) is a reasonable measure of *Daphnia* physiological time. Accordingly, the infection period in all treatments lasted 150 degree-days,

i.e. 6 days at 25 °C, 7.5 days at 20 °C and 10 days at 15 °C (Mitchell *et al.*, 2005; Little *et al.*, 2007a). During the infection period, all replicates were stirred daily and fed low amounts of chemostat-grown *S. obliquus* algae (1.5×10^6 cells per *Daphnia* per day). All replicates at a particular temperature treatment were grouped within the same incubator, and thus we cannot exclude the possibility that uncontrolled incubator effects have confounded the effects we attribute to temperature. However, we consider it reasonable to assume that 'effects of incubators set at a particular temperature' are essentially 'temperature effects', and previous experiments that tested for consistency among incubators (Mitchell *et al.*, 2005) support this assumption.

After the infection period, all replicates were transferred to jars with 60 mL of clean medium. Food levels were increased and remained in excess of what *Daphnia* can consume daily: (algae cells per *Daphnia* per day) 15 °C: 2×10^6 ; 20 °C: 3.5×10^6 ; 25 °C: 6×10^6 (Mitchell *et al.*, 2005). Females that produced clutches were changed into clean medium, or changed three times a week regardless of producing a clutch. The number of offspring produced was counted at every clutch. The experiment lasted 900 degree-days and during this time jars were distributed randomly within trays of 24 jars and the position of the trays was changed regularly to equilibrate any positional effects within the incubators. Hosts that showed signs of infection (no eggs in the brood chamber and red colour) were observed under a dissecting microscope for symptoms consistent with *P. ramosa* infection (sterilization, bacterial growth in the haemolymph). Hosts that died during the experiment were individually frozen in 1.5-mL Eppendorf tubes at -20 °C until needed. Counts of *P. ramosa* transmission stages were obtained by homogenizing the dead host with a sterile pestle in 100 μ L of ddH₂O, and counting two independent samples of this solution in a Neubauer (improved) counting chamber [$0.0025 \text{ mm}^2 \times 0.100 \text{ mm}$ (depth)]. The number of transmission spores per *Daphnia* was used as a measure of transmission potential.

Data analysis

All data analysis was carried out using the statistical software package JMP 7 (SAS Institute Inc., Cary, NC, USA). We focused first on the cost of parasitism to the host in relation to temperature. We determined how the response variables host fecundity (total number of offspring produced) and mortality differed with infection status in hosts exposed to the different parasite genotypes at the three temperatures tested. Square root-transformed 'total number of offspring' data were analysed with a generalized linear model assuming normally distributed residuals. Mortality was analysed using proportional hazards and the timescale used was always degree-day to allow comparisons between temperature treatments. Both models were constructed by including

'infection status', 'temperature' and 'parasite genotype' in a full factorial model and then removing the highest order nonsignificant term in the model until only significant terms were present in the model.

Next, we studied how infectivity (the proportion of hosts that became infected) and the number of transmission stage spores produced on the day of death once infection was achieved were affected by temperature, parasite genotype and the parasite genotype-by-temperature interaction. For infectivity, a binomial distribution of error terms was assumed (Logit function). Parasite transmission was log transformed and a normally distributed error term was used.

Finally, we tested whether the relationships between host and parasite fitness components (production of transmission stage spores, host fecundity and mortality) were modified by temperature. We controlled for the effect of spore genotype by including it as a main effect in a linear model with either the parasite transmission or host fitness components as response variables. We then used the residuals from these models (the total variation not explained by spore genotype) to test if the relationship between parasite and host fitness components changed with temperature. We used a general linear model with 'spore per *Daphnia*' residuals as the response variable, and either the residuals for time to host death (for mortality) or residuals for total number of offspring (for fecundity) and temperature in a full factorial model. The effect of interest for our purposes is a significant interaction between the host fitness component and temperature, suggesting that the amount of variation in the number of spores produced per *Daphnia* can be explained by the host fitness component, but in a temperature-dependent manner.

Results

A total of 240 female *Daphnia* were individually exposed to infection (four parasite genotypes \times three temperature treatments \times 20 replicates), of which 26 were lost either due to premature death during the exposure period or due to handling error. Overall, all treatments remained with between 17 and 19 replicates. In all analyses, including replicate as a random variable did not change the outcome, confirming the role of our acclimation period in reducing between replicate variation.

General and parasite-specific costs of parasitism under temperature variation

Daphnia experience sterilization and reduced survival when infected with *P. ramosa* (Ebert *et al.*, 1996). Our experimental design aimed to test whether these costs differed at different temperatures. Fecundity (the total number of offspring produced during the experiment) differed, as expected, between infected and uninfected hosts (Table 1; Fig. 1a). Temperature alone did not have

Fecundity (all data)	d.f.	L-R χ^2	P
Temperature	2	2.648	0.2660
Infection status	1	171.018	< 0.0001
Parasite genotype	3	21.506	< 0.0001
Temperature × infection status	2	15.844	0.0004
Parasite × temperature	6	18.940	0.0043
Fecundity (infected hosts only)			
Temperature	2	4.678	0.1969
Parasite genotype	3	3.129	0.2091
Parasite × temperature	6	7.358	0.2890
Fecundity (uninfected hosts only)			
Temperature	2	17.020	0.0007
Parasite genotype	3	39.016	< 0.0001
Parasite × temperature	6	13.280	0.0388
Mortality			
Temperature	2	10.105	0.0064
Infection status	1	4.053	0.0441
Parasite genotype	3	0.341	0.9521
Temperature × infection status	2	6.511	0.0386
Parasite × temperature	6	3.390	0.7585

The effect of temperature, infection status, parasite genotype and their interactions were tested on host fitness traits (fecundity – the total number of offspring – and host mortality). For fecundity, we also present the summary statistics from the separate analysis of hosts that became infected or remained uninfected after exposure. These analyses were identical to the full model, but infection status was not included as an effect. Fecundity analyses were carried out on square root-transformed total number of offspring in a generalized linear model assuming normal residuals. Mortality was analysed using proportional hazards. L-R χ^2 is the likelihood ratio chi-squared test.

a significant effect on the total number of offspring, but we found a significant interaction between infection status and temperature, showing that being infected will have different costs depending on the temperature at which infections occur (Table 1; Fig. 1a). The significant parasite genotype-by-temperature interaction appears to be due to the fact that hosts exposed to Sp1 have particularly low fecundity at 20 °C (data not shown), but this is true for both infected and uninfected hosts (hence the three-way interaction was not significant). Both a cost of resisting infection and a cost of parasitism could contribute to this pattern. By studying the fecundity of infected and uninfected hosts separately, it is apparent that parasite genotype and temperature have little effect on the fecundity of infected hosts (Table 1, fecundity of infected hosts), but they do effect the fecundity of hosts that remained uninfected following exposure to parasites. The possibility of genotype and temperature-specific costs of resisting infection will be addressed in subsequent experiments (see also Little & Killick, 2007).

Mortality analysis also indicated temperature-dependent costs of infection. In particular, an infection status by temperature interaction was evident (Table 1), being driven by the relatively higher mortality among infected hosts at 25 °C (Fig. 2). However, parasite genotype-specific effects on host mortality were not detected (Table 1; Fig. 2).

Table 1 Summary statistics from generalized linear models testing the cost of parasitism under temperature variation.

Parasite genotype and temperature interactions

Variation in infectivity showed significant main effects of both temperature and parasite genotype (Table 2). The highest proportion of infected hosts occurred at 20 °C (46% on average), followed by 25 °C (36%) and 15 °C (18%) (Fig. 1b). Exposure to parasite isolate Sp1 achieved the highest levels of infectivity (60% on average), whereas isolate Sp8 gave the lowest amount of infection (15% on average) (Fig. 3a). Differences between parasites were consistent across temperatures, i.e. parasite isolate-by-temperature interactions were not evident for infectivity (Table 2). Among infected hosts, spore production was influenced by temperature main effects (Table 2; Fig. 1c), and showed a parasite-by-temperature interaction (Table 2; Fig. 3b).

Does temperature modify relationships between host and parasite fitness traits?

