doi:10.1111/j.1558-5646.2011.01345.x

FOOD-ENVIRONMENT MEDIATES THE OUTCOME OF SPECIFIC INTERACTIONS BETWEEN A BUMBLEBEE AND ITS TRYPANOSOME PARASITE

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Received August 23, 2010
Accepted April 29, 2011
Data Archived: Dryad doi:10.5061/dryad.t903h

Specific host–parasite interactions, where the outcome of exposure to a parasite depends upon the genotypic identity of both parties, have implications for understanding host–parasite coevolution and patterns of genetic diversity. Thus, grasping the extent to which these interactions are mediated by environmental changes in a spatially and temporally heterogeneous world is vital. In this study, it is shown that the environment can influence specific host–parasite interactions in the well-studied system of the bumblebee *Bombus terrestris* and its trypanosome parasite *Crithidia bombi.* Naturally relevant variation in the quality of the food environment formed a three-way interaction with both host and parasite identity in determining the outcome of infection, with regard to the resistance of the host and the transmission of the parasite. The demonstration of such a host-genotype by parasite-genotype by environment interaction (G_H x G_P x E) shows the importance of considering environmental variation when investigating host–parasite interactions. Moreover, such interactions may to some extent explain levels of genetic diversity in natural host–parasite systems owing to the fact that they will create selection mosaics when environments are heterogeneous.

KEY WORDS: ***Bombus terrestris*, *Crithidia bombi*, food-environment, genotype by genotype by environment, host–parasite.**

It is undeniable that the environments in which organisms live vary over space and time. This is true for broad-scale environmental dynamics such as climate, but important elements such as temperature, food, and the biotic environment will also vary on a local scale. Fitness-relevant variation in the environment will potentially create a mosaic of selection and add complexity to dynamic interactions between organisms. The process of a selection mosaic is central, for example, to the idea of the geographic mosaic theory of coevolution (GMTC: Thompson 2005; Gomulkiewicz et al. 2007).

It is becoming increasingly apparent that host–parasite interactions are not impervious to environmental variation (Lazzaro and Little 2009; Wolinska and King 2009). Knowledge of spatial and other variation influencing host–parasite interactions and their outcomes is important for understanding such important topics as virulence (Regoes et al. 2000) and genetic diversity (Haldane 1949), among others. Furthermore, the manner of interactions between hosts and parasites and their coevolution is important for evolutionary phenomena, such as Red-Queen dynamics, which are key to one explanation for the maintenance of sexual reproduction and recombination (Jaenike 1978; Hamilton 1980; Salathe et al. 2008). One prerequisite is the existence of specific host–parasite interactions, where the outcome of infection depends on the genotypic identity of both host and parasite ($G_H \times G_P$). Such specific interactions have been demonstrated in a number of invertebrate host systems (reviewed in Sadd and Schmid-Hempel 2009).

Typically however, these experiments do not investigate the influence of environmental variation on the outcome. Yet, the sensitivity of the interactions to relevant environmental variation is likely to alter important characteristics of host parasite dynamics (Mostowy and Engelstädter, in press).

If and how the environment interacts with host and parasite genotypes will determine the consequences of environmental variation for coevolution. The environment can be an overall main effect, thus altering the strength of selection (Wolinska and King 2009). In these cases, the same hosts and parasites will be favored across spatially variant environments, yet the variation in the strength of selection will result in a patchwork where reciprocal selection is taking place to different extents. Alternatively, more complex interactions can take place between the environment and host genotype ($G_H \times E$) or parasite genotype ($G_P \times E$), altering the specificity of selection, which denotes the infection profiles of host genotypes when exposed to a range of distinct parasite genotypes. The extreme case is where environmental variation, host genotype, and parasite genotype interact to determine infection outcome ($G_H \times G_P \times E$), thus altering the patterns of observed host–parasite specificity. In these more complex cases, spatial variation in the environment will mean reciprocal selection between host and parasite results in divergent patches where different host and parasite genotypes are favored. Likewise, temporal variation in the environment has the potential to maintain polymorphisms in hosts and parasites in situations where polymorphism would be lost should the environment either be stable or not influence host and parasite specificity (Mostowy and Engelstädter in press).

