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Measuring parasite fitness under genetic and thermal variation

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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 the 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. In addition, 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.
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Introduction

Parasite growth has commonly been used as a parasite fitness correlate, particularly in empirical work testing the evolution of virulence theory (for example, 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 (reviewed in 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 (for example, 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 minimize environmental variation but such variation are widespread in the wild and could affect genotypes differently, leading to genotype-by-environment interactions ($G \times E$, 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 $G \times E$ interactions affecting infection outcomes in the pea aphid–fungal system *Acyrthosiphon–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 (reviewed in 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, 1999), further shaping the temporal and spatial distribution of genetic diversity and patterns of local adaptation (Kaltz and Shykoff, 1998; Laine, 2008). In all these cases, a correct assessment of whether environmental variability can maintain polymorphism clearly relies on an accurate measurement of fitness. Although

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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.

Earlier 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*., 2004; Duncan and Little, 2007). Experimental studies in this system have found evidence for host genotype × temperature interactions ($G_H \times E_T$) affecting the probability of infections becoming established (Mitchell *et al*., 2005), parasite genotype × temperature interactions ($G_P \times E_T$) affecting parasite growth (Vale *et al*., 2008), as well as strong $G_H \times G_P$ interactions for infectivity (Carius *et al*., 2001). Given that *Daphnia* inhabits 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, 20 and 25 °C. In doing so, 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 (that is, $G_H \times G_P$ interactions) can be altered under thermal variation ($G_H \times G_P \times E_T$ interaction).

Materials 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 and 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 genotype (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

Before infection, host maternal lines were acclimatized for three generations to equilibrate maternal and environmental effects. Twelve replicate jars of each isofemale line (five *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 h. Medium was changed with every clutch or every 3–4 days regardless of a clutch being present. Although infections were carried out at three different temperatures, all host lines were acclimatized at 20 °C. Earlier experiments have shown that the temperature of acclimation of the maternal generation does not affect susceptibility to infection in their offspring (Mitchell and Read, 2005). Acclimation at the same temperature allows clutch production to be synchronized and infections to be performed on the same day. After acclimation, infections were carried out at 15, 20 and 25 °C by splitting replicates among three incubators at these temperatures. Incubators do not vary substantially apart from the temperature at which they are set; earlier experiments (for example, Mitchell *et al*., 2005) have shown that host phenotypes are consistent with the temperature treatment, regardless of which incubator they were studied in.

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, 1-day-old (<24 h) female, placed in a jar containing 60 ml of artificial *Daphnia* medium and sterile sand. These jars were placed in an incubator at the appropriate temperature overnight before 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 suspensions were originally obtained by homogenizing infected *Daphnia*, and these suspensions were stored at −20 °C until required. Before infection, spore suspensions were thawed, shaken thoroughly and counted using a Neubauer counting chamber ($0.0025\,mm^2 \times 0.1\,mm$ depth) (Hawksley, Sussex, UK). *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; Vale *et al*., 2008), *Daphnia* were exposed to parasites for 150 degree-days, that is, 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 *S. obliquus* algae (1.5×10^6 cells per *Daphnia*). The combination of stirring and low food increases the likelihood of *Daphnia* encountering transmission spores and becoming infected.

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 2×10^6 at 15 °C, 3.5×10^6 at 20 °C and 6×10^6 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 suspension in a Neubauer (improved) counting chamber ($0.0025\,mm^2 \times 0.1\,mm$ 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., Cary, NC, USA) and R (R, 2005, Vienna, Austria). 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 generalized 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 were analysed using analysis of variance.

The number of spores produced per infected host was analysed, first, 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). Second, we controlled for variation due to differential survival by analysing the number of spores produced per degree-day a host remained alive (that is, spores per host per 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 the infection status (hosts that did not become infected contributed zero spores and thus parasite genotypes produce, on average, a number of spores that are 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 to be 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.

Although 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 divided by the total sum of squares in the model. For survival, to enable straightforward calculation of the effect sizes of each term, we analysed 'day of death' using analysis of variance and calculated variance as for spore production. The reduced model in both analysis of variance and formal survival analysis (proportional hazards) did not yield different qualitative results.

Results

We exposed a total of 576 individual *Daphnia* to infection (12 replicates of four host genotypes exposed to four parasite genotypes at three temperatures), of which 38 replicates were lost during the experiment, mainly due to death during the infection period. Owing 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 nine 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 (18%) and at 25 °C (16%) (Figure 1). The reduced model (Table 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 1). The three-way interaction was not significant in the full model ($\chi^2_{18} = 8.271$, $P = 0.974$).

Temperature influenced the survival of infected hosts, explaining 23% of the variation in the time to death (Table 1; Figure 2a). The direction and magnitude of this effect depended on the parasite genotype that was involved in the infection. This parasite genotype-by-temperature interaction explained 8% of the variation for the time to host death (Table 1; Figure 2b). In some cases, we observed a complete switch in the rank order of parasite genotypes between temperatures (Figure 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).