As indicated above, the cost of being parasitized (i.e. virulence) varied with temperature, with higher temperatures leading to greater costs whether measured as mortality or fecundity. Theory on the evolution of virulence assumes particular relationships between parasite transmission and virulence depending on the cost–benefit relationship of host exploitation strategies. Although our experiments were not explicitly designed

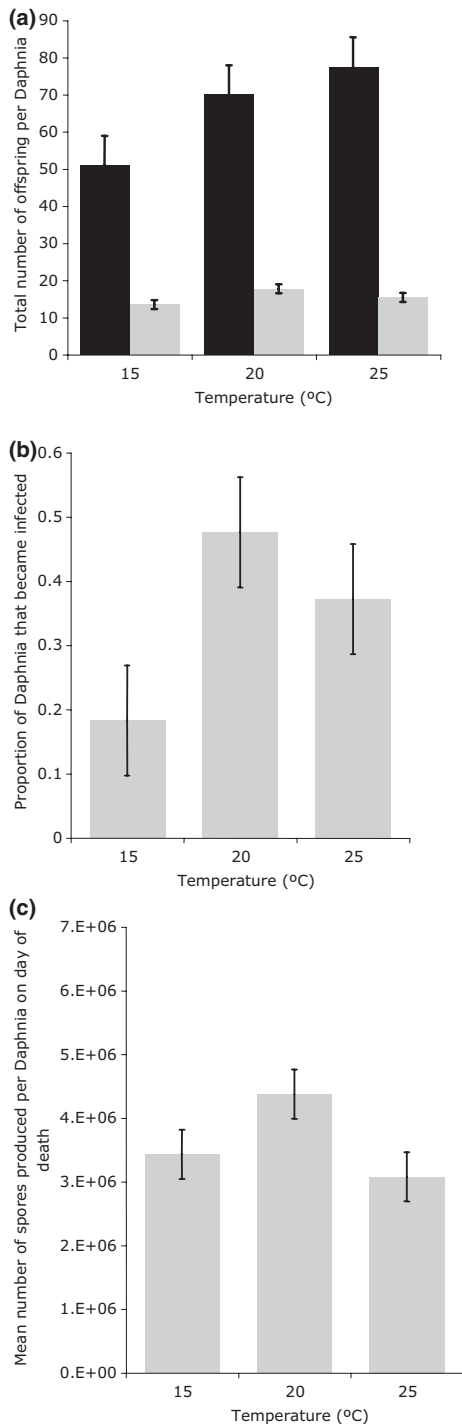


Fig. 1 The general effects of temperature on infection outcomes. (a) The effect of temperature on the total number of offspring produced. Infected hosts (grey bars); noninfected hosts (black bars). (b) General effects of temperature on infectivity (the proportion of hosts that developed infection) and on (c) the mean number of transmission stage spores produced by each *Daphnia magna* during experimental infection with *Pasteuria ramosa* at 15, 20 and 25 °C. See Tables 1 and 2 for statistical details.

to study the nature of these relationships, our data can assess if any relationships exist, and if temperature can modify them. We used a general linear model to determine how transmission potential was related to virulence, and if temperature altered that relationship (virulence-by-temperature interaction). No significant relationship between spore production and fecundity was found (Table 3; Fig. 4a), whereas, for host mortality, we found a significant relationship with spore production (Table 3; Fig. 4b). Virulence-by-temperature interactions were not evident for fecundity or mortality, indicating that the relationship between virulence and parasite transmission is relatively stable to changes in temperature within the range we tested.

Discussion

Genetic variation in traits relevant to infection is often attributed to balancing selection resulting from host-parasite coevolution (Haldane, 1949; Anderson & May, 1982; Woolhouse *et al.*, 2002). However, the maintenance of genetic polymorphism can also occur due to context-dependent selection, as a variable environment can result in changes in the direction and strength of selection on different traits (reviewed in Byers, 2005). Most host-parasite systems experience environmental heterogeneity, and it is therefore highly relevant to enquire about the pervasiveness of genotype-by-environment interactions. The present study showed that the cost of infection to hosts increased with increasing temperature, and that parasite G × E interactions were present for parasite transmission stage production. Other traits (infectivity, host mortality and fecundity) did not show clear evidence of genotype-by-environment interactions. With reference to the trade-off hypothesis for virulence, we also explored if temperature changed the relationship between host and parasite fitness traits, but our results indicated that, when this relationship existed, it was stable across temperatures.

Thermal optima and the cost of parasitism

Both the proportion of infected hosts and the number of transmission stages produced were higher at 20 °C (Fig. 1b,c). Although we do not know the mechanism by which temperature affects the probability of acquiring infection (whether it affects host resistance directly, induces behavioural changes that affect infectivity indirectly or reflects a thermal optimum of the parasite), we can speculate on its effects given what we know regarding *Daphnia* physiology and *P. ramosa* infection biology. Transmission of *P. ramosa* occurs horizontally through the release of water-borne spores from dead infected hosts (Ebert *et al.*, 1996), and infection takes place when spores are filtered by *Daphnia* during feeding. If the likelihood of a host acquiring an infection

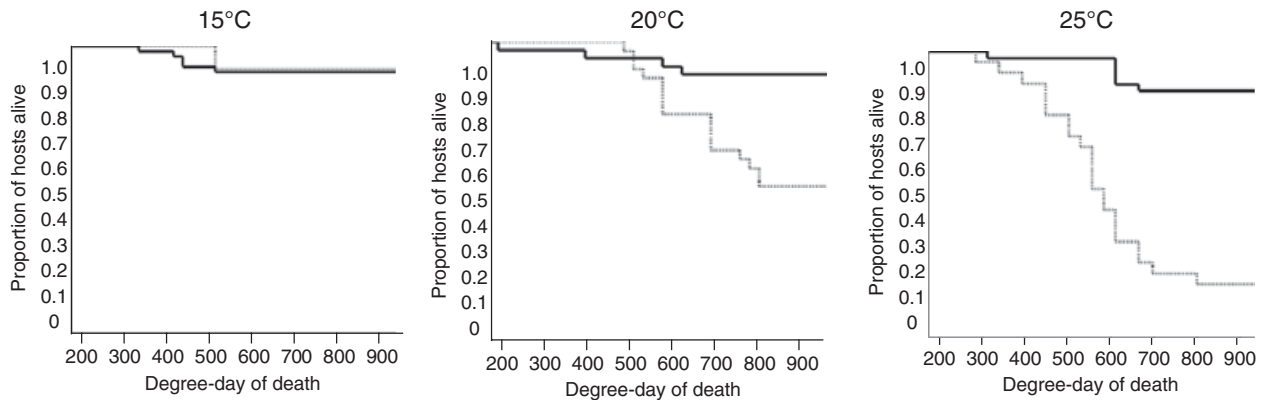


Fig. 2 Survival curves showing the proportion of *Daphnia magna* exposed to *Pasteuria ramosa* during the experiment at 15, 20 and 25 °C. Infected (dotted line); not infected (full line). Timescale is *Daphnia* physiological time (degree-day) to allow comparisons between temperature treatments (see Materials and methods for details). See Table 1 for statistical details.

Table 2 Summary statistics from generalized linear models testing the effects of parasite genotype, temperature and their interaction on parasite fitness traits (infectivity and production of transmission spores).

	d.f.	L-R χ^2	P
Infectivity			
Parasite genotype	3	28.09	< 0.0001
Temperature	2	15.98	0.0003
Parasite \times temperature	6	3.13	0.7930
Number of spores per infected <i>Daphnia</i>			
Parasite genotype	3	1.03	0.7950
Temperature	2	12.22	0.0022
Parasite \times temperature	6	16.52	0.0112

L-R χ^2 is the likelihood ratio chi-squared test.

correlates positively with its filtration rate, we should expect the highest infectivity to occur for maximized filtration rates. Indeed, previous studies on the effect of temperature on *Daphnia* filtration rate have found a maximum close to 20 °C (Lampert 1987). Additionally, spore production in infected individuals was also the highest on average at 20 °C (Fig. 1c), suggesting a possible optimum for parasite growth once successful infection has occurred. However, it is difficult to disentangle this hypothesis from the effect of a larger establishing population due to higher host filtration rate. Estimates of the rate of parasite growth across temperatures would shed light on these issues.

G \times E interactions and the maintenance of polymorphism

The number of transmission stages produced per infection is likely to be an important parasite fitness component. Support for this idea comes from numerous studies showing that dose (i.e. the number of parasite spores a

host is exposed to) strongly influences the likelihood of achieving a successful infection (Ebert *et al.*, 2000; Little & Ebert, 2000; Regoes *et al.*, 2003; Ebert, 2004; Little & Killick, 2007; Ben-Ami *et al.*, 2008). We found that the production of transmission stages was influenced by temperature, but its effect depended on the parasite genotype involved in the infection, i.e. genotype-by-environment (G \times E) interactions were present. For the specific parasite genotypes we tested, there was a switch in the rank order of transmission stage production. For example, infections with isolates Sp1 and Sp8 yielded the most spores at 20 °C but produced the least number at 25 °C, where parasite isolate Sp13 was most productive (Fig. 3b). Given that no parasite isolate outperformed all other isolates across all temperatures, this switching can presumably promote the co-occurrence of distinct isolates in environments where temperature fluctuates.