$G \times G \times E$ interactions have been demonstrated for an aphid herbivore (G), host plant (G), rhizosphere bacteria (E) system (Tetard-Jones et al. 2007), and recently for infection by a hyperparasitic virus of a pathogenic fungus of chestnut trees across different temperatures (Bryner and Rigling 2011). Furthermore, $G \times G \times E$ have been found to be present within interactions considered traditionally to be mutualistic in plants systems (Piculell et al. 2008; Heath et al. 2010). Within animal–parasite systems, environmental influences on the outcome of host–parasite interactions, $G_H \times E$ and $G_P \times E$, have been investigated in the system of *Daphnia* and its parasites (e.g., Mitchell et al. 2005; Vale et al. 2008; Vale and Little 2009; Schoebel et al. 2011). Further, $G_H \times E$ has been demonstrated with regard to temperature and food environment in a number of other systems (Ferguson and Read 2002; Blanford et al. 2003; Bedhomme et al. 2004; Lambrechts et al. 2006; Restif and Kaltz 2006) (further examples are comprehensively reviewed in Wolinska and King (2009)). However, widespread evidence for the existence of $G_H \times G_P \times E$ in animal systems is lacking, and in fact has only been formally tested in the case of *Daphnia* (Vale and Little 2009).

The trypanosome *Crithidia bombi* is a prevalent parasite infecting the guts of bumblebees, *Bombus* spp. (Lipa and Triggiani 1988). After establishing a chronic infection within the hind-gut of these social insects, transmission of the parasite to nest-mates or unrelated individuals, which pick up the parasite while foraging on flowers, takes place through cells shed in the faeces (Durrer and Schmid-Hempel 1994). Infection by *Crithidia* has a number of fitness-relevant consequences for bumblebees, including impaired foraging ability (Gegear et al. 2005), reduced individual survival under harsh conditions (Brown et al. 2000), and reduced colony founding success and subsequent overall fitness in queens (Brown et al. 2003). Specific host–parasite interactions have been demonstrated between *Bombus terrestris* and *C. bombi* (Schmid-Hempel 2001; Mallon et al. 2003). Given that colonies of this social insect are made up of closely related sister workers originating from a mating between their mother and a single haploid male (coefficient of relatedness, $r = 0.75$, [Hamilton 1964]), all individuals sourced from a colony can be regarded as being from a single genotypic unit (G_H). These experiments of host–parasite specificity have typically, and for good reason, been carried out in situations that ameliorates environmental variation as much as possible. However, environmental conditions in the field will rarely show such stability. One important environmental factor that will vary spatially and temporally is the quantity and quality of the food-environment available to a colony. Quality of the food available to bumblebees will vary depending on the location, season, and presence or absence of particular flowering plants (e.g., Wykes 1953; Wright 1988; Herrera et al. 2006). Because of this variation, food-environment will be a relevant factor for nectar foraging organisms such as *B. terrestris*. With this in mind, the aim of this study was to investigate the stability of specific interactions between host and parasite in the bumblebee–*Crithidia* system in the context of ecologically relevant variation in the food-environment. Given that food quality will be linked to host-condition, it is not unreasonable to expect an influence of food-environment on the outcome of host–parasite interactions. A naive expectation, based on hosts only having limited resources available to partition between defense against parasites and other traits, would be that hosts become more susceptible to infection as the food-environment quality decreases. However, many unknowns relating to underlying mechanisms make it difficult to make specific predictions ab initio.

