Activation of host constitutive immune defence by an intestinal trypanosome parasite of bumble bees

M. J. F. BROWN[1,2]*, Y. MORET[1,3] and P. SCHMID-HEMPEL[1]

[1] *Ecology and Evolution, ETH-Zürich, ETH-Zentrum NW, CH-8092 Zürich, Switzerland*
[2] *Department of Zoology, Trinity College Dublin, Dublin 2, Ireland*
[3] *Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield S10 2TN, UK*

SUMMARY

Many parasites, including important species that affect humans and livestock, must survive the harsh environment of insect guts to complete their life-cycle. Hence, understanding how insects protect themselves against such parasites has immediate practical implications. Previously, such protection has been thought to consist mainly of mechanical structures and the action of lectins. However, recently it has become apparent that gut infections may interact with the host immune system in more complex ways. Here, using bumble bees, *Bombus terrestris* and their non-invasive gut trypanosome, *Crithidia bombi*, as a model system we investigated the effects of parasitic infection, host resources and the duration of infections on the host immune system. We found that infection doubled standing levels of immune defence in the haemolymph (the constitutive pro-phenoloxidase system), which is used as a first, general defence against parasites. However, physical separation of the parasite from the haemolymph suggests the presence of a messenger system between the gut and the genes that control the pro-phenoloxidase system. Surprisingly, we found no direct effect of host resource-stress or duration of the infection on the immune system. Our results suggest a novel and tactical response of insects to gut infections, demonstrating the complexity of such host–parasite systems.

Key words: trypanosomes, *Crithidia bombi*, bumble bees, *Bombus terrestris*, immune response, pro-phenoloxidase system.

INTRODUCTION

Many parasites, including species of *Trypanosoma*, *Crithidia*, *Leishmania* and *Plasmodium*, and filarial nematodes, must overcome the inhospitable environment of the insect gut for successful reproduction and transmission (Kaslow & Welburn, 1996). Given the major impact that such parasites can have on human and animal health, understanding how insects defend themselves against such parasites and prevent the establishment of infections is important from both a pure and applied perspective. The main defences of insects against such parasites are thought to be lectins, the peritrophic matrix, lysis, and melanization and encapsulation above the basal lamina of the gut (reviewed by Kaslow & Welburn (1996)). Recent work, however, suggests that interactions between gut parasites and the invertebrate immune system may be more complex than previously suspected (Kaaya, Flyg & Boman, 1987; Richman *et al.* 1997; Lowenberger *et al.* 1999; Boulanger *et al.* 2001, 2002; Hao *et al.* 2001).

Invertebrates possess an innate immune system, which has been the focus of much recent study (for reviews, see Hoffmann, Reichhart & Hetru (1996); Hoffmann & Reichhart (1997); Hoffmann *et al.*

(1999)). It is based on different types of haemocytes that are involved in (i) recognition of non-self surfaces, (ii) the organization of the immune response and, (iii) in nodule formation and encapsulation (Rattcliffe & Rowley, 1979; Gupta, 1991). Invertebrates also produce anti-microbial substances, lectins, and other immune factors, such as lysozymes. In addition, the phenoloxidase activating system plays a pivotal role in the organization and effectiveness of immune defence (Söderhall & Cerenius, 1998).

Functionally, the invertebrate immune response can, therefore, be broken down into 2 main elements, a constitutive and an inducible part (Hultmark, 1993; Hoffmann *et al.* 1996; Söderhall & Cerenius, 1998). Although the distinction is not always clear-cut and the terminology is not universally accepted, we here call constitutive responses those that utilize or activate immune factors that are present without previous contact with a parasite. Typically, in insects these are the circulating haemocytes and the pro-phenoloxidase system. Inducible responses, in contrast, rely on the *de novo* synthesis of anti-parasite factors e.g. anti-microbial peptides, upon recognition of an appropriate antigenic signal. Constitutive responses provide a first line of defence effective against a broad spectrum of parasites (i.e. they are non-specific), while coexisting with the inducible responses that provide a second, more specific (i.e. effective against a narrow range of parasites) defence (Hultmark, 1993; Brey & Hultmark, 1998).

