JOURNAL OF Evolutionary Biology

doi: 10.1111/j.1420-9101.2012.02507.x

Robustness of the outcome of adult bumblebee infection with a trypanosome parasite after varied parasite exposures during larval development

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Keywords:

Bombus terrestris;
bumblebee;
Crithidia bombi;
environmental dependence;
genotype-by-genotype interaction;
host–parasite interaction;
immune priming;
social immunity.

Abstract

The outcome of defence by the invertebrate immunity has recently been shown to be more complex than previously thought. In particular, the outcome is affected by biotic and abiotic environmental variation, host genotype, parasite genotype and their interaction. Knowledge of conditions under which environmental variation affects the outcome of an infection is one important question that relates to this complexity. We here use the model system of the bumblebee, *Bombus terrestris*, infected by the trypanosome, *Crithidia bombi*, combined with a split-colony design to test the influence of the parasite environment during larval rearing on adult resistance. We find that genotype-specific interactions are maintained and adult resistance is not influenced. This demonstrates that environmental dependence of bumblebee–trypanosome interactions is not ubiquitous, and yet unknown constraints will maintain standard coevolutionary dynamics under such environmental deviations.

Introduction

Insect (and more generally invertebrate) immunity was traditionally considered to be relatively simple and not matching any of the complex performance elements of the immune system in the jawed vertebrates. However, in recent decades, especially since the advent of the field of ecological immunology (Sheldon & Verhulst, 1996; Schmid-Hempel, 2003), studies have demonstrated that the outcome of immune responses and parasite infection is not only dependent on the environment (E) but also shows complex phenomena such as priming and memory. This complexity has many ramifications for how insect hosts and parasite interact. With regard to the environment, a change in condition, mechanistic or other constraints may mean either that environmental variation has little or no influence or that the subsequent outcome of host–parasite interactions is altered. For example, previous exposure to parasites is an environmental factor that might change the subsequent conditions in important ways. Therefore, knowledge of which naturally occurring factors alter host–parasite outcomes is important for judging the general relevance of environmental fluctuations for host–parasite systems.

The relevant factors include both abiotic and biotic ones. For example, food availability has repeatedly been proven to be an important environmental factor in relation to immune responses. For instance, bumblebees show a decreased immunocompetence in food-deprived workers (Schmid-Hempel & Schmid-Hempel, 1998) and increase their food uptake when immune-challenged (Tyler *et al.*, 2006). A comparable effect on immunity has been found in *Drosophila melanogaster* (Valtonen *et al.*, 2010). Similarly, immune priming (Little & Kraaijeveld, 2004), that is, the prior experience with parasites (a biotic factor in the hosts environment), matters (Rosengaus *et al.*, 1999; Rahman *et al.*, 2004; Witteveldt *et al.*, 2004; Eleftherianos *et al.*, 2006). Immune priming can furthermore be specific (only being relevant to the same parasites as in the first exposure) at the level of parasite

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species (Sadd & Schmid-Hempel, 2006; Pham *et al.*, 2007) or even strains (Roth & Kurtz, 2009; Roth *et al.*, 2009). There is also evidence for immune priming within individuals (Sadd & Schmid-Hempel, 2006; Pham *et al.*, 2007; Roth *et al.*, 2009), between generations (Little *et al.*, 2003; Sadd *et al.*, 2005; Roth *et al.*, 2010) or even potentially for social priming between individuals sharing the same social environment (Traniello *et al.*, 2002; Ugelvig & Cremer, 2007). However, priming is not ubiquitous, even within a host species (Pham *et al.*, 2007).

In addition to general environmental influences on the outcome of infection, host genotypes (G_H) have been shown to differ in their susceptibility to parasites (Henter & Via, 1995; Ebert *et al.*, 1998; Little & Ebert, 2000, 2001; Baer & Schmid-Hempel, 2003; Lambrechts *et al.*, 2006a), and different parasite genotypes vary in their infectivity (G_P) (Henter, 1995; Little *et al.*, 2006). In some cases, the interaction of host with parasite genotypes ($G_H \times G_P$) determines the outcome of infection (Carius *et al.*, 2001; Lambrechts *et al.*, 2005, 2006b; Luijckx *et al.*, 2011). Such $G_H \times G_P$, referred to as specific host–parasite interactions, have the potential to maintain diversity in host and parasite populations through frequency-dependent selection and the emergence of Red Queen dynamics (e.g. Decaestecker *et al.*, 2007).