Materials and Methods

BUMBLEBEE MAINTENANCE AND PARASITE CULTURING

Worker bees, *B. terrestris*, used in the preliminary condition and food-environment tests originated from second-generation laboratory-raised colonies. The mother queens of these colonies

and the males that they were mated with were sourced from colonies set up from queens collected in Northern Switzerland in the spring of 2008. The colonies used in the main experiment were raised by queens of *B. terrestris* caught in the spring of 2009 in Northern Switzerland, and were chosen haphazardly from a larger sample of successfully founded colonies. Regular faecal checks from both the queens and subsequently produced workers confirmed that these colonies were healthy. Callow workers were removed on the day of emergence, isolated, and assigned randomly to one of the experimental groups. Postexperimentation the length of the radial cell of the forewing was taken as a measure of body size (Muller et al. 1996; Schmid-Hempel and Schmid-Hempel 1996). All colonies were kept at 28 ± 2°C under red light illumination, with pollen and sugar water (ApiInvert®) provided ad libitum. Individually isolated bees were kept at 28 ± 2°C under red light illumination with the appropriate feeding regime required for their particular treatment group (see below).

The parasite strains used in this experiment were isolated from spring queens collected in Northern Switzerland in the spring of 2008. The distinctness of the four strains is likely as they showed variation in their infection outcome in earlier work (unpublished data), where eight randomly sampled field derived strains were screened. The strains also produced a significant $G_H \times G_P$ in this earlier work, but the host colonies were however different from those used here. The strains used are hereafter referred to as strains 1 through 4, but their laboratory specific codes are 08.068, 08.075, 08.091, and 08.161, respectively. These single strain isolates were produced from single infective cells that had been isolated using a fluorescence-activated cell-sorter and subsequently cultured and maintained clonally in liquid medium at 27°C and 3% CO_2 (Salathé et al., unpubl. ms.).

PRELIMINARY CONDITION AND FOOD-QUALITY TESTS

To establish appropriate food-environment treatments, initial tests were carried out on worker bees from two colonies. Callow workers were removed from the colonies and housed individually with ad libitum access to one of six sugar-water concentration treatments. The treatments consisted of 0, 1, 5, 12, 20, or 50 percent ApiInvert® (in distilled water). The feeders providing these treatments were renewed every six days. Survival was recorded daily until all bees had died.

IMPACT OF FOOD QUALITY ON THE OUTCOME OF HOST–PARASITE INTERACTIONS

Based on the preliminary experiments described above and natural ranges of sugar concentrations found in nectar (Wykes 1953), three food-environment treatments were chosen for the main experiment investigating the influence of food-environment on the outcome of host–parasite interactions in the *Bombus–Crithidia* system. These food-environments were designated as high (50% ApiInvert®), medium (20% ApiInvert®), and low (12% ApiInvert®). Callow worker bees were assigned to a treatment group on the day of eclosion and provided with sugar-water at the appropriate concentration for the entirety of the experiment. The provided sugar water was replaced following the parasite exposure that took place at five days posteclosion.

Within each host-colony (genotypic unit) individual infections of each of the four parasite strains (see above) were carried out in individuals exposed to each food-environment treatment, with a minimum of three replicates for each parasite and environment combination. Of nine initial host-colonies that were set up for this experiment, six colonies (subsequently referred to as colonies A through F) produced a sufficient number of workers to achieve the minimal experimental coverage and replication. Therefore, a total of 72 unique $G_H \times G_P \times E$ treatment combinations were included. Infections were carried out five days posteclosion with 10,000 infective parasite cells suspended in 10 μl of a 50% sugar–water solution. Bees were deprived of access to sugar–water for 2 h before being presented with the freshly prepared suspension for them to take up per os. Imbibition of the entirety of the infective solution was confirmed by eye, and any bees that had not taken up all 10 μl within 40 min of the time that it was provided to them were removed from the experiment. After the parasite exposure, bees were once again provided with sugar–water appropriate to their food-environment treatment.