Although the occurrence of G \times E interactions is generally interpreted as evidence that genetic variation could be maintained due to context-dependent selection (Gillespie & Turelli, 1989), direct evidence linking levels of environmental variation and levels of genetic diversity are not conclusive (Maynard Smith & Hoekstra, 1980; Bell & Rebound, 1997; Byers, 2005). Without prior knowledge of the selection history or past levels of genetic variation, the cause-effect relationship is uncertain (Byers, 2005). Many of the quantitative models developed to examine the conditions that would favour such a link have also reached mixed conclusions (Levene, 1953; Gillespie & Turelli, 1989; Sasaki & de Jong, 1999), and tend to require strict conditions; specifically, that the contrast in the fitness effects of traits in different environments needs to be considerable in order for genetic variation to be maintained (Maynard Smith & Hoekstra, 1980).

Maintenance of polymorphism by environment-dependent selection requires fitness differences between genotypes across environments. The production of transmission stages (a reflection of within-host growth) is an

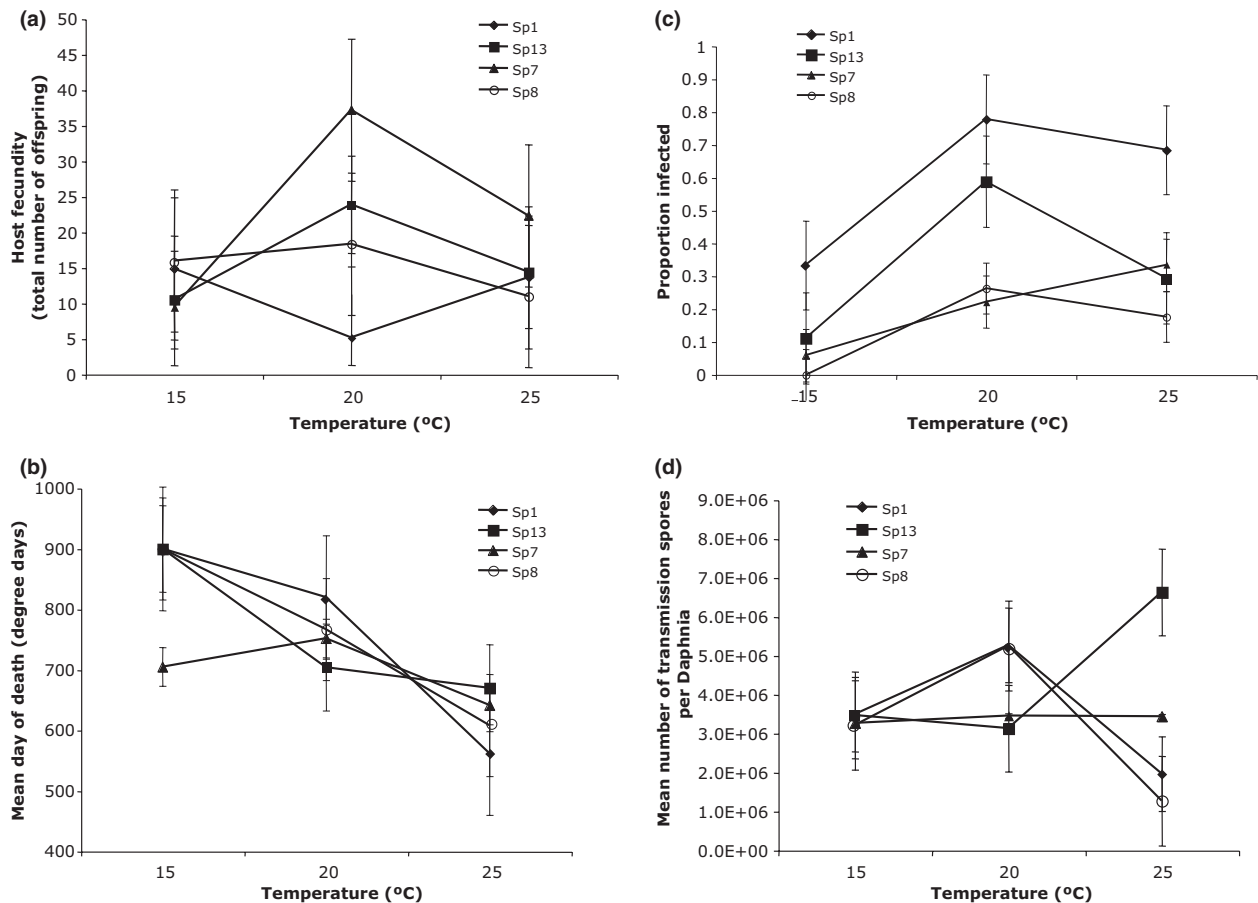


Fig. 3 Reaction norms for infection-related traits when infected with each spore type. (a) Infectivity (the proportion of exposed hosts that developed infection) and (b) the number of transmission spores per infected *Daphnia*. Error bars are standard error of the mean. See Table 2 for statistical details.

Table 3 Summary statistics from generalized linear models testing if the relationship between parasite transmission (the number of spores per *Daphnia*) and virulence (measured in terms of host fecundity and mortality) was modified by temperature (virulence-by-temperature interaction).

	d.f.	F ratio	P
Temperature	2	1.172	0.3205
Fecundity (residuals)	1	0.005	0.9424
Temperature × fecundity (residuals)	2	0.625	0.5407
Temperature	2	0.227	0.7979
Mortality (residuals)	1	13.365	0.0008
Temperature × mortality (residuals)	2	0.7640	0.4726

The analysis was performed on residuals after controlling for spore genotype (see data analysis section in methods).

important parasite fitness component as it strongly influences the likelihood of subsequently transmitting to and infecting susceptible hosts. Although the $G \times E$ interaction we found for this trait across temperatures

would suggest that parasite genotypes differ in fitness across temperatures, producing transmission stages is not the only trait relevant for parasite fitness. For example, being able to infect a host is most likely a strong determinant of parasite fitness, as without gaining entry into the host, no transmission stages are produced. Infectivity is a trait that has been shown in previous work in this system (Carius *et al.*, 2001) to be determined by the specific combinations of host and parasite genotypes (genotype-by-genotype interactions). Therefore, experiments incorporating genetic variation for both host and parasite would provide a more complete picture of how parasite fitness is affected by environmental variation.

Understanding which fitness traits are important and how environmental variation affects them in the wild is necessary to fully comprehend how genetic variation is maintained. Testing for differences in fitness effects in natural populations presents a formidable challenge, as it requires information about genetic variation and

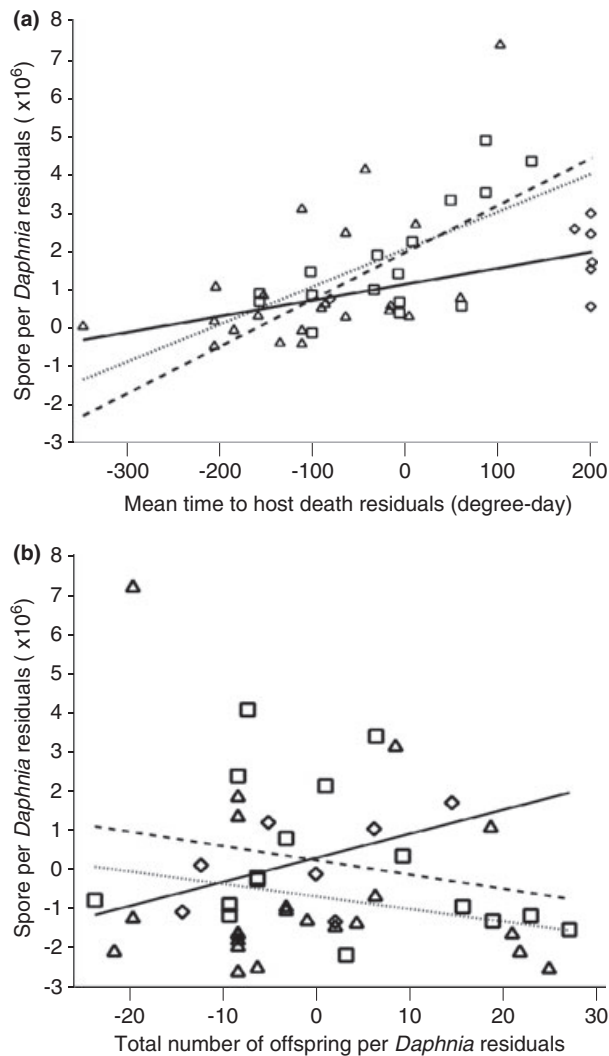


Fig. 4 Scatterplots of parasite and host life-history traits at 15 °C (\diamond – black full line), 20 °C (\square – black dashed line) and 25 °C (\triangle – dotted line), after correcting for the effect of parasite genotype (residuals shown). (a) Number of spores produced plotted against host fecundity (the total number of offspring), per infected host. (b) Number of spores produced plotted against host mortality (the mean time to host death). Only data for infected hosts that died before the end of the experiment are included. See Table 3 for statistical details.

how frequencies of specific genotypes change in response to environmental variation. Tracking parasite genotype frequencies and temperature in the wild, as has been achieved for hosts (Little & Ebert 1999, Carvalho 1987), would provide an idea of how parasite fitness varies under thermal variation. This could be complemented with laboratory experiments using the same parasite genotypes under controlled temperature treatments to test their effect on parasite fitness components. However,

although there is evidence for *P. ramosa* genetic variation (Carius *et al.*, 2001; Jensen *et al.*, 2006; Little *et al.*, 2007b), and some *P. ramosa* genetic markers have been developed (Mouton *et al.*, 2007; Mouton & Ebert, 2008), these have not yet revealed substantial *P. ramosa* within-population genetic variation.