Bringing together environmental and genetic influences, the abiotic environment can influence the outcome of an infection by interacting either with the host ($G_H \times E$) (Blanford *et al.*, 2003; Bedhomme *et al.*, 2004; Schoebel *et al.*, 2011) or with the parasite genotype ($G_P \times E$) (Ferguson & Read, 2002; Mitchell *et al.*, 2005; Vale *et al.*, 2008; Vale & Little, 2009). By favouring different genotypes in different environments, genetic polymorphism can be maintained (Gillespie & Turelli, 1989). Furthermore, recent studies have demonstrated that the outcome of specific host–parasite interactions may, in some cases, be mediated by the environment ($G_H \times G_P \times E$) (Heath & Tiffin, 2007; Tétard-Jones *et al.*, 2007; Piculell *et al.*, 2008; Bryner & Rigling, 2011; Sadd, 2011). For example, in the bumblebee *Bombus terrestris* L., the $G_H \times G_P$ interactions determining infection outcome between host genotypes and genotypes of the gut parasite *Crithidia bombi* (Lipa & Triggiani, 1988) can be altered by food quality (Sadd, 2011). While outcomes of three-way interactions are more complex to formulate, $G_H \times G_P \times E$ will have important implications for coevolution, because of a mosaic of selection pressures across environments (Lazzaro & Little, 2009; Wolinska & King, 2009). Moreover, Mostowy & Engelstädter (2011) have theoretically shown that the impact of environmental dependence on coevolution can largely inverse the parameter space under which coevolution is expected to select for sex and recombination. Of course, exact outcomes will depend upon the frequency and amplitude of environmental variation across space and/or time (Wolinska & King, 2009).

It is likely that although present in some cases, environmental dependence in the outcome of specific host–parasite interactions will not always be present due to yet unknown constraints within the particular systems. For instance, another experiment looking at $G_H \times G_P \times E$ interactions in an animal system could not detect any change in the genotype-specific interaction between *Daphnia magna* and its parasite *Pasteuria ramosa* across three different temperatures (Vale & Little, 2009), although they did detect $G_P \times E$ for parasite transmission stage production.

The system of the bumblebee *B. terrestris* L. and its trypanosomatid parasite *C. bombi* (Lipa & Triggiani, 1988) is a relevant and interesting system in which to study the importance of environmental variation for the outcome of host–parasite interactions. Strains of *C. bombi* show an immense genetic diversity (Schmid-Hempel & Funk, 2004), and cultures of single clones can be grown for controlled infections. The system shows marked $G_H \times G_P$ interactions under standardized conditions (Schmid-Hempel, 2001), but as outlined above, naturally relevant variation in food quality may alter these interactions (Sadd, 2011). Relating to parasite experience, specific immune priming within an individual (Sadd & Schmid-Hempel, 2006), as well as immune priming between individuals (Moret & Schmid-Hempel, 2001) and across generations, has been demonstrated (Sadd *et al.*, 2005; Sadd & Schmid-Hempel, 2007). However, the latter results on immune priming have all been obtained by priming with bacterial based challenges, not by naturally infecting bumblebees. Furthermore, bumblebees are social insects, and therefore, there is a potential for a social response and immune priming (Cremer *et al.*, 2007). This may have particular relevance for *C. bombi*, as while adult workers rearing the future cohorts of offspring can get infected, these future cohorts themselves are thought not to be susceptible to infection until adulthood. Such workers-to-larvae effects have indeed been demonstrated in this system before, at least for a 'bacterial' challenge (Moret & Schmid-Hempel, 2001). Influences on offspring immunity due to larval parasite rearing environment could be twofold. On the one hand, continued exposure to albeit currently noninfective parasite cells in contaminated food throughout larval rearing could lead to direct immune priming. Alternatively, immune experience of infected adult workers rearing the larvae could be transmitted in the manner of social immune priming.

Using a split-colony design (Schmid-Hempel & Schmid-Hempel, 1998), we investigate whether parasite pressure and identity in the rearing environment influences parasite resistance when offspring reach adulthood. Indicative of the absence of a strong influence, we show that the genotype-specific host–parasite interactions found in the *B. terrestris* – *C. bombi* host–parasite system are robust to environmental variation relating to the surrounding parasite environment during larval rearing.