Faecal samples were collected from the bees seven days after infection, following which they were immediately sacrificed and frozen until dissection. The volumes of the faeces samples were measured, and parasite cells in the faeces were quantified by placing the samples in counting chambers (Fast-Read 102®) (Madaus Diagnostik, Köln, Germany). The total number of parasite cells being shed was calculated based on the concentration of parasite cells in the faeces and the volume collected. In addition, the guts of the sacrificed bees were dissected out and infection intensities were subsequently determined using qPCR (Ulrich et al. 2011). Briefly, the primers "CriRTF2" (GGCCACCCACGGGAATAT) and "CriRTR2" (CAAAGCTTTCGCGTGAAGAAA) were used to amplify a 56-bp fragment of the *C. bombi* 18sRNA gene. The qPCR reactions were performed on a ABI 7500 Real-time PCR System (Applied Biosystems, Rotkreuz, Switzerland) in 20 μL reaction volumes containing 2 μL eluted DNA, 0.3 μM of each primer, and 1× Power SYBR Green Universal MasterMix (Applied Biosystems), according to the following thermal profile: 10-min preincubation at 95°C followed by 40 cycles of amplification with 15 s denaturation at 95°C, and 1-min simultaneous annealing and extension at 60°C. Negative controls of double-distilled H_2O and parasite-free bumblebee DNA were included in every run. Sample DNA quantities were measured using a Nanodrop 8000 (Thermo Scientific, Wilmington, NC).

Analyses

The influence of food-environment on the longevity of bees was analyzed using a linear model. Infection intensities measured by qPCR were corrected for differences in DNA extraction efficiency by dividing the raw value by the measured DNA concentration to create a corrected infection intensity that was used in further analyses. Body size of individuals did not have a significant effect on the amount of DNA extracted from the gut ($F_{1,328} = 2.7$, $P = 0.10$), so was not included in any correction calculation. The responses of corrected infection intensity and parasite cell numbers shed in the faeces were analyzed using generalized linear models fitted with quasi-Poisson error distributions and log link functions to account for overdispersion. Full models were fitted with food-environment, host colony, parasite strain, and all interactions between them, and date of infection and worker size as further factors. For gut infection intensities, models were also fitted with DNA concentration as a factor to ensure patterns observed were not driven by variation in DNA concentration between samples. This was not the case. To check for the existence of apparent host-genotype by parasite-genotype interactions within food environments, models were further fitted for each food environment individually. Terms were removed from models in a stepwise fashion in all cases until the best fitting minimal model remained. Only minimal models are reported here. The relationship between estimated parasite infection intensities in the gut and total cells shed in the faeces was tested with a linear model with both variables being transformed ($\log(y + 0.5)$). All analyses were carried out in R 2.92 for Mac (R Development Core Team 2008).

Results

FOOD-ENVIRONMENT AND CONDITION

The concentration of sugar-water in the food-environment treatment had a significant effect on the lifetime survival of workers ($F_{5,92} = 70.34$, $P < 0.001$). Those bees receiving food-treatments with a higher sugar-water concentration survived longer, demonstrating that the food-environments the bees were subjected to can be equated with their subsequent condition (Table 1).

IMPACT OF FOOD-ENVIRONMENT ON INFECTION INTENSITY AND PARASITE TRANSMISSION

Food-environment, host colony, and parasite strain were significant main effects with regard to infection intensity measured in the guts (Fig. 1A and Table 2A). Infections were highest in the medium food-environment and lowest in the low. Colony "A" showed a general pattern of resistance against all strains irrespective of the food-environment. Parasite strain "3" likewise showed little variation across host colonies and environments, showing

Table 1. Available food environment, defined by sugar-water concentration, and mean survival time (days) of worker bees. Different superscripts next to means denote significant statistical differences based on sequential Bonferroni-corrected pairwise *t*-tests ($P < 0.05$).

Sugar water concentration (percent ApiInvert®)	*N*	Mean (±standard deviation) survival in days
0	15	2.27 (±0.59)[a]
1	16	3.50 (±1.41)[a]
5	17	12.29 (±2.87)[b]
12	17	14.53 (±3.95)[b]
20	17	19.12 (±5.82)[c]
50	16	22.44 (±5.28)[d]

Table 2. Predictors and their respective statistical values for the minimum adequate models (Generalized linear models fitted with a quasi-Poisson distribution) for *Crithidia* infection intensity within the gut (A) and the number of parasite cells in the faeces seven days after infection (B). Significant predictors ($\alpha = 0.05$) are highlighted with asterisks.