* Corresponding author: Department of Zoology, Trinity College Dublin, Dublin 2, Ireland. Tel: +353 (0)1 608 1627. Fax: +353 (0)1 677 8094. E-mail: mabrown@tcd.ie

The application of evolutionary concepts demonstrated that the invertebrate immune system is costly to evolve (Kraaijeveld & Godfray, 1997) and utilize (Moret & Schmid-Hempel, 2000). In consequence we would expect induction of immune defences, and levels of constitutive response to be appropriate to the threat of parasitism. Indeed, the induction of anti-bacterial peptides has been shown to be specific, with different types of pathogen inducing the production of different peptides (Lemaitre, Reichhart & Hoffmann, 1997; Engstrom, 1999). Furthermore, recent work suggests that invertebrates can alter the constitutive defence response of their offspring with respect to current levels of perceived parasite threat (Moret & Schmid-Hempel, 2001), and, similarly, that host populations can vary the constitutive defence according to population density, which acts as a correlate for the threat of infections (Wilson & Reeson, 1998; Barnes & Siva-Jothy, 2000; Wilson et al. 2001).

Apart from the perceived risk of infection, host condition and environmental stress, e.g. resource limitation, temperature, and humidity, are well-known factors that explain variation in the level of the constitutive immune response (e.g. Ferguson & Read (2002)). As a consequence, otherwise benign parasites may have a large impact when their host is stressed (Jokela et al. 1999; Brown, Loosli & Schmid-Hempel, 2000), and consequently allocation of resources to immune function and the ability to resist stress may trade-off against each other.

Here we examine how the non-invasive, intestinal trypanosome gut parasite, *Crithidia bombi*, affects the immune system of its host, the bumble bee *Bombus terrestris*, and how this effect is moderated by environmental stress. *C. bombi* has a high prevalence in bumble bees (Shykoff & Schmid-Hempel, 1991*c*). The life-cycle is simple – transmission stages (amastigotes and choanomastigotes; personal observation) are ingested from bumble bee faeces and then pass into the hindgut. New transmission stages are released into the host's faeces starting about 3–4 days post-infection (Schmid-Hempel & Schmid-Hempel, 1993). Several studies, using both cross-infection experiments and genetic analyses, demonstrate strong genetic interactions between the parasite and its host (Shykoff & Schmid-Hempel, 1991*a*, *b*; Schmid-Hempel & Schmid-Hempel, 1993; Imhoof & Schmid-Hempel, 1998; Schmid-Hempel et al. 1999). While normally benign, in food-stressed bees the parasite may impose a 50% increase in background mortality rate (Brown et al. 2000).

We examined the effects of infection, food-stress and the duration of these treatments (days post-inoculation) on immune parameters of the bumble bee host. As the parasite is non-invasive and thus restricted to the gut lumen, we expected that the infection would have a marginal or no effect on the immune responses located in the haemolymph, such as the pro-phenoloxidase activating system or the

number of circulating haemocytes. In contrast, we predicted that under food-stress the parasite should cause a decrease in the level of the constitutive immune defence due to the cost of the use of host's resource by the parasite, which would be hard to compensate for under stressful conditions. We also predicted that this decrease of constitutive defence should be more marked the longer the duration of the infection. Surprisingly, we found that infection by this non-invasive parasite produced an increase in the constitutive pro-phenoloxidase immune system present in the haemolymph, a previously unreported aspect of the invertebrate immune function in this particular *in vivo* context. However, in contrast to other recent studies (Boulanger et al. 2001, 2002; Hao et al. 2001), we found no evidence for a similar increase in the production of inducible anti-bacterial peptides.