The lifetime transmission potential of parasites could be explained by temperature, and also by an interaction between parasite genotype and temperature (nearly 30%

15°C

	GG3	GG4	GG7	GG13	Mean
Sp1	0.75	0.82	0.00	0.00	0.39
Sp13	0.27	0.00	0.00	0.25	0.13
Sp7	0.25	0.25	0.00	0.00	0.13
Sp8	0.17	0.00	0.00	0.08	0.06
Mean	0.36	0.27	0.00	0.08	0.18

20°C

	GG3	GG4	GG7	GG13	Mean
Sp1	1.00	1.00	0.08	0.00	0.52
Sp13	0.75	0.00	0.09	0.25	0.27
Sp7	0.67	0.17	0.20	0.00	0.26
Sp8	0.75	0.08	0.00	0.20	0.26
Mean	0.79	0.31	0.09	0.11	0.33

25°C

	GG3	GG4	GG7	GG13	Mean
Sp1	0.70	0.82	0.00	0.00	0.38
Sp13	0.09	0.10	0.00	0.00	0.05
Sp7	0.42	0.18	0.09	0.00	0.17
Sp8	0.00	0.08	0.00	0.00	0.02
Mean	0.30	0.30	0.02	0.00	0.16

Figure 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 with combinations with little or no infectivity. See Table 1 for statistical details.

Table 1 Summary of statistical analyses testing the effects of temperature, host and parasite genotypes on infectivity and host survival

Source	*d.f.*	*L-R* χ^2	P-*value*	*% Variance full model*
Infectivity				
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 × Parasite	9	62.28	<0.0001	12.58
Host × Temperature	6	13.56	0.0350	2.82
Parasite × Temperature	6	8.68	0.1924	1.61
Host × Temperature × 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 × Parasite	—	—	NS	5.15
Host × Temperature	—	—	NS	2.13
Parasite × Temperature	6	14.30	0.0265	8.10
Host × Parasite × Temperature	—	—	NS	0.93
Error	93			52.07
Total	120			100

Abbreviation: NS, not significant.
Infectivity was modelled 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. d.f. is the degrees of freedom for the reduced model. L-R χ^2 is the likelihood ratio χ^2. Terms are significant for $P>0.05$. See Materials and methods section for statistical details.

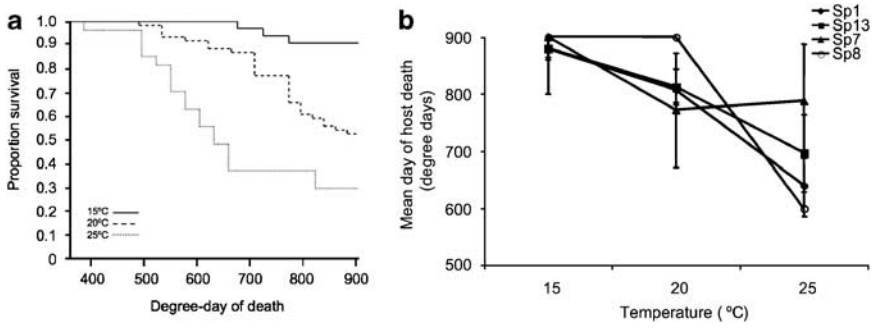

Figure 2 Host survival under thermal variation. (**a**) Survival curves for infected hosts at 15 °C (full black line), 20 °C (dashed line) and 25 °C (dotted line). (**b**) Reaction norms for the mean time to host death in infected hosts. Each data point is the mean trait value for each parasite genotype. Error bars are standard error of the mean. See Table 1 for statistical details.

of the variance, combined; Table 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 3a). Removing host genotype as a main effect did not improve the overall model fit. After controlling for differential survival (by studying spores per *Daphnia* per degree-day, which is a measure of parasite growth rate), we found that the parasite × temperature interaction was no longer sig-

nificant, 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 2). Incorporating information on the infectivity of each parasite genotype also dissipated the parasite genotype-by-temperature interaction (Table 2; Figure 3b).

Table 2 Summary of statistical analyses testing the effects of temperature, host and parasite genotypes on different measures of parasite growth

	Reduced model			*Full model*	
Lifetime transmission potential					
Source	d.f.	F ratio	F	d.f.	% Var
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 × Parasite	—	—	NS	6	2.42
Host × Temperature	—	—	NS	4	1.23
Parasite × Temperature	6	2.258	0.043	6	7.75
Host × Parasite × 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 × Parasite	—	—	NS	6	1.44
Host × Temperature	—	—	NS	4	0.88
Parasite × Temperature	6	1.2771	0.274	6	3.53
Host × Parasite × Temperature	—	—	NS	3	1.06
Model				27	50.66
Error				93	49.34
Total				120	100
Spores on all hosts (infected+not infected)					
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 × Parasite	9	15.9416	<0.0001	9	14.05
Host × Temperature	6	9.0014	<0.0001	6	5.29
Parasite × Temperature	—	—	NS	6	0.44
Host × Parasite × Temperature	—	—	NS	18	2.36
Model				47	52.60
Error				490	47.40
Total				537	100

Abbreviation: NS, not significant.
We present the test statistics for all the terms in the reduced model. % Variance explained by each term refers to the full model. d.f., degrees of freedom for the reduced model. F is the F-ratio test statistic. Terms are significant for $P>0.05$. See Materials and methods section for statistical details.