Predictor	df	*F*-value	*P*-value
(A) *Crithidia* infection intensity in the gut			
Food-environment	2,330	3.87	0.022*
Host colony	5,325	11.69	<0.001*
Parasite strain	3,322	19.12	<0.001*
Food-environment × host colony	10,312	1.12	0.349
Food-environment × parasite strain	6,306	0.48	0.821
Host colony × parasite strain	15,291	1.39	0.154
Food-environment × host colony × parasite strain	30,261	2.56	<0.001*
(B) *Crithidia* cells shed in the faeces			
Food-environment	2,309	2.99	0.052
Host colony	5,304	3.44	<0.001*
Parasite strain	3,301	10.31	<0.001*
Food-environment × host colony	10,291	0.92	0.514
Food-environment × parasite strain	6,285	1.36	0.230
Host colony × parasite strain	15,270	0.69	0.794
Food-environment × host colony × parasite strain	30,240	1.67	0.019*

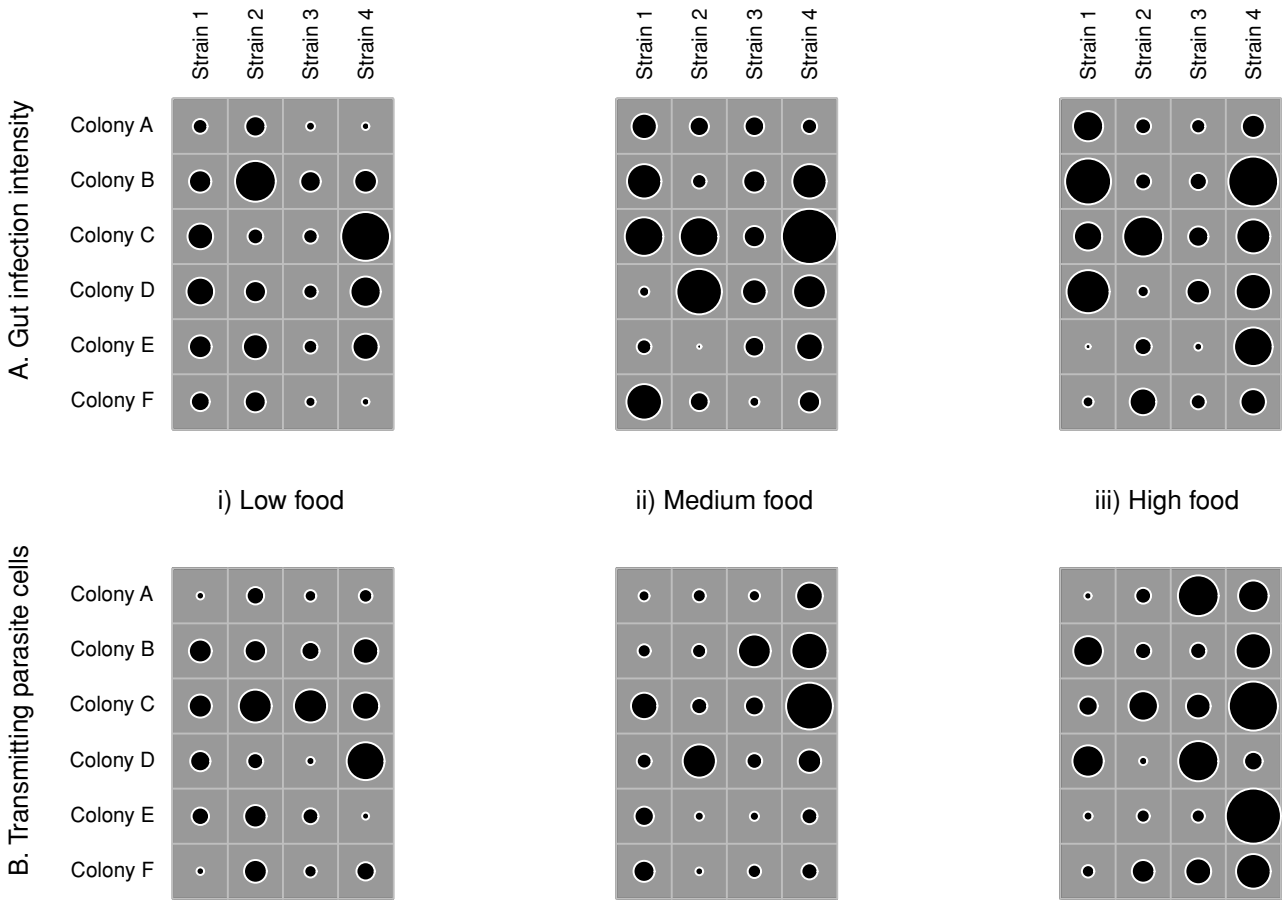

Figure 1. Matrices representing host–parasite combinations across, from left to right, low (i), medium (ii), and high (iii) food environments. Rows within each matrix represent each of the six the distinct bumblebee host colonies, and columns represent the four strains of *Crithidia bombi* used in experimental infections. The bubbles for each combination are representative of the mean corrected infection intensity in the guts (A, bubbles represent a range from 3.35 to 542.79) or the mean number of parasite cells shed in the faeces (B, bubbles represent a range from 474 to 29,348) with each value comprising of measurements from a minimum of three replicate bees.

general low infectivity. Further to the main effects, an interaction between the food-environment, host colony, and parasite strain had a significant influence on the measured infection intensity (Table 2A), and explained 15% of the deviance (deviance explained by the complete model = 46%). Looking at a snapshot of parasite transmission, by sampling the faeces of the bees seven days after infection, a similar pattern was observed to that seen for infection intensity in the gut (Fig. 1B and Table 2B). The similarity between the measurements was also confirmed by a linear regression between parasite cells counted in the faeces and the infection intensities measured in the gut (Linear regression: $F_{1,364} = 230.9$, $P < 0.001$, $R^2 = 0.39$).

Both the gut infection intensities and transmitting cells in the faeces were also analyzed separately for each food environment to investigate if patterns indicative of host-genotype by parasite-genotype were present within a particular environmental level. Significant interaction terms were present in four of the six analyses (Host colony × Parasite strain within low food: $F_{15,88} = 1.93$, $P = 0.029$ [gut infection], $F_{15,79} = 0.82$, $P = 0.655$ [faeces counts]; Host colony × Parasite strain within medium food: $F_{15,81} = 2.35$, $P = 0.007$ [gut infection], $F_{15,75} = 1.31$, $P = 0.217$ [faeces counts]; Host colony × Parasite strain within high food: $F_{15,92} = 2.20$, $P = 0.011$ [gut infection], $F_{15,86} = 1.82$, $P = 0.044$ [faeces counts]).