MATERIALS AND METHODS

Bees for the experiment were taken from 3, unrelated laboratory-reared colonies of *B. terrestris*, first-generation descendants of queens collected in the field around Zürich, Switzerland in spring 2000. Prior to the experiment, bees were reared at 29 °C and 60% relative humidity and supplied with *ad libitum* pollen and sugar water.

Bees from each colony were randomly assigned to 1 of 8 treatment groups, for a total of 10 bees per group. Treatments were as follows: (A) control versus infected, to test for the effect of *Crithidia bombi* infection on the immune system of the bees, (B) pollen versus no pollen, to test for the effect of resource-stress (pollen is needed for both ovary and fat body development, with the fat body playing an essential role in the invertebrate immune system), (C) measurement after 7 days versus 14 days, to test for the effect of the duration of infection, as well as a simple age effect (see Table 1 for the full crossed design). Bees in the infected treatment were starved for 3–4 h and then fed a standardized inoculum of 5000 *C. bombi* cells (from at least 5 different parasite strains) in sugar water. Control bees were similarly starved, but then fed normal sugar water. Bees in the pollen treatment were kept with *ad libitum* pollen and sugar water for the duration of the treatment, while bees in the no pollen treatment had their pollen source removed at day 5 of the experiment and were then kept on sugar water. Bees in the 7 days treatment were frozen on the 7th day of the experiment for measurement of the experimental variables (see below), and bees in the 14 days treatment were frozen on the 14th (and last) day of the experiment.

We measured 5 variables in each experimental animal – haemocyte concentration, anti-bacterial activity, phenoloxidase activity, fat body size and ovary development. Of these, the first 4 are directly relevant to immune function (the insect fat body is functionally

Table 1. *Experimental design of the infection with Crithidia bombi for each of the 3 colonies of Bombus terrestris*

(For each colony a group of 10 workers was assigned to 1 of the 8 treatment groups according to infection treatment (control vs. infected), resource treatment (fed with vs. without pollen) and duration of the infection where the workers were tested for various measures at 7 vs. 14 days post-infection.)

	Control for infection	Infection with *C. bombi*
Fed with pollen	Tested at 7 days post-inoculation ($N = 10$)	Tested at 7 days post-inoculation ($N = 10$)
	Tested at 14 days post-inoculation ($N = 10$)	Tested at 14 days post-inoculation ($N = 10$)
Starved (without pollen)	Tested at 7 days post-inoculation ($N = 10$)	Tested at 7 days post-inoculation ($N = 10$)
	Tested at 14 days post-inoculation ($N = 10$)	Tested at 14 days post-inoculation ($N = 10$)

analogous to the vertebrate liver, and responsible for the production of antibacterial peptides), whilst ovary development is a proxy for reproductive capacity and known to trade-off with allocation of resources to the fat body. Each worker was bled to measure the haemocyte concentration, the antibacterial activity and the phenoloxidase (PO) activity of their haemolymph. For this, each insect was chilled 10 min on ice and the pleural membrane between the 5th and the 6th sternite of the abdomen was punctured with a sterile hypodermic needle. The droplet of haemolymph that came out of the wound was collected into a sterile and pre-chilled glass capillary. For each insect, 10 μl of haemolymph was collected and flushed into a 1·5 ml Eppendorf tube containing 50 μl of cold sodium cacodylate/CaCl$_2$ buffer (0·01 M Na-Cac, 0·005 M CaCl$_2$, pH 6·5). Bees were immediately freeze-killed and stored at -80 °C for remaining analyses (see below). A 10 μl sample of the diluted haemolymph was immediately used for the estimation of the haemocyte concentration using an improved Neubauer haemocytometer. Another 10 μl sample was kept in a 0·5 ml Eppendorf tube and stored at -20 °C for later examination of the antibacterial activity using a zone-of-inhibition test. This test consists of adding a 2 μl drop of haemolymph to a plate of bacteria and measuring the radius of the zone where antibacterial peptides in the haemolymph inhibit bacterial growth. The methods for this test were the same as described by Moret & Schmid-Hempel (2000) except that the assay was performed using haemolymph diluted 6 times with sodium cacodylate/CaCl$_2$ buffer. The rest of the haemolymph solution was diluted with an additional volume of 100 μl of cold sodium cacodylate/CaCl$_2$ buffer and immediately stored at -80 °C for later measurement of the PO activity. For the PO activity assay, samples of frozen haemolymph solution (dilution 1/21; haemolymph/sodium cacodylate/CaCl$_2$ buffer) were thawed on ice and then centrifuged (3800 *g*, 15 min, 4 °C). The supernatant fraction was removed and vortexed,