Discussion

A growing body of empirical work, mostly on plant and invertebrate host–pathogen systems, has shown that infection is frequently dependent on the host and parasite genotypes involved ($G_H \times G_P$) (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 ($G_H \times E$, $G_P \times E$ and $G_H \times G_P \times E$, for example, Mitchell *et al*., 2005; Laine, 2007b; reviewed in 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 *D. magna* is exposed to the bacterium *P. ramosa*. Our experiment included four genotypes of the host and four genotypes of the parasite that were earlier established to show substantial differences in infection-related traits as well as genetic specificity for infectivity ($G_H \times G_P$ 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 ($G_H \times E_T$) 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 ($G_P \times E_T$) 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, $G_H \times G_P \times E$, interactions were not evident.

Infectivity

The $G_H \times E_T$ 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 1). Although both scenarios will influence the strength of selection, the long-term maintenance

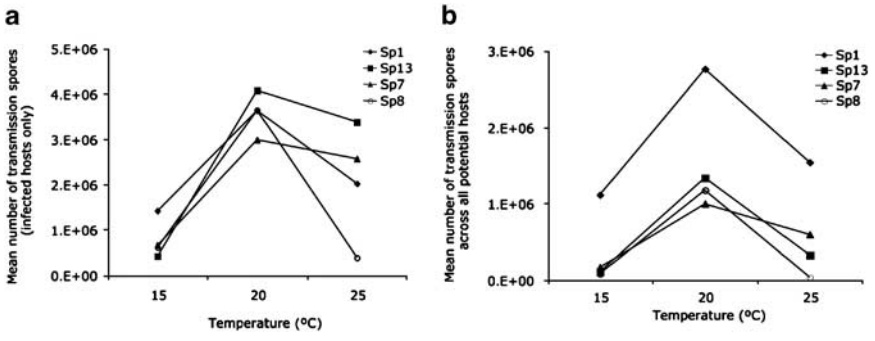

Figure 3 Measures of parasite fitness considering spore production among infected hosts only or incorporating the ability of parasite genotypes to infect. (**a**) The average number of spores produced during the experiment across all infected hosts is shown for three temperatures (parasite lifetime transmission 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 1 for statistical details.

of polymorphism through $G \times E$ 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). Indeed, a general issue in studies of $G \times E$ is that interactions may come about not because of a change in the rank order of genotypes (changes in the mean), but simply because genotypes have unequal variances across environmental treatments. 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 that infectivity is a trait robust to thermal variation, as was recently reported in a plant–pathogen system (Laine, 2007b, 2008). In an earlier 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 two experiments) than this study, 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 (for example, 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 1, Figure 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 2b) and produced the least number of spores compared with 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 3a). Results from both demographical and epidemiological models show that selection favours early over late births in expanding populations, 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 and 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. Instead, 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 2, Figure 3a). This interaction may be due to slightly divergent slopes of the reaction norms rather than due to a change in the rank order of genotypes between treatments. Again, care should be taken when interpreting $G \times E$ interactions where a clear change in the rank order of genotypes is not observed. In this case, however,
the genotype-by-temperature interaction follows earlier reports on the effect of temperature on parasite growth and transmission potential in this (Vale *et al*., 2008) and other systems (for example, Blanford *et al*., 2003; Laine, 2007b). 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 $G_P \times E_T$ interaction for parasite growth rate (Table 2), suggesting that variation in survival substantially contributed to $G_P \times E_T$ interaction 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 $G_P \times E_T$ was more important). Therefore, we were interested in determining if the $G_P \times E_T$ interaction 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 contributed zero spores. The $G_P \times E_T$ interaction that was significant for lifetime transmission potential among infected hosts (Table 2; Figure 3a) was no longer evident once this information about parasite infectivity was incorporated into parasite fitness (Table 2; Figure 3b). Notably, parasite genotype Sp1 is by far the genotype with highest (estimated) fitness at all temperatures (Figure 3b), even though it was not the genotype producing the highest number of spores (Figure 3a) during infections. This is a reflection of it having the highest mean infectivity across all host genotypes (Figure 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 this 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 the growth within the host cell (Abedon, 2008).

However, this does not necessarily mean that the observed $G_P \times E_T$ interaction for spore production is inconsequential. Earlier studies have shown that the number of *P. ramosa* spores a host is exposed to (that is, dose) strongly influences the likelihood of achieving a successful infection (for example, 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 *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 $G_H \times G_P \times E_T$ interaction 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 (for example, Tetard-Jones *et al*., 2007), whereas other workers (for example, Heath and Tiffin, 2007; Laine, 2007b) 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, 2007a), 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. $G_H \times G_P$ interaction indicates 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.

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