The relationship between host and pathogen fitness traits and the evolution of virulence

The evolution of life-history traits depends on the expression of heritable genetic variation (Falconer & Mackay, 1996). Several studies of genotype-by-environment interactions have shown that such expression differs with the environment (Falconer & Latyszewski, 1952; Ebert *et al.*, 1993), with implications for the expression of host and parasite life-history traits (Laine, 2007). However, traits are also correlated with each other, possibly leading to constraints on their evolution imposed by trade-offs, a key component of life-history evolution theory (Stearns, 1976; Falconer & Mackay, 1996). Such theory is at the basis of the mathematical framework developed to understand virulence evolution that assumes that: (a) transmission and virulence are coupled; and (b) pathogen evolution will proceed by a trade-off between within-host growth (and the consequent fitness cost to the host) and between-host transmission (Anderson & May, 1982; Frank, 1996). It is possible that the strength and direction of such relationships are environment specific (Sgro & Hoffmann, 2004). In particular, changing the relationships between host and parasite traits could modify the costs associated with exploiting the host and potentially alter the course of virulence evolution.

We found a positive relationship between parasite transmission (measured as the number of spores produced during an infection) and the survival time of infected individuals, and this relationship was robust to changes in temperature (Table 3; Fig. 4b). Such a relationship is expected, as there is a direct cost to the parasite in killing its host too quickly as it will have less time to produce spores. For obligate killing parasites (where host death is required for transmission to occur), theory suggests that virulence should evolve such that parasites maximize their use of host resources, killing them around the time when host growth decelerates (Ebert & Weisser, 1997). Empirical support for such an optimum for virulence has been found in this system, where parasite lifetime transmission was maximal when hosts died between 50 and 55 days, coinciding with a phase of decelerating growth (Jensen *et al.*, 2006). In comparison with our study, this work was performed at 20 °C, hence 50–55 days corresponds to approximately 1000 degree-days. As our experiment only continued until degree-day 900, we are most likely on the left-hand side of the hump-shaped optimum curve, where the relationship between lifetime transmission and host survival is positive.

For another measure of host fitness (fecundity), there was an unexpected lack of relationship with parasite spore production (Table 3; Fig. 4a). Host resources that are not allocated by the host to reproduction are available to the parasite; hence, parasites should evolve to castrate hosts as soon as possible to maximize resource availability (Ebert *et al.*, 2004). Therefore, in the absence of any constraint, we should expect a strong negative relationship between parasite fitness and host fecundity as they both compete for the same resources, and this has indeed been shown in previous *Daphnia*–*Pasteuria* studies (Ebert *et al.*, 2004). Possibly, in our experiment, resources were abundant to the point where parasite growth was maximized while hosts were still able to allocate resources toward reproduction, thereby weakening the expected negative relationship between parasite and host fitness. Although the food levels we used were similar to the ones reported in Ebert *et al.* (2004), it is still possible that food quality differed between the two studies. Although we have no way of testing this possibility, we find no alternative explanation why parasite fitness should show no relationship with host fecundity. Interestingly, the slopes diverge between temperatures and the negative slope for 20 and 25 °C is the expected pattern (Fig. 4a). Relationships between host and parasite fitness traits have been shown to change depending on the host and parasite genotypes (Salvaudon *et al.*, 2005, 2007), but further experimentation is needed to ascertain if these relationships also change with abiotic environmental variables, such as temperature.

In summary, we have presented evidence for temperature-dependent costs of parasitism and for the potential maintenance of genetic variation in parasite infection traits through the presence of G × E interactions for spore production. We also explored the possibility that temperature could alter the relationships between host and parasite traits that could direct virulence evolution, but found that when these relationships existed they did not change across temperatures. Although our experimental system certainly does not capture either the full levels of host and parasite genetic variation or the environmental heterogeneity found in the wild, it adds strength to the increasing realization that knowledge of how environmental variation affects infection parameters is essential for our understanding of disease ecology and evolution.

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The role of the environment in the evolutionary ecology of host parasite interactions

Meeting report, Paris, 5th December, 2007

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1. Introduction

It has long been recognised that the expression of quantitative traits will be different depending on the environment (Falconer, 1952). If traits affecting fitness are expressed differently in different environments, this could lead to changes in the direction and strength of selection on these traits. If the sign and magnitude of fitness differences between genotypes changes across environments (termed genotype-by-genotype ($G \times E$) interactions), this could promote the co-occurrence of different genotypes through heterogeneous selection (Gillespie and Turelli, 1989; Byers, 2005).

Over the past few years, the application of this idea to host–parasite systems has generated work addressing the role of environmental variation on the expression of traits involved in infection (Ferguson and Read, 2002; Mitchell et al., 2005; Lambrechts et al., 2006a; Salvaudon et al., 2007), and on the general impact that context-dependent selection may have on the coevolutionary process (Thompson, 1994, 1999). The environment of parasites is made up of many factors. First, the genotype of the host can be considered an “environment” in which the parasite must survive, and interactions between host and parasite genotypes ($G \times G$ interactions) can result in

shared control of epidemiological traits by both host and parasite, with implications for evolutionary trajectories of virulence and resistance (Restif and Koella, 2003; Lambrechts et al., 2006b; Salvaudon et al., 2007). Moreover, various biotic or abiotic factors may affect the expression of host and parasite traits, thereby adding another level of complexity ($G \times G \times E$ interactions).

What are the consequences of this complexity? From a standard quantitative genetics point of view, the efficiency of selection on host and parasite genotypes will depend on the expressed genetic variance, and such expression of variance is known to be environment-dependent (Falconer, 1981). Consequently, environmental variation may influence the intensity of coevolution, potentially creating coevolutionary cold and hot spots in different environments (Thompson, 1994, 1999). Further, selection may favour different (combinations of) host and parasite genotypes in different environments, thus shaping the geographic distribution of genetic diversity and patterns of local adaptation in host and parasite. A strong impact of the environment may even alter coevolutionary trajectories, thereby generating different evolutionary optima for attack and defence (Hochberg and van Baalen, 1998) or changing the nature of the interaction (e.g., from mutualistic to antagonistic). In this context, it is important to remember that evolution is a population-level process and that the effect of selection will depend on several aspects of population genetics, such as population size, spatial structure or migration, all of which are themselves potentially influenced by environmental conditions.

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Despite the increasing number of examples from laboratory host–parasite systems indicating the occurrence of environment-dependent interactions, their importance in changing the strength and direction of selection in the field remains obscure. Thus, perhaps the most challenging question is then to ask how robust genotype interactions are against environmental variation in the wild: does the “E” in $G \times G \times E$ really matter? Answers to this question will not only provide important insights in the coevolutionary process and the causes of the maintenance of genetic diversity, but also matter from an applied perspective. Indeed, being able to predict the fate of particular genes or genotypes (e.g., introduced resistance genes) in variable environments is of extreme importance for disease control programs. A 1-day meeting (5th December 2007), organized by the *Laboratoire de Parasitologie évolutive* (Université Pierre & Marie Curie, Paris, France) and *Laboratoire Ecologie, Systématique et Evolution* (Université Paris-Sud 11–CNRS, France), brought together researchers from Europe and the USA, working on a variety of microbial, animal and plant systems, to discuss the role of the environment on the evolutionary ecology of host–parasite interactions. Here follows a report on the proceedings of this meeting.

2. Summary of presentations

The meeting was divided into two sessions, each concluding with a general discussion. The morning session focused on the coevolutionary process and how experimental studies can contribute to the understanding of evolution in variable environments. Michael Hochberg (University of Montpellier) highlighted the importance of considering variation in ecological processes. Describing an experimental coevolution approach using the *Pseudomonas fluorescens*-phage Phi2 host–parasite system (Buckling and Rainey, 2002), he explored how varying levels of disturbance could affect the evolution of resistance to parasites. His findings indicated that the highest levels of resistance occur at intermediate levels of disturbance, presumably because the force of infection is highest at intermediate levels.