after which 20 μl of the supernatant was added to a microplate well containing 140 μl of distilled water, 20 μl of phosphate-buffered saline (PBS: 8·74 g NaCl; 1·78 g Na$_2$HPO$_4$, 2H$_2$O; 1000 ml distilled water; pH 6·5) and 20 μl of L-Dopa solution (4 mg per millilitre of distilled water). The reaction was allowed to proceed at 30 °C in a microplate reader (SpectraMAX-340PC) for 20 min. Readings were taken every 10 sec at 480 nm and analysed using SOFTmax®PRO 3.1 software. The enzyme activity was measured as the slope of the reaction curves during the linear phase of the reaction (between 10 and 20 min after the reaction mix was made; Y. Moret, personal observation).

Frozen bees were dissected blind with respect to treatment and we recorded presence/absence of a *C. bombi* infection (under $\times 400$ magnification), ovary development (the mean length of the 3 terminal oocytes), body size (length of the radial cell of the right forewing), and fat body size. To measure the amount of fat present we followed the protocol of Ellers (1996). For this, the abdomen was dried at 70 °C for 3 days and then weighed with a precision balance. The abdomen was then placed in 2 ml of ether for 24 h to extract the fat. After rinsing with fresh ether, the abdomen was again dried for 3 days and weighed again. The amount of fat is indicated by the difference between these two measures. We divided fat mass by body size to get an estimate of relative fat body size for use in the statistical analyses.

The ideal analysis for this experiment would be a 4-way MANOVA with colony as a random factor, and infection, pollen, and time treatments as fixed factors. Unfortunately, the data failed to meet the assumptions for such an analysis (the structure of variation across treatment groups made it impossible to transform the data to meet the assumptions of homogeneity and normality when colony was included in the design). Consequently, we took 2 complementary approaches to analyse these data. Firstly, using the untransformed data, we conducted

Table 2. Mean ± S.E. of the 5 dependent variables haemocyte number, phenoloxidase activity, anti-bacterial activity, relative fat content (in mg/mm) and oocyte length (in mm) for the 8 treatment groups by colony

(Numbers 1, 2, 3 represent 3, unrelated laboratory-reared colonies.)