Materials and methods

Insects and parasites

Laboratory colonies of *B. terrestris* were started from field-caught queens collected in the spring of 2010 in north-western Switzerland. *Crithidia bombi* strains used in this experiment came from single cells isolated from faeces of naturally infected queens collected in 2008 from the same population, stored in liquid nitrogen as clonal cultures and subsequently revitalized. To maximize the probability of seeing any strain-specific responses, we selected two strains (nos. 08.226 and 08.261, subsequently labelled A and B, respectively) that had relatively distinct infectious rates across several bumblebee colonies (i.e. showing the signature of host–genotype-by-parasite–genotype interaction) in a pilot study (data not shown).

Split-colony design and infection protocol

When brood size reached a sufficient size to be split into three parts, three groups of three workers from each of these parts per colony were isolated. Two of these groups were infected *per os* (after a starvation period of 2 h) with 10 μL cell suspension of 2000 cells μL^{-1} of either parasite strain A or B in 50 per cent sugar water (the adult treatments at this stage translate into the future larval parasite treatment groups, with the strains being used subsequently called the 'larval exposure strain'; a given group only received A or B). The third, control group, was fed only sugar water without parasite cells. These workers were kept separate as groups until being merged with their nest mates as follows. After 7 days, the brood of each colony was split into three parts, which were subsequently kept separately (without the queen) as 'mini-colonies', and randomly assigned to one of the previously defined treatment groups (larval exposure strain A, larval exposure strain B and larval exposure control). The workers that had been separated and exposed earlier, together with five nonmanipulated workers from the original colony, formed each of these mini-colonies. The queen and a small amount of brood were left in the original colony to continue laying eggs. The eggs subsequently produced by the queen were used to replenish the mini-colonies with fresh offspring where needed. This split-colony design was carried out for a total of nine colonies, thus yielding a total of 27 mini-colonies.

In the mini-colonies, the infection was left to spread for 7 days, at which point we started to remove and individually isolate callow workers (i.e. newly hatched individuals), which are typically uninfected. In fact, from our tests, we found that a very small proportion (0.034%) of 59 bees removed as callows to check for residual infections tested positive for *C. bombi* in a PCR-based assay. Residual infections are thus unlikely to have contributed to the main results below relating to adult infection.

After seven more days, these removed workers were in turn infected as explained previously with either strain A or B (the strain used to infect these workers being subsequently called 'infective strain') or left naïve (to confirm noninfection of freshly removed callows). The bees were then snap-frozen in liquid nitrogen 7 days post-infection and stored at −80 °C until inspection. Infection intensity was measured in these bees with quantitative real-time PCR (qPCR) following the protocol of Ulrich *et al.* (2010).

Statistical analyses

Unlike Ulrich *et al.* (2010), qPCR assays of infection intensities were not corrected for DNA concentration in the sample, as it has been subsequently shown that raw measurements ($R^2 = 0.998$) provide a slightly better fit in controlled tests than measurements corrected for DNA concentration ($R^2 = 0.992$) (B. M. Sadd, unpublished data). Five of nine colonies, for which the following statistical analyses were carried out, yielded a sufficient level of replication in all treatments (colonies were removed that did not produce enough offspring after the formation of mini-colonies to have at least two individuals per treatment combination), and these results are reported here. Three models were fitted in R2.10.1 (R Development Core Team, 2009) to analyse infection intensity (i) with larval exposure strain and subsequent adult infective strain as fixed factors; (ii) with the relationship (heterologous, homologous and control) between the larval exposure strain and the adult infective strain as a fixed factor; and (iii) whether there was any general influence of larval parasite exposure, a fixed factor with larval exposure strains A and B pooled and controls. Colony identity was included in all models as fixed factor. All models were GLMs with a quasi-Poisson error structure. Statistical values reported are from the models with all two-way interactions, but with any nonsignificant three-way interaction removed.