Fabrice Vavre (University of Lyon) continued the session by discussing work from several insect systems where vertically transmitted bacterial symbionts (among which *Wolbachia*) are present. He described work showing that cost of *Wolbachia* infections and bacterial load is not only temperature dependent (Mouton et al., 2006) but sometimes depends on the specific combinations of genotypes of *Wolbachia* that are present and on the host genotype (Mouton et al., 2004; Mouton et al., 2007). More generally, it was shown that the co-occurrence of different genotypes or species of symbionts within the same host species was different from random. This suggests that the presence of a particular symbiont can create an extended host phenotype protecting the host against infection by other symbionts, against attack by other enemies (Oliver et al., 2003) or allowing the extension of the host niche (Tsuchida et al., 2004).

This was followed by an example of how environmental variation can be an important factor when applied to agricultural systems. Mamadou Mboup (INRA-AGROPAR-

ITECH, Grignon) spoke about his work on the plant pathogen Wheat Yellow Rust (*Puccinia striiformis*). In France, this pathogen exhibits a geographical structure, with some isolates only existing in the North or only in the South. Controlled greenhouse and field experiments revealed temperature-dependent variation in germination and infection rates among pathotypes, likely to confer a selective advantage to Southern pathotypes to the higher temperatures in the South. Conversely, Southern pathotypes are not found in the North because they cannot infect Northern wheat cultivars.

The morning session finished with Pedro Vale (University of Edinburgh) describing work on the freshwater crustacean *Daphnia magna* and its naturally occurring bacterial parasite *Pasteuria ramosa*. In experiments that included both host and parasite genetic variation and thermal variation, he showed evidence for temperature-dependent costs of parasitism, and for the presence of $G \times G$ interactions for infectivity and $G \times E$ interactions for parasite transmission stage production and time to host death. Although there was no evidence in these experiments that $G \times G \times E$ interactions occurred (i.e. patterns of infectivity were generally robust to environmental variation), variation in the number of transmission stage spores produced could alter infectivity levels in subsequent infection cycles, as spore dose has been shown to affect infectivity in this system.

The afternoon session was aimed at discussing how to integrate environmental fluctuations into theoretical models of host and parasite evolution. Curiously, only two out of the five speakers described work using a mathematical modelling approach. This is possibly a reflection of how the theoretical tools to study these effects still lag behind the experimental evidence for their occurrence. Olivier Restif (Cambridge University) alluded to this problem, saying that traditionally, ecological interactions such as competition, predation, and parasitism have all been studied separately and as such have their own theoretical frameworks. He attempted to integrate at least two of these interactions (competition and parasitism) by developing a model where hosts vary in their resistance (reduced likelihood of becoming infected) and tolerance (reduction in the detrimental effects of infection) under varying levels of migration and fragmentation. He showed how this approach could be useful to understand under what conditions we can expect variation in resistance and tolerance to coexist. Building upon existing parasite-mediated competition models (Miller et al., 2005), he suggested that coexistence depended strongly on the degree of fragmentation of the host population. Nevertheless, when the same questions were investigated with stochastic simulations, transient dynamics associated with small population sizes modified the outcome of competition in the presence of a shared parasite. This illustrates the great importance of the modelling methodology.

Benjamin Roche (Institut de Recherche pour le Développement, Montpellier) also presented a mathematical modelling approach to elucidate the most likely transmission routes of avian influenza. He hypothesised that water-borne transmission was a likely transmission route and supported this by fitting data collected from bird populations in Southern France to a Susceptible-Infected-Removed epidemiological model, to

which he incorporated an additional class of water-borne transmission.

The remainder of speakers described results from experimental systems. Peter Tiffin (University of Minnesota) added an interesting twist by describing $G \times G \times E$ interaction not in a host–parasite system but in a Legume–*Rhizobium* mutualism. From the plant perspective, the rhizobia are beneficial because they provide nitrogen to the plant but the mutualism involves a cost of carbon needed to maintain the rhizobia. He argued that the unstable dynamics observed in antagonist coevolution between hosts and parasites could also be expected in mutualisms if sub-optimal rhizobia genotypes (or “cheaters”) are common and plant hosts evolve to preferentially associate with or reward rhizobia genotypes that are more beneficial. The experiments he described with a genetically variable *Medicago truncatula*–*Sinorhizobium medicae* system showed evidence for $G \times G$ interactions. Moreover, mixed inoculations by two *Sinorhizobium* genotypes were more costly to the host than single inoculations, but only when nitrogen was added to the soil—suggesting that the selection acting on species involved in mutualisms will depend on both the abiotic and biotic environment (Heath and Tiffin, 2007).

Richard Preziosi (University of Manchester) followed, using a community genetics approach to study the interaction of barley and aphids in the absence and presence of rhizosphere bacteria. Community genetics aims to ascertain how much genetic variation in one species affects other species in the community. Within this framework he provided a further example of $G \times G \times E$ interactions, and in one example these explained almost 40% of the variation in host fitness (Tetard-Jones et al., 2007). R. Preziosi emphasised the need to quantify the effect sizes of these interaction effects if we are to gain insight into their relevance in the wild.

Lastly, Oliver Kaltz (University Pierre & Marie Curie, Paris) also described experimental work carried out by his research group. He asked the question “What if hosts and parasites have different thermal optima?” He provided an answer using a model system consisting of the protozoan *Paramecium caudatum* and its bacterial parasite *Holospora undulata*. By conducting experimental infections at 23 and 35 °C, he found that infection increased host survival at high temperature, possibly due to parasite-induced over-expression of heat-shock proteins. However, in experimental populations, prevalence rapidly declined at 35 °C, indicating that the parasite cannot survive at this temperature. Despite these general effects, the amount of genetic variation in tolerance expressed in the host varied between temperatures, suggesting that strength of selection could be environment-dependent.

3. Discussions and perspectives

This meeting provided a forum for stimulating discussion regarding the relevance of interactions between genotypes and environment in natural host–parasite systems. A positive aspect was the presence of researchers working on a broad spectrum of interactions – from mutualists to obligate killing parasites – in both animal and plant systems. Bringing together this diverse

expertise served to highlight that $G \times G$ and $G \times E$ interactions are ubiquitous in host–parasite systems, at least when assessed in experimental settings. Below we highlight some of the questions addressed during the open discussion sessions.

3.1. Does environmental variation affect coevolutionary outcomes?

It is still unknown whether the interactions between genotypes and with the environment are important factors driving the evolution of host–parasite relationships, or if they are mainly noise, introducing some variation in the expression of traits, but not enough to override the main genotypic effects. Indeed, if the contrast in fitness effects due to environmental variation were not large, this would reduce the relevance of such interactions in affecting the coevolutionary process, and question their importance in the maintenance of genetic variation (Maynard Smith and Hoekstra, 1980; Gillespie and Turelli, 1989; Byers, 2005). The general opinion was that any attempt to answer this question would require more sampling of natural populations in order to gain information on levels of genetic variation in traits involved in the infection process. This can be complemented by experimental evolution approaches that test specific hypotheses about how coevolution could be affected by environmental variation, and using modelling approaches are instrumental in generating testable hypotheses.

3.2. Testing the effect of environmental variation on coevolution

Apart from identifying genotypes, the challenge in natural populations is equally to identify the relevant environmental factors at play and design the appropriate experiments to validate their effects. Experimental evolution provides an approach to address the role of the environment. By manipulating the relevant factors in experimental microcosms, we can explore the impact of environmental conditions on real-time coevolutionary change. However, it was generally agreed that this potentially very powerful approach has nonetheless important limits. While it can help us to generate hypotheses or to validate specific predictions of theoretical models, it remains restricted to particular model systems and simple experimental communities. Simply identifying that traits are expressed differently in different environments only tells us that $G \times E$ interactions can occur, but without quantifying the effect sizes of these interactions in natural populations we can say little about how or if they will change evolutionary outcomes. Thus, clearly, experimental evolution cannot replace studies on $G \times G \times E$ in natural populations. To this end, P. Tiffin raised the potential for testing whether local adaptation has altered the relative costs and benefits in the *Medicago*–*Sinorhizobium* mutualism by examining populations growing in environments with differing levels of abiotic N availability—such as those near and far from agricultural fields. Studying environmental variation and measuring selection coefficients in natural populations still presents a formidable challenge.