		Uninfected Pollen 7 days	Infected Pollen 7 days	Uninfected No pollen 7 days	Infected No pollen 7 days	Uninfected Pollen 14 days	Infected Pollen 14 days	Uninfected No pollen 14 days	Infected No pollen 14 days
(1)	Haemocyte number	85·3±17·14	59·1±8·28	52·4±9·06	66·2±10·54	52·4±15·33	25·25±8·05	33·2±7·39	24·1±6·97
	Phenoloxidase activity	410·3±188·84	611·2±123·15	229·0±79·59	400·7±94·17	813·5±183·77	718·8±191·50	479·7±111·72	1300·8±265·71
	Anti-bacterial activity	0·0±0·00	1·7±1·20	1·9±1·20	0·6±0·56	0·0±0·00	3·4±2·26	2·2±1·18	3·1±1·27
	Relative fat body	0·064±0·0087	0·056±0·0084	0·049±0·0051	0·040±0·0063	0·075±0·0109	0·074±0·0086	0·067±0·0113	0·073±0·0123
	Oocyte length	1·5±0·26	1·4±0·23	0·5±0·26	0·8±0·24	0·2±0·15	0·8±0·34	0·2±0·13	0·3±0·15
(2)	Haemocyte number	60·6±6·39	29·3±9·81	38·3±5·87	20·0±6·82	25·3±5·29	34·2±8·30	35·4±11·53	47·8±13·66
	Phenoloxidase activity	432·3±83·10	318·4±58·57	160·3±59·83	565·5±291·16	886·8±437·21	1235·2±315·62	216·8±71·33	1366·6±263·83
	Anti-bacterial activity	0·0±0·00	0·0±0·00	2·1±1·40	0·0±0·00	0·0±0·00	5·9±2·70	1·7±1·70	1·3±1·31
	Relative fat body	0·068±0·0072	0·051±0·0054	0·065±0·0117	0·092±0·0360	0·070±0·0119	0·064±0·0134	0·064±0·0124	0·051±0·0095
	Oocyte length	0·5±0·21	0·6±0·39	0·7±0·23	0·2±0·11	0·0±0·00	0·1±0·11	0·1±0·13	0·0±0·00
(3)	Haemocyte number	29·4±6·61	36·6±6·40	42·6±9·75	47·4±9·94	41·5±7·51	24·7±2·86	43·3±11·70	35·0±8·29
	Phenoloxidase activity	661·3±265·21	847·0±191·13	138·1±55·52	625·8±259·19	30·5±15·11	742·4±126·22	10·4±8·10	748·0±174·67
	Anti-bacterial activity	3·5±1·93	5·3±2·24	3·4±1·74	3·4±1·88	1·09±1·094	2·9±1·49	0·0±0·00	1·3±1·33
	Relative fat body	0·075±0·0084	0·071±0·0060	0·060±0·0071	0·057±0·0087	0·071±0·0065	0·071±0·0077	0·083±0·0155	0·068±0·0069
	Oocyte length	2·0±0·11	1·3±0·32	1·5±0·27	1·4±0·32	0·9±0·37	1·6±0·27	0·3±0·14	0·1±0·09

4-way MANOVA analyses for each of the variables independently (that is, using the error terms assigned for MANOVA univariate tests). We followed these analyses with 3-way MANOVAs on the transformed data for each colony individually (phenoloxidase activity was square-root transformed to meet the assumptions of the analyses). Because the results of the two analyses were qualitatively the same, we report here the results of the 4-way analyses. The untransformed data are presented in Table 2.

Of the 5 dependent variables, anti-bacterial activity and oocyte length could not be transformed to meet the assumptions of MANOVA analyses, due to a large number of zero values, and the complexity of the experiment prevented a comparable non-parametric analysis. Consequently, to analyse these data we converted the variables into 0/1 categorical variables ('0'=no activity or no egg development, '1'=activity or egg development) and entered them into logistic regression analyses. We used a forward stepwise likelihood ratio design, with colony given deviation coding and infection, food and life-treatments coded as indicator variables.

All analyses were done on SPSS 6.1.1 and SPSS 10 for the Macintosh, and for the MANOVA we report the results of the multivariate and univariate tests (all P values are two-tailed). Data are reported as mean ± standard deviation.

RESULTS

Direct effects of infection

Infection by *Crithidia bombi* had a positive effect on the level of phenoloxidase activity, with infected bees having, on average, twice the level of activity of uninfected bees (MANOVA, $F_{1,2}=27·3$, $P=0·035$; Fig. 1). In contrast, infection had no direct effect on the haemocyte concentration of bees ($F_{1,2}=7·83$, $P=0·108$), their relative fat content ($F_{1,2}=1·23$, $P=0·383$), anti-bacterial activity (control bees, 21/118 exhibited activity, $1·5±3·60$; infected bees, 26/116 exhibited activity, $2·4±4·72$; no significant regression model, Score $=2·708$, $P=0·1$) or ovary development (not in the final model, Score $=0·358$, $P=0·550$).