Results and discussion

The *C. bombi* strains A and B differed in their general level of infectiousness ($F_{1,350} = 67.33$, $P < 0.001$), whereas *B. terrestris* colonies were different from one another as to their general resistance to the parasite as indicated by their infection intensities ($F_{4,346} = 14.04$, $P < 0.001$). The interaction between larval exposure strain and adult infective strain was not significant ($F_{2,344} = 2.13$, $P = 0.12$), indicating no specific influence of larval parasite exposure on adult resistance (Fig. 1). Grouping the data according to the relatedness between the larval exposure strain and the adult infective strain (i.e. homologous, heterologous and control; $F_{2,351} = 0.18$, $P = 0.84$) or general larval parasite

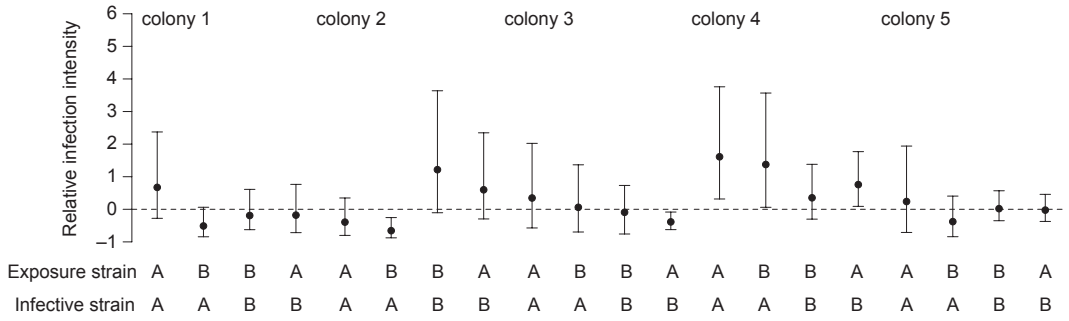

Fig. 1 Infection intensity of bees from larval parasite exposures A or B (first letter) when infected as adults with parasite strains A or B (second letter), relative to bees reared in a control (parasite-free) environment. For example, bees reared by workers infected by strain A, and subsequently infected with the same strain A, are compared to bees reared by noninfected workers, and subsequently infected with strain A. Values are average bootstrap values (±95% CI).

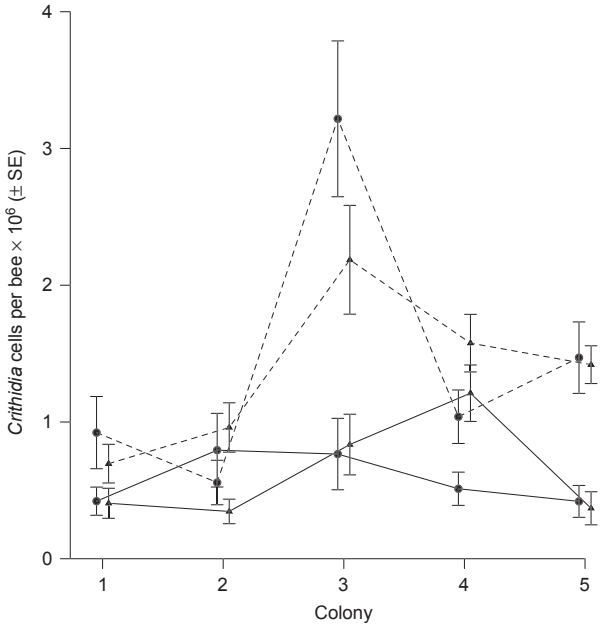

Fig. 2 Infection intensity in adult bees infected with strain A (solid line) or strain B (dashed line) for individuals from the larval control exposure treatment (circles) or the larval parasite exposure (triangles; larval exposure strains A and B pooled; see Materials and Methods). Data points represent average infection intensity (±SE).

exposure (pooled larval exposures vs. control; $F_{1,352} = 0.18$, $P = 0.67$) also did not reveal any effect. There was, however, a parasite–genotype-by-host–genotype interaction, as shown by the adult infective strain × colony interaction ($F_{4,332} = 3.15$, $P = 0.015$; Fig. 2). There was also a weaker larval exposure strain × colony interaction ($F_{8,336} = 1.99$, $P = 0.047$), suggesting that different colonies react differently (in direction and/or intensity) to the larval exposure. This interaction was also present in the analysis looking at the effect of priming (of any type) vs. control ($F_{4,344} = 2.94$, $P = 0.021$).