3.3. How does environmental spatiotemporal variation affect coevolutionary processes?

Many factors could modify the effects of $G \times G \times E$ interactions host–parasite coevolution and several speakers underlined the important role of explicit spatial structure and temporal variation. In particular, gene flow due to migration could disturb adaptation to local environments, or make it difficult to identify adaptations to particular environments, thereby increasing the spatial scale of field studies needed to identify $G \times E$ interactions. This relates directly to the issue of discerning the spatial scale at which environmental variation occurs (e.g. microclimate in the immediate neighbourhood of a plant vs. regional average temperature). The relevance of considering the temporal scale of fluctuations was also discussed. Generally, if the environment varies very quickly (daily variation in temperature, for example) then there might not be enough time for selection to produce specific adaptations to any one environmental condition. In such cases, instead of maintaining genetic variation through heterogeneous selection, this could select for phenotypic plasticity and generalist strategies. What this means in terms of host–parasite interactions remains unclear. To our knowledge, no one has explicitly integrated environmental fluctuations in the theoretical framework of host–parasite coevolution.

3.4. Implications for health and disease

The importance of interactions between host and parasite genotypes and with their environment is not only important for our understanding of evolution, but could also have applied consequences for health policies. If nothing else, it emphasizes the need to consider the role of heterogeneity – genetic and environmental – in host–parasite systems. Indeed, if infection outcomes are context-dependent, anti-parasitic intervention strategies could be thwarted when the environmental variation encountered in the wild is not taken into account.

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Parasite transgenerational effects on infection

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ABSTRACT

Question: Do past conditions experienced by parasites mediate current levels of infectivity and virulence in the host–parasite combination of *Daphnia magna* and *Pasteuria ramosa*?

Methods: We varied either temperature (three levels: 15, 20 or 25°C) or food supplied to the host (two levels) during a primary infection event, and then harvested parasites and measured their infectivity during a secondary infection event that was subject to the same environmental variation.

Result: Past temperatures did not influence any of the infection-related traits measured. By contrast, past food conditions appeared to impact infection, with parasite spores originating from well-fed hosts generally being more harmful. There was no indication that parasites had become specialized to their past environment. Four host genotypes were included in the experiment, and there was evidence that one of four was more sensitive to the environmental history of parasites than were the other hosts, i.e. there was an interaction between host genotype and parasite treatment effects.

Conclusion: Overall, parasite transgenerational effects appear to influence the level of harm parasites cause.

Keywords: *Daphnia*, genetic variation, genotype × environment interaction, maternal effect, parasitism, *Pasteuria*, pathogen, virulence.

INTRODUCTION

Parasites are ubiquitous and are important factors in host population dynamics and community structure (e.g. Anderson and May, 1982; Hudson *et al.*, 1998; Boots and Sasaki, 2003) and may even be responsible for the maintenance of genetic diversity and sexual reproduction in their hosts (e.g. Hamilton, 1980; Lively, 1987). Infection outcomes are, like all traits, dependent on the genotype, the environment they experience, and genotype × environment interactions. An additional aspect of environmental variation that is increasingly recognized to have a profound impact on the expression of disease, among other traits (see Mousseau and Fox, 1998), is the condition experienced by the parental or previous generation.

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Transgenerational effects on resistance have been demonstrated in a wide range of vertebrate and invertebrate taxa. For example, in mammals, nutrient deficiencies in mothers limit the development of the offspring immune system (Gershwin *et al.*, 1985), while food stress in the crustacean *Daphnia* appears to enhance parasite resistance in their offspring (Mitchell and Read, 2005). Comparable cross-generational effects in parasites have received less attention. Certainly, the evolution of parasites/pathogens has been widely studied (Ebert, 1998, 1999; Mackinnon and Read, 2004; McClelland *et al.*, 2004; Read *et al.*, 2004), and there is ample evidence for environmentally dependent expression of parasite traits, such as life history (Buckling *et al.*, 1997; Gemmill *et al.*, 1997; Kaltz and Koella, 2003; Poulin, 2003; Schjorring, 2004) and virulence (Wedekind *et al.*, 1998; Bedhomme *et al.*, 2004). However, limited data are available regarding one clearly important circumstance: how the environmental conditions parasites experience at one point in time determines their subsequent infectivity or virulence. There are two such studies we are aware of: Little *et al.* (2006) conditioned a bacterial parasite of *Daphnia* in a range of different host genotypes and showed that this experience did not influence the outcome of later exposures, while Tseng (2006) reared a protozoan mosquito parasite in hosts fed either high or low food to show that parasites originating from well-fed hosts were more virulent to subsequent hosts.

In the present study, we examined the infectivity and virulence of parasites that were conditioned in a range of host environments (high or low food, or under a range of temperatures). We specifically sought to determine whether the condition of the host populated by one generation of parasites could mediate susceptibility and infection outcomes in the next, which would shed light on the speed with which parasites can become specialized to their environments in the short term. The parasite we studied is a bacterium and as such has the potential to mutate and evolve rapidly. Thus, it is not possible to resolve whether any conditioning effects were due to genetic change or changes in expression patterns (plasticity), although over the short time-frames we studied plasticity was expected to dominate. Nevertheless, we were motivated to determine if environmental variation impacted, whatever the mechanism, transmissibility and virulence across a transmission event.

METHODS

Study system

Daphnia magna is a planktonic crustacean found in still freshwater bodies and is host to many bacterial, microsporidian, and fungal parasites (Green, 1974; Stirnadel and Ebert, 1997; Little and Ebert, 1999). *Pasteuria ramosa*, the best studied of the *Daphnia* parasites, is a bacterial, spore-forming, obligate endoparasite of *D. magna* that greatly reduces host fecundity. Transmission is horizontal, achieved by the release of spores from the decomposing cadavers of previously infected hosts (Ebert *et al.*, 1996).

The *D. magna* and *P. ramosa* used in the present study were collected from a farm pond at Leitholm, Scottish Borders (2°20.410'W, 55°42.131'N) in the summer of 2003 and maintained in the laboratory in a state of clonal reproduction. Both experiments presented here used the same four host clones, which were originally isolated as isofemale lines and subsequently shown to differ at allozyme loci. Initial infections in the F₀ generation were performed using a diverse mixture of parasite spores (see Mitchell and Read, 2005; Mitchell *et al.*, 2005).

Parasite conditioning in the F₀ generation

For the temperature studies, parasites were passaged through hosts at 15, 20 and 25°C and harvested from infected hosts sacrificed on degree day 600 as described in a previous study (Mitchell *et al.*, 2005) and which is long enough for the development of transmission spores (Ebert *et al.*, 1996). Degree day is simply the actual number of days multiplied by the appropriate temperature and is used to standardize comparison of organisms studied at different temperatures. Mitchell *et al.* (2005) have shown that degree days accurately represent physiological time in *Daphnia*. For the food level studies, we grew parasites on hosts kept at 20°C and fed them either high food (3.5×10^6 algal cells per *Daphnia*) or low food (1.5×10^6 algal cells per *Daphnia*), and harvested parasite spores on day 30 for subsequent use in the F₁ generation.

Infection assays in the F₁ generation

Host standardization

The food and temperature experiments were conducted and analysed separately, although every effort was made to make them comparable by keeping methods similar. Before the experiments, replicates of each of the four *D. magna* clones were acclimated under standardized conditions at a 14:10 hour light:dark cycle in controlled climate chambers at 20°C. Regarding temperature, prior acclimation conditions have been shown to have little impact, at least compared with current conditions, on *Daphnia* life history and susceptibility (Mitchell *et al.*, 2005). Prior food conditions can affect susceptibility, but interactions between prior conditions and current conditions are not evident for either food (Guinee *et al.*, 2007) or temperature (Mitchell *et al.*, 2005). *Daphnia* were kept in synthetic pond medium (Klüttgen *et al.*, 1994), and were fed exclusively on *Scenedesmus obliquus*, a green algae cultured in chemostats with Chu B medium. During acclimation, clonal replicates were fed 3.5×10^6 algal cells per *Daphnia* per day and maintained as 5 females in 200 ml (temperature experiment) or 20 females in 2000 ml (food experiment) that was changed three times a week. Host acclimation lasted for three generations, where each generation was started using second or third clutch neonates. Acclimating all replicates for three generations is a process designed to ensure that each replicate is independent, enabling a split-brood experimental design (see Ebert *et al.*, 1998), where replicate need not be entered into statistical models.