Direct effects of food deprivation

Surprisingly, the food treatment (*ad libitum* pollen versus no pollen) had no direct effect on haemocytes ($F_{1,2}=0·06$, $P=0·831$), phenoloxidase activity ($F_{1,2}=6·22$, $P=0·13$), fat content ($F_{1,2}=0·00$, $P=0·98$) or anti-bacterial activity of the experimental animals (pollen-fed bees, 22/115 exhibited activity, $2·0±4·45$; pollen-starved bees, 25/119 exhibited activity, $1·9±3·97$; no significant regression model, Score $=0·114$, $P=0·736$). As expected, pollen-starved bees
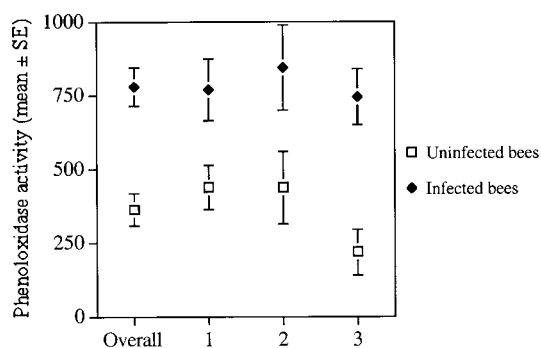
Fig. 1. The effect of infection by *Crithidia bombi* on phenoloxidase activity, across all bees and within each colony (colony number is on the *x*-axis).
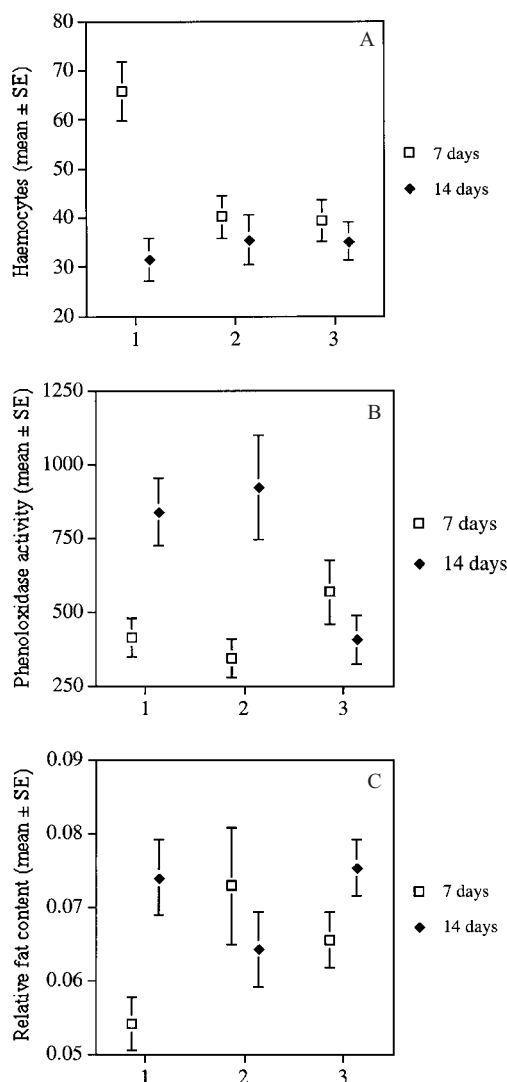

Fig. 2. The interaction between colony identity and duration of the experiment on the haemocyte counts, phenoloxidase activity and relative fat content of bees (colony number is given on the *x*-axis).

were less likely to exhibit ovary development (Wald-statistic $= 8 \cdot 749$, D.F. $= 1$, $P = 0 \cdot 003$).