Discussion

Changing something as simple as the food-environment, which almost certainly varies in nature, can have notable, while not necessarily intuitive, effects on the outcome of host–parasite interactions. Although the food-environments used influenced the condition of healthy bees, as measured by long-term survival, resistance of hosts did not decrease in low-quality environments as may have been expected. Importantly however, there is a three-way interaction between food-environment, host-genotype, and parasite genotype that accounts for the greatest proportion of the explained deviance in infection intensity and number of transmitting parasite cells. This result represents one of the first demonstrations

of a $G_H \times G_P \times E$ in an animal–parasite system, and illustrates that specific host genotype by parasite genotype interactions can be modified by the environment. Given that the host colonies were chosen haphazardly, and the parasite genotypes, while selected, represented half of those screened, it seems that $G_H \times G_P \times E$ may be widespread within this particular host–parasite system.

In the bumblebee–*Crithidia* system, previous work has shown that the food-environment can influence the population of parasites that can be harbored by an individual host. Parasites infecting hosts provided with pollen had greater population growth and higher final numbers (Logan et al. 2005). However, this experiment did not investigate the impact of the environment on the specific interactions between *B. terrestris* and *C. bombi* genotypes. The study reported here suggests that, in addition to food-environment being a main effect, the specificity of the interaction in the *Bombus–Crithidia* system can be altered by the food-environment of the host. This means that the genotypes of hosts do not necessarily equate to a set level of resistance when faced with a particular parasite genotype (and vice versa for parasite genotypes and their infectivity), with genotype and phenotype in essence being decoupled (Lazzaro and Little 2009). The very existence of $G_H \times G_P \times E$, as shown here, suggests that resistance and specific interactions between host and parasite types are not wholly determined by constitutive genetically encoded mechanisms. Instead, an infection dependent on the pattern of expression of a suite of interacting components (e.g., see Riddell et al. 2009) that vary with the environment, would fit this model.