The results show that the parasite exposure during larval development does not noticeably influence adult resistance, in the manner that social or individual immune priming would be expected to. Indeed, the host–parasite genotype interaction between workers of *B. terrestris* colonies and *C. bombi* strains is robust against variation in the parasitic environment experienced during larval rearing. The three-way interaction between host colony, parasite strain and larval environment was not significant ($F_{8,324} = 1.19$, $P = 0.3$) and thus removed from the final model. The fact that we did not find any transfer of protection from rearing workers to developing larvae contrasts to some degree with the results of Moret & Schmid-Hempel (2001). In their experiment, they found that male bumblebees reared by workers immune-challenged with bacterial lipopolysaccharides (LPS) showed higher phenoloxidase (PO) activity, an enzyme in the melanization cascade, compared to those that had been reared by control workers. While it has not been demonstrated that this increased immunity would necessarily translate into increased resistance against *C. bombi* (like the link between infection and immune measurements in Ayres & Schneider, 2008), it has been shown that infection with *C. bombi* induces in the host an up-regulation of the PO activity (Brown *et al.*, 2003a) and leads to the expression of antimicrobial peptides (Riddell *et al.*, 2009). The contrast with the results of Moret & Schmid-Hempel (2001) could be due to different immune challenges (an immune elicitor in the haemocoel vs. a live parasite in the gut).

Although evidence is accumulating that invertebrate immunity and parasite resistance can be strongly influenced by environmental variation, it is not known what the limits of the system are. Bumblebees, like other organisms, are host to numerous parasites, from viruses to bacteria, trypanosomes, protozoa, nematodes and parasitoid flies (Schmid-Hempel, 1998; Goulson, 2010). Different parasite types and their infections may be differentially influenced by environmental variation.

While more and more evidence for immune priming in invertebrates is accumulating (Sadd & Schmid-Hempel, 2006; Pham *et al.*, 2007; Schulenburg *et al.*, 2007; Roth & Kurtz, 2009; Rodrigues *et al.*, 2010), it is likely that mechanistic and other constraints mean that immune priming will not be ubiquitous for all parasite types and situations. For example, in *Drosophila*, Pham *et al.* (2007) found evidence of immune priming to *Streptococcus pneumoniae* and *Beauveria bassiana*, but not to *Salmonella typhimurium*, *Listeria monocytogenes* and *Mycobacterium marinum*. *Crithidia bombi* seems not to be a parasite the bumblebee immune system can be primed against under the conditions of the current study.

That exposure to *C. bombi* does not lead to immune priming is perhaps surprising given the high fitness cost of an infection by *C. bombi* (Brown *et al.*, 2000, 2003b) and given that *C. bombi* does elicit the immune system of the host (Brown *et al.*, 2003a; Riddell *et al.*, 2009; Schlüns *et al.*, 2010). Furthermore, living in societies of closely related individuals favours the spread of infectious diseases (Schmid-Hempel, 1998), and research on social immunity has shed light on the various ways of how these organisms cope with this situation (reviewed in Cremer *et al.*, 2007). Very little is known about the mechanism by which workers could directly up-regulate the immune response of the developing larvae (Moret & Schmid-Hempel, 2001) to be better prepared against later infections. Hamilton *et al.* (2011) found that immunized workers of the carpenter ant *Camponotus pennsylvanicus* both increased trophallactic behaviour (mutual feeding of nest mates through regurgitation) and transferred food droplets with higher antimicrobial activity. However, the functional implications of this behaviour are not known. Further, whether any kind of priming of bumblebee larvae would persist through metamorphosis has never been formally tested. In other holometabolous insects, a study with the wax moth *Galleria mellonella* (Meylaers *et al.*, 2007) could not find any effect of an immune challenge during the larval or pupal stages on adult immunocompetence; however, effects were found in studies on beetles (Roth *et al.*, 2009; Thomas & Rudolf, 2010). In the hemimetabolous insect *Gryllus campestris*, a challenge in juveniles had an effect in adults (Jacot *et al.*, 2005).

Our study is another step towards a better understanding of how invertebrate immunity and the outcome of infection depend upon environmental variation. The study suggests that although bumblebees, their immunity and resistance to parasites have been shown to be relatively malleable upon environmental change (Moret & Schmid-Hempel, 2001; Sadd & Schmid-Hempel, 2006, 2007; Sadd, 2011), not all environmental variation will be relevant. It is yet to be seen whether these instances are due to adaptive differences and the evolution of environmentally dependent plasticity or due to constraints within the particular system.

Acknowledgments

We thank Y. Ulrich and M. Jales Hon for their help during the experiment. The real-time PCR data were generated in the Genetic Diversity Center (GDC) of ETH Zurich. This work was supported by the Swiss NSF (nr. 31003A-116057 to PSH).

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Received 10 January 2012; revised 29 February 2012; accepted 7 March 2012