Temperature

For the temperature experiment, there were five replicates of each clone. From each replicate, groups of five female offspring less than 24 hours old were assigned to a treatment and placed in a jar containing 60 ml of *Daphnia* medium. Each jar contained a teaspoon of purified sand at the bottom, which tends to reduce variation in infection levels and increases the incidence of infection (Mitchell *et al.*, 2004). To each jar, we added 1×10^5 *P. ramosa* transmission spores. These spores were from the F₀ generation of parasites, and thus there were three types of 'parasite history': previously grown at 15, 20 or 25°C. The infection period was 150 degree days (i.e. 6 days at 25°C, 7.5 days at 20°C, and 10 days at 15°C). Every day, until degree day 150, each jar was stirred to increase chances of contact with parasite spores. One degree day after degree day 150, each group of five *Daphnia* were transferred to a jar containing 200 ml of *Daphnia* medium. Each jar was checked for newborns daily. When

newborns were present, the adult females were moved to a new jar and the offspring counted. In the absence of any clutches, *Daphnia* were transferred to a new jar with fresh medium every 60 degree days. The experiment finished on degree day 600. During the infection period, *Daphnia* were fed 3.5×10^6 algal cells per *Daphnia* every other day, but this was increased to 3.5×10^6 algal cells per *Daphnia* per day afterwards until the end of the experiment. The comparatively low level of food during the infection period encourages the *Daphnia* to graze the sand, increasing contact with the parasite.

Food

For the food experiment, there were six replicates of each clone. From each replicate, groups of five newborns less than 24 hours old were assigned to a treatment and placed in a jar containing sand and parasite spores. These spores were from the F_0 generation of parasites, and thus represented two types of ‘parasite history’: they were parasites previously propagated through hosts fed either low food (3.5×10^6 algal cells per *Daphnia* every other day) or high food (3.5×10^6 algal cells per *Daphnia* every day). The infection period was 7 days and each jar was stirred daily. On day 8, each group of five *Daphnia* was transferred to a jar containing 200 ml of *Daphnia* medium. Each jar was checked for newborns daily, and when present the adult females were moved to a new jar and the offspring counted. In the absence of any clutches, *Daphnia* were transferred to a new jar with fresh medium every 3 days. The experiment finished on day 30. During the infection period, all *Daphnia* were fed 3.5×10^6 algal cells per *Daphnia* every other day. The low and high food treatments, which were identical to those used in the F_0 generation, were realized during the post-infection period: *Daphnia* assigned to the low current food treatment were fed 3.5×10^6 algal cells per *Daphnia* every other day, while *Daphnia* assigned to the high current food treatment saw their food levels double to 3.5×10^6 algal cells per *Daphnia* per day. The food experiment was conducted at 20°C.

Analysis

We used general linear models as implemented in SAS procedure GENMOD to determine how the response variables infectivity (the proportion of hosts infected), host offspring production per host, and survivorship (the proportion of hosts surviving in each jar at the end of the experiment) were affected by the ‘parasite history’, ‘current conditions’, and ‘host clone’ in a fully factorial model. Offspring production largely mirrors infectivity (the main effect of infection is a loss of reproduction) but may reflect additional information, for example if the length of the pre-patent period varies among treatments. For the two response variables based on proportions (infectivity and survivorship), we defined the error distribution to be binomial (DIST = BIN, LINK = LOGIT options in GENMOD), while offspring production was analysed using basic analysis of variance (ANOVA). The response variable age at first reproduction (which is the first day that offspring were observed in a jar) was analysed using proportional hazards analysis (age at first reproduction is essentially a ‘time to event’ variable). For the temperature experiment, ‘parasite history’ and ‘current conditions’ were entered as continuous variables, while for the food experiment the two food levels were fixed factors. Host clone was a fixed factor. Two manipulations of the data were required. First, the offspring counts were square root transformed to meet the assumptions of ANOVA. Second, for the temperature experiment, we converted the time-scale from real time to degree days to enable direct comparison among temperatures. Although our three

generations of host acclimation were designed to make replicates truly independent, we nevertheless repeated the analysis with replicate (nested with host clone and treated as a random effect) as a further explanatory variable, but this had no effect on the outcome of the statistical analyses (data not shown).

RESULTS

Temperature

Forty-five percent of hosts became infected in the temperature experiment. 'Current temperature' had a strong impact on infectivity and host reproduction in the face of parasitism (Table 1, Fig. 1). However, 'parasite history' did not have a significant influence on these response variables either as a main effect or through an interaction with current conditions. Host clone effects were highly significant, although they showed no interaction with either 'current temperature' or with 'parasite history' (Table 1). Age at first reproduction was not affected by 'parasite history', 'current conditions', clone effects or any interactions between them. Survivorship was significantly influenced by 'current temperature' and also differed among clones (Table 1).

Food

Thirty-two percent of hosts became infected in the food experiment. 'Current food' had a strong impact on both the proportion of hosts infected and host reproduction in the face of parasitism (Table 1 Fig. 2). 'Parasite history' had no effect on infectivity or offspring production, and there was no interaction between 'parasite history' and 'current conditions' (Table 1, Fig. 2). Host clone effects were significant for both the proportion of hosts infected and offspring production (Table 1). For offspring production, interactions between host clone and both 'current food' and 'parasite history' were evident (Table 1, Fig. 3). Age at first reproduction was influenced by current food conditions, but not affected by 'parasite history', clone effects or any interactions between them. Survivorship was influenced by 'current food', clone effects and, notably, 'parasite history' where hosts exposed to parasites previously grown on poorly fed hosts survived longer (proportion surviving = 0.79, standard error = 0.031) than those exposed to parasites previously grown on well-fed hosts (proportion surviving = 0.66, standard error = 0.038).

DISCUSSION

This study propagated parasites for one host generation in one of a range of environments, and then for a second generation propagated them in either the same or a different environment. These experiments were designed to study how conditions experienced by the previous generation of parasites might mediate current levels of infectivity and virulence. Present temperature clearly influences infection outcomes in our study system (see also Mitchell *et al.*, 2005), but past temperatures experienced by parasites appeared not to be important for current infection. This pattern is similar to that in a previous study that showed that past exposure to different host genotypes did not affect current patterns of infectivity (Little *et al.*, 2006). In the present study, the amount of food currently available to hosts had a strong impact on infection outcomes, and, in contrast to past temperatures, past food conditions

Table 1. Results of statistical analyses relating four host traits to the effect of current environment and the environment parasites experienced one generation previously

	Test statistic	d.f., error d.f.	<i>P</i>
TEMPERATURE			
Infectivity			
Current temperature	6.90	1, 161	0.009
Parasite history	0.11	1, 161	n.s.
Host clone	40.80	3, 161	<0.0001
Offspring			
Current temperature	8.60	1, 158	0.004
Parasite history	1.10	1, 158	n.s.
Host clone	19.10	3, 158	<0.0001
Age at first reproduction			
Current temperature	1.80	1, 158	n.s.
Parasite history	1.10	1, 158	n.s.
Host clone	4.90	3, 158	n.s.
Survivorsip			
Current temperature	17.10	1, 174	<0.0001
Parasite history	0.12	1, 174	n.s.
Host clone	12.23	3, 174	0.007
FOOD			
Infectivity			
Current food	7.58	1, 74	0.006
Parasite history	2.62	1, 74	n.s.
Host clone	25.79	1, 74	<0.0001
Offspring			
Current food	42.54	1, 71	<0.0001
Parasite history	0.98	1, 71	n.s.
Host clone	3.89	2, 71	0.012
Current food*host clone	5.46	3, 71	0.002
Parasite history*host clone	3.05	3, 71	0.034
Age at first reproduction			
Current food	5.31	1, 70	0.021
Parasite history	0.16	1, 70	n.s.
Host clone	5.02	3, 70	n.s.
Survivorsip			
Current food	13.77	1, 75	0.034
Parasite history	5.35	1, 75	0.021
Host clone	13.77	3, 75	0.003

Note: We ran two experiments, one involving temperature variation, the other involving variation in the amount of food supplied to hosts. Each experiment used multiple host clones that were also tested for differences in the response variables. Test statistics are *F*-ratios for offspring counts, but a likelihood ratio χ^2 for the other response variables.

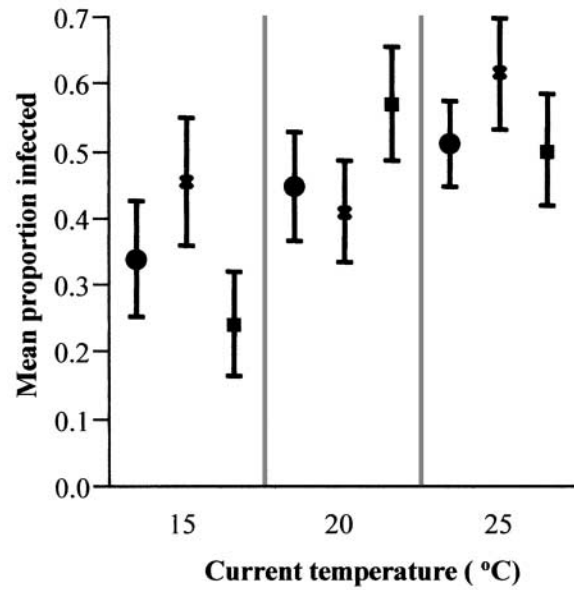


Fig. 1. Infection levels for *Daphnia* hosts reared at three temperatures when parasites had, one generation previously, been propagated at three temperatures (●, 15°C; ×, 20°C; ■, 25°C). Bars are standard errors.