The implications of the results presented here for coevolution between host and parasite are hard to predict exactly without a comprehensive model mirroring the natural situation and including the parameters measured here and their component fitness consequences. However, it is possible to make some broader conjecture building on existing theories and studies. A simplistic assumption could be that environmental intervention in the outcome of host–parasite interactions will promote genetic diversity in both host and parasite populations. Heterogeneous environments, over either space or time, will create heterogeneous selection pressures that do not favor one particular host or parasite genotype. Heterogeneity in experienced environments could thus explain to some degree the high levels of diversity that are found in natural infections of *C. bombi* (Schmid-Hempel and Reber Funk 2004). Along similar lines, persistent spatial variation in the environment tied with the existence of $G_H \times G_P \times E$ will provide the basis for a selection mosaic. Selection mosaics are one of the three fundamental processes central to the Geographic Mosaic Theory of Co-evolution (GMTC, Thompson 2005), and the fact that $G_H \times G_P \times E$ underlie selection mosaics (Piculell et al. 2008) means the existence of such three-way interactions is significant. The patterns characteristic of the GMTC could potentially occur through processes unrelated to selection mosaics and the GMTC in general (Gomulkiewicz et al. 2007). Therefore, demonstrating $G_H \times G_P \times E$ in various systems strengthens the evidence for, and increases the plausibility of, important components of the GMTC, and thus, the GMTC itself.

Particularly in the context of the GMTC, a number of theoretical studies have investigated the impact of environmental heterogeneity on the coevolutionary process. For example, geographic structure of coevolutionary dynamics through spatial environmental heterogeneity will have an important role in maintaining allelic polymorphisms within interacting species (Nuismer et al. 1999). In addition, Mostowy and Engelstädter (in press) have recently modeled the impact of temporal environmental heterogeneity and $G_H \times G_P \times E$ on coevolutionary dynamics. Importantly, they showed that oscillatory dynamics of allele frequencies, important for the Red Queen Hypothesis (Jaenike 1978; Hamilton 1980; Salathe et al. 2008), are inhibited in situations where they would be present under a stable environment, while being triggered in situations where they would otherwise be absent. $G_H \times G_P \times E$ interactions represent a basic element behind selection mosaics. However, their exact impact on the coevolutionary process will depend on a number of other factors, such as the frequency of environmental fluctuation and gene flow in geographically structured populations (Nuismer et al. 1999, 2000; Gomulkiewicz et al. 2000; Mostowy and Engelstädter, in press).

The study presented here adds to previous demonstrations of $G \times E$ interactions in animal–parasite systems (reviewed in Wolinska and King 2009), by showing that the environment can interact in an even more complex manner to determine the outcome of infection. Further work, including theoretical treatises, will be required to exactly comprehend how these complex interactions will shape dynamics of host–parasite evolution, patterns of genetic diversity, and epidemiology. However, what is clear is that knowledge of the environmental context will be vital for understanding and making predictions about the outcome of host–parasite interactions and their evolutionary dynamics. This is not merely an academic exercise, with host–parasite interactions contingent on the environment having applied consequences, influencing for example biocontrol strategies across different locations, and linking the spheres of climate change and human disease (Wolinska and King 2009).

ACKNOWLEDGMENTS

I thank M. Jales Hon and D. Heinzmann for help with laboratory work. P. Schmid-Hempel gave helpful feedback on the completed manuscript. Additionally, S. Nuismer, as associate editor, and two anonymous reviewers provided critiques that helped improve the manuscript. Facilities of the Genetic Diversity Centre at the ETH Zürich were used for this project.

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Associate Editor: S. Nuismer