Direct effects of duration of the experiment

Similarly, the duration of the experiment did not affect haemocytes ($F_{1,2} = 1 \cdot 43$, $P = 0 \cdot 355$), phenoloxidase activity ($F_{1,2} = 1 \cdot 28$, $P = 0 \cdot 376$), fat content ($F_{1,2} = 0 \cdot 61$, $P = 0 \cdot 517$) or anti-bacterial activity of the experimental animals (7-day bees, 22/120 exhibited activity, $1 \cdot 9 \pm 4 \cdot 33$; 14-day bees, 25/114 exhibited activity, $2 \cdot 1 \pm 4 \cdot 09$; no significant regression model, Score $= 0 \cdot 082$, $P = 0 \cdot 774$). In contrast, bees from day 14 of the experiment were less likely to exhibit ovary development (Wald-statistic $= 8 \cdot 749$, D.F. $= 1$, $P = 0 \cdot 003$).

Direct effects of colony identity

There were significant differences among colonies in their haemocyte concentrations (mean $\pm$ S.E. of counts: colony $1 = 50 \pm 4 \cdot 3$, colony $2 = 38 \pm 3 \cdot 3$, colony $3 = 37 \pm 2 \cdot 9$; $F_{2,180} = 4 \cdot 91$, $P = 0 \cdot 008$). Otherwise, colony identity only had a direct effect on ovary development, with bees from colonies 1 and 3 being more likely to have developed ovaries than bees from colony 2 (Wald-statistic $= 25 \cdot 833$, D.F. $= 2$, $P < 0 \cdot 001$).

Interactions among experimental treatments

There were no significant 2-way interactions between the infection treatment and any other treatment. This indicates that, regardless of colony identity, duration of the infection, or nutrient intake (*ad libitum* pollen versus pollen-starved), all bees responded similarly to the infection.

The only significant 2-way interaction was between colony identity and the duration treatment (Fig. 2). There were significant effects on haemocyte concentration ($F_{2,180} = 6 \cdot 72$, $P = 0 \cdot 002$), phenoloxidase activity ($F_{2,185} = 8 \cdot 09$, $P < 0 \cdot 001$), and relative fat content ($F_{2,180} = 4 \cdot 43$, $P = 0 \cdot 013$). These effects

were due to a larger decline in haemocyte concentration across time in colony 1 versus colonies 2 and 3 (Fig. 2A), an increase in phenoloxidase activity across time in colonies 1 and 2 versus a decrease in colony 3 (Fig. 2B), and an increase in relative fat content across time in colonies 1 and 3 versus a decrease in colony 2 (Fig. 2C).

DISCUSSION

The main result from our experiment is that a non-invasive gut parasite effectively doubled the standing-level of immune response in the haemolymph of infected bees as measured by the activity of the pro-phenoloxidase system. Previous reports suggest that trypanosome parasites are able to activate the pro-phenoloxidase system in haemolymph upon direct contact *in vitro* and that this activation is crucial to contain the infection (Nigam *et al.* 1997). However,

here the *in vivo* increase in phenoloxidase activity was found in the samples taken from the host haemolymph, and was thus physically separated from the site of infection. Infection had no direct effect on any of the other measures, which was in line with previous work (Brown *et al.* 2000).

Three separate lines of evidence demonstrate that *C. bombi* is indeed a non-invasive parasite. (1) In over 10 years of study in this system, *C. bombi* parasites have never been seen to break through the host gut and invade the haemolymph (personal observations). (2) During this study we microscopically examined the haemolymph of infected animals for our haemocyte counts and never observed parasites or their encapsulated remains. (3) The absence of an anti-bacterial response to infection, which we would expect if the gut wall was broken by the passaging of parasites, again indicates no invasion of the body cavity. Consequently, our results suggest the existence of a messenger system between the gut and the genes that control pro-phenoloxidase activity in the haemolymph, as has recently been suggested for the inducible immune system of invertebrates (Hao *et al.* 2001).