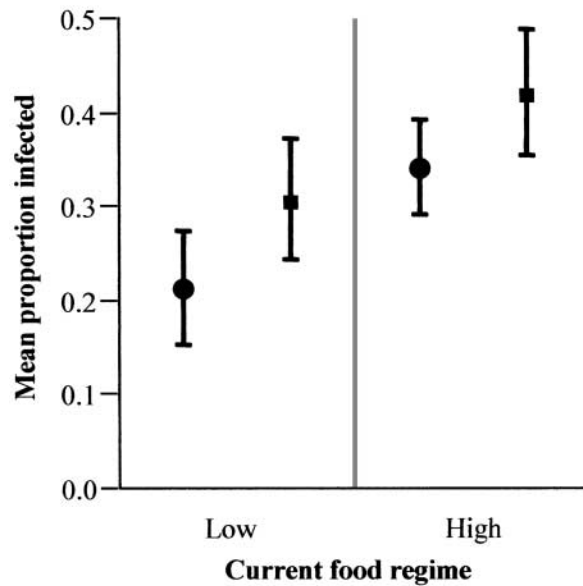


Fig. 2. Infection levels for *Daphnia* hosts reared at two food levels when parasites had, one generation previously, been propagated at different food levels (●, low food; ■, high food). Bars are standard errors.

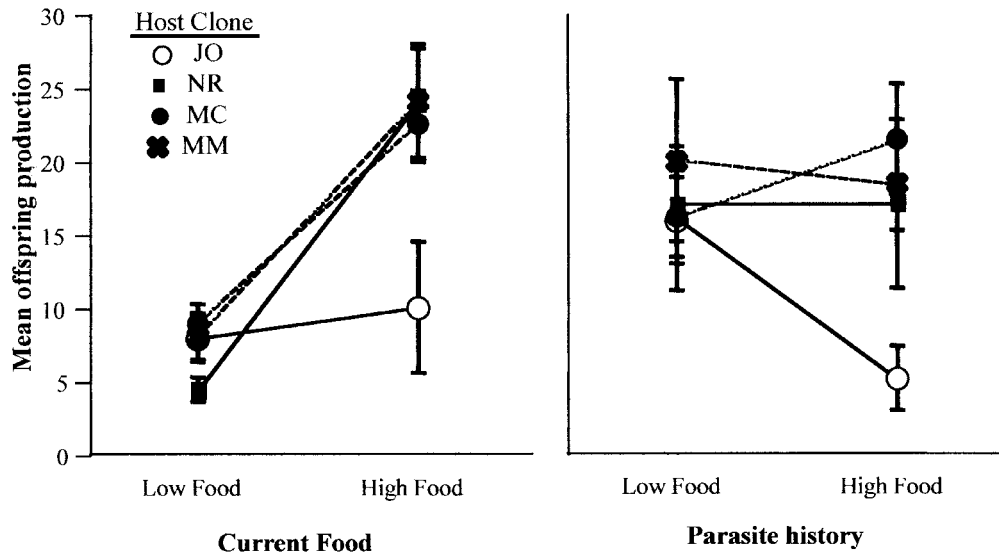


Fig. 3. Left panel shows a genotype \times environment ($G \times E$) interaction regarding offspring production under parasitism in a low or high current food environment. Right panel shows a $G \times E$ interaction where E is the environmental history (food level) of the parasite. JO, NR, MC, and MM are different host clones. Bars are standard errors

experienced by parasites did impact current infections. In particular, parasites propagated on relatively poorly fed hosts harmed (in terms of host survival) new hosts less than did parasites previously propagated on well-fed hosts. Additionally, parasite history impacted the level of reproduction hosts attained, although the direction of this effect was variable and dependent on host genotype (Fig. 3). Thus, our data indicate that, while not a pervasive factor, past environments experienced by parasites can influence the harm that parasites cause.

Although we do recommend caution in interpreting these results (this study incorporated a large number of statistical tests and significance levels for tests involving parasite history were not overwhelmingly strong), the potential effects of parasite conditioning raise some interesting points for discussion. In particular, the 'parasite history' \times host clone interaction we observed potentially adds a complex context to the host–parasite interaction. This pattern implies that the conditions experienced by the previous generation of parasites will impact some host clones to a greater degree than others, raising the possibility that past parasite environments impact parasite-mediated selection. This situation is analogous to that found in a study of maternal effects on host resistance: the conditions experienced by the maternal host generation determined host resistance in the current generation, but some host genotypes appeared to be more sensitive than others (Mitchell and Read, 2005). In general, such transgenerational effects are likely to exhibit unique evolutionary features (Mousseau and Fox, 1998). For example, a time lag between previous selection, acting on the previous generation, and the evolutionary response, which occurs in the current generation, may be counterproductive if current conditions are markedly different from maternal conditions (Kirkpatrick and Lande, 1989). Adapting this thinking to co-evolutionary interactions will require

the incorporation of a complex array of host and parasite factors and their interaction with a developing environment.

That parasites previously propagated on poorly fed hosts caused relatively little mortality compared with parasites previously propagated on well-fed hosts could indicate that low resource environments affect the quality of parasite transmission spores. Previous studies have clearly established that poor environments impact the quantity of spores produced (Ebert *et al.*, 2004). We carefully controlled for this effect in the second round (the F₁ exposure) of infections by standardizing spore doses to the same level regardless of parasite history, and thus the present data, in principle, show that the cost of inhabiting poorly fed hosts is not only the production of a small number of transmission spores but also their relative lack of effectiveness. The issue of spore quality could, however, be confounded by incomplete knowledge regarding when a spore is mature and capable of transmission. We diagnose the life stage of spores based on their morphology, and although this has been studied in depth (Ebert *et al.*, 1996), we cannot be certain that what we identify as a mature parasite spore is indeed yet capable of initiating infection. Our results could therefore reflect slow parasite growth, and especially slow maturation rate, in a low food environment. This hypothesis should be testable by harvesting spores over time and comparing infectivity of standardized doses.

Effects of host genetic background (host clone) were ubiquitous in this and other studies of this host–parasite system (Little and Ebert, 2000; Carius *et al.*, 2001; Duncan *et al.*, 2006). Here, genetic background (clone) also showed strong interactions with current food levels (i.e. a standard genotype \times environment interaction) but somewhat surprisingly, host clone did not interact with current temperature. In a previous study (Mitchell *et al.*, 2005) on host and parasites from the same source populations, such a genotype \times (temperature) environment interaction was clearly evident. However, Mitchell *et al.* (2005) studied a relatively large number of host clones (eight vs. four in the present study), which offered substantial additional power for the detection of genotype-specific effects.

We might also have expected significant ‘parasite history’ \times ‘current environment’ interactions if parasites had become adapted, or acclimated, to their previous environment. In this case, we would have observed, for example, that parasites previously propagated at high temperatures would perform well at high current temperatures. Acclimation has been demonstrated, for example, in studies of other microorganisms (Bennett and Lenski, 1997; Dillon *et al.*, 2003; Rainey, 2004) or in plant–herbivore interactions where insect herbivores acclimate to the chemical environment of their hosts (Jermy, 1990; Akhtar and Isman, 2004). Acclimation, being based on phenotypic plasticity, can be expected to occur quite rapidly, and it thus was not unreasonable to expect a bacterium such as *P. ramosa* to have done so over the time-frames provided by this study.

Given longer time-frames, evolutionary events could drive adaptations and specialization, as they probably have in other systems (Mackinnon and Read, 2004; McClelland *et al.*, 2004) and indeed in other studies on the *Daphnia*–*Pasteuria* system (Little *et al.*, 2006). However, even in these cases, it remains difficult to determine when specialization is due to genetic differences (evolution) or differential patterns of expression (acclimation/phenotypic plasticity). In general, the process of specialization is of considerable interest to the field of parasitology and evolutionary thinking on disease. For example, an understanding of specialization can shed light on what limits the host range of certain diseases or help to explain why the spread of disease is sometimes associated with increasing virulence (Read *et al.*, 1999). We speculated that parasite specialization via acclimation to host environments could contribute to this

process, but our data indicate that it does not, at least over ecological time-scales. Perhaps the significant interactions we observed with host clone indicate that longer-term evolutionary interactions are of greater importance.

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