Given the specific and inducible nature of anti-bacterial peptides, the absence of an effect of infection on anti-bacterial activity is perhaps unsurprising. This result is unlikely to be due to a lack of sensitivity in the test, as (i) 20% of the study animals showed measurable anti-bacterial activity (see Results section) and (ii) previous studies using this test have found statistically significant differences between induced and control bees in anti-bacterial activity (Moret & Schmid-Hempel, 2000, 2001). However, our results differ sharply from those of Boulanger *et al.* (2001) and Boulanger *et al.* (2002), who found specific production of inducible anti-microbial peptides both when *Drosophila melanogaster* were infected *per os* by *C. bombi*, and when *Glossina mortisans* were infected by *Trypanosoma brucei brucei*. These differences may reflect the parasite pressure felt by the different species. The pro-phenoloxidase system acts as a general, non-specific protection and may be energetically cheaper and more efficient than the production of specific peptides when the particular threat cannot be predicted. In contrast, Boulanger *et al.* (2002) suggested that the presence of numerous bacterial symbionts in the digestive tract of tsetse flies might explain the induction of anti-microbial peptides in their experiment. Whether *D. melanogaster* or *G. mortisans* produce an increase in the constitutive pro-phenoloxidase system after infection remains unknown.

The general absence of a direct effect of resource-stress on measures of the immune system suggests either that immune function is a priority for resource allocation, or that bees were simply not stressed enough. Given that pollen-starved bees exhibited less ovary development, and that previous work also suggested priority allocation of resources to immune function over survival (Moret & Schmid-Hempel, 2000), we believe that the former explanation is most likely. In 1 colony, pollen-starvation did reduce phenoloxidase activity when compared to control bees of the same age, suggesting that a reduction in resource availability can decrease the immune function of invertebrates, as has also recently been found in the mealworm beetle, *Tenebrio molitor* (Siva-Jothy & Thompson, 2002). The magnitude and occurrence of this effect probably depends upon the initial host condition, as has been seen in vertebrates (Nordling *et al.* 1998; Fair, Hansen & Ricklefs, 1999).

We examined the effect of the duration of an infection on immune function by sacrificing host and control bees at 7 and 14 days post-infection. In general, longer infections produced a greater increase in the standing immune response (as shown by increased phenoloxidase activity), although this was true for only 2 of the 3 colonies. While infection had no effect on haemocyte concentration, the longer-lived and therefore older bees had a reduced haemocyte complement, reflecting a general effect of age on the immune function of individuals. This matches results from previous work in bumble bees, where encapsulation ability and haemocyte number also declined with increasing age (Doums *et al.* 2002). A surprising result of the life-treatment was the effect that it had on fat-body size. The increase in fat body size from days 7 to 14 seen in 2 out of 3 colonies suggests that bees may be re-allocating resources from the ovary (which regressed over time) to the fat body.

To conclude, we have demonstrated a novel response *in vivo* of the constitutive invertebrate immune system to infection by a non-invasive, intestinal parasite. This adds to recent evidence that such infections can induce the production of anti-bacterial peptides (Boulanger *et al.* 2001, 2002; Hao *et al.* 2001). There is increasing evidence that local infection of the gut triggers a systemic immune response and, vice versa, a systemic response induces a local response (Lehane, Wu & Lehane, 1997; Nakajima *et al.* 2001). It has been suggested that immune messengers connecting gut tissue and the fat body (a prime site of the immune system) include nitric oxide or cytokine-like molecules (Hao *et al.* 2001), perhaps in the form of TNF-homologues as recently discovered in *Drosophila* (Kanda *et al.* 2002; Moreno, Yan & Basler, 2002). Hence, the invertebrate immune system may also play an important role in the control of intestinal parasites. The exact function of the enhanced immune response to *Crithidia* infection remains to be determined.

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