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A field study on the influence of food and immune priming on a bumblebee–gut parasite system

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Abstract Laboratory experiments are often preferred over field experiments because they allow the control of confounding factors that would otherwise influence the causal effect of a particular focal experimental factor. These confounding factors can, however, significantly alter the response of an organism confronted with a particular situation, which can have great implications. In a field experiment with a bumblebee host–parasite system, we looked at the influence of additional food supply and immune challenge on various colony fitness values and parasite traits. We could confirm the importance of food on the colony fitness, but not on parasite infection probability or parasite genetic diversity. In contrast to the findings of laboratory experiments of this system, challenge of the immune system had no significant effect on colony fitness or parasite infections. These results likely reflect an overriding effect of environmental variation without disproving the concept of a cost of defence per se. But the results also demonstrate that confounding factors purposely controlled for in the laboratory have to be weighed against their ecological relevance, and stress the need for careful analysis before any direct transfer is made of laboratory results to field situations.

Keywords *Bombus terrestris* · *Crithidia bombi* · Host–parasite interaction · Cost of immunity · Ecology

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Introduction

The general decline of natural insect pollinators due to modifications of the landscape or the intensive use of pesticides (Murray et al. 2009) is a major concern. Moreover, today's intensive agriculture (and the use of greenhouses) makes the pollination capacity by wild pollinator populations insufficient (Klein et al. 2007; Williams and Osborne 2009); the use of commercially reared pollinators has thus become unavoidable. Morse and Calderone (2000) estimated the value of honeybee pollination to be worth \$14 billion in the United States alone, and the worldwide value of the tomato crops pollinated by bumblebees, for instance, is already approximated to \$17 billion per year (Velthuis and van Doorn 2006). However, even commercial populations of pollinators are at risk. This is demonstrated by honeybees suffering from the so-called Colony Collapse Disorder (CCD), where affected hives are suffering from adult bees deserting their colony. Although the exact cause of this collapse is currently unknown and many leads are being followed, parasites are suspected to contribute to this problem (Cox-Foster et al. 2007; Oldroyd 2007; vanEngelsdorp et al. 2009; Ratnieks and Carreck 2010).

Similar concerns apply to bumblebees (*Bombus* spp.), which are also suffering from alarming declines and where pathogens such as *Crithidia bombi* and *Nosema bombi* have also been implicated as possible sources of their demise (Otti and Schmid-Hempel 2008; Cameron et al. 2011). Hence, understanding the interplay between insect hosts, especially pollinators, and their parasites is of great importance not the least for its applied perspective. From this viewpoint, research on the bumblebee *Bombus terrestris* L. and one of its major parasites, the genetically highly diversified trypanosome *Crithidia bombi* (Lipa and Triggiani 1988; Schmid-Hempel and Funk 2004), has

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proved to be of great interest and is now a well-established system to investigate host–parasite interactions.

Infections by *C. bombi* reduce the life span of workers under otherwise stressful conditions (Brown et al. 2000), and reduce founding success and life-time fitness of infected spring queens (Brown et al. 2003; Yourth et al. 2008). In addition, the activation (or challenge) of the immune system has a cost for individual survival in poor, but not in good, environments, a cost most likely to be energetic (Moret and Schmid-Hempel 2000; Brown et al. 2003). This cost also has repercussions on the fitness and life history of the whole colony (Moret and Schmid-Hempel 2004). Furthermore, a general defence (encapsulation) is maintained even under adverse environmental conditions (Schmid-Hempel and Schmid-Hempel 1998; Brown et al. 2003), although strong general defence implies susceptibility against a broader range of strains of *C. bombi* (Mallon et al. 2003); high general defence is furthermore traded-off with antimicrobial activity (Moret and Schmid-Hempel 2001).

Many—but not all (e.g. König and Schmid-Hempel 1995)—of these results originate from laboratory experiments. Such experiments have the advantage that possible confounding effects on the outcome can be controlled much more easily. However, while laboratory experiments allow identification of direct causal effects, this approach has limitations when it comes to understanding the significance of the results in the "real world". This issue was of course already recognised long ago, for example, in the context of interspecific competition (Connell 1961, 1983) and ecosystem ecology (Carpenter 1996). As an example of more recent work, Calisi and Bentley (2009) reviewed endocrinological and behavioural experiments with vertebrates that yielded different results in the laboratory as compared to the field. They argue that the underlying reason is a change in the titre of various endocrinous hormones caused by environmental conditions or social interactions, hence causing variation in the stress response, reproduction, circadian rhythm or immune function. It is thus not surprising that the outcome in the field may be very variable, and different from the laboratory, depending on the organism's physiology. But regardless of the cause of the differences, it seems typically very difficult to generate general predictions. Therefore, the insight for laboratory–field comparisons is that these have probably to be done on a case-by-case basis. The necessity for confirmation of laboratory-derived results in a field setting is particularly important when it comes to applications of ecological principles such as host–parasite interactions, for instance, in relation to pest control (Georgis et al. 2006) or host immunity (Tripet et al. 2008; Boughton et al. 2011).

The aim of this study is to determine whether the earlier, laboratory-derived results hold in field conditions. In particular, the main questions addressed here are: (1) Does immune challenge with its demonstrated protective effect reduce *C. bombi* and *N. bombi* load in field-housed *B. terrestris* colonies? (2) Does it therefore increase fitness of a colony, or is the stimulated defence so costly in the field that it decreases fitness? (3) Does immune challenge lead to an increase of foraging activity of workers to compensate its cost? And (4) how does immune challenge influence the strain diversity of *C. bombi* infections?

Materials and methods

Bumblebee rearing and experimental treatments

Field-caught spring queens of *B. terrestris* were collected in the field in Neunforn (Thurgau, northeastern Switzerland), brought back to the laboratory and allowed to found a colony as described in Gerloff and Schmid-Hempel (2005). When four colonies (one experimental block) had reached a size of approximately ten workers each, they were brought back to the field, and one of the four following treatments was randomly assigned to a given colony, so that each colony was assigned to a different treatment: (1) no treatment (Nt), (2) additional food supply (60 mL 50 % Apiinvert sugar water per week) (Fs), (3) a weekly immune challenge of all workers with 2 μL of a mixed bacterial solution of *Arthrobacter globiformis* and *Escherichia coli* in insect Ringer injected in the abdomen between the second and third tergites with a pulled glass micro-capillary needle (Ch), and (4) both additional food supply and immune challenge (FsCh). For practical reasons under the more difficult field conditions, the control for the immune challenge in treatments Nt and Fs was simply pricking with a micro-capillary, without injecting insect Ringer. Based on previous experiments, strong differences in the strength and persistence of immune system activation can be expected between the immune-challenged and simply pricked individuals (Korner and Schmid-Hempel 2004; Sadd and Schmid-Hempel 2007). Treatment began after the colonies had been in the field for 1 week. Colonies were grouped in experimental blocks to randomise any environmental variation among treatments. All blocks were put in the field within a week and were only a few hundred metres from one another, within foraging distance for bumblebees. In total, ten colonies were assigned to each treatment. Hence, the experimental design was fully factorial with factors "immune challenge" (yes/no) and "food addition" (yes/no).

Every week, colony size (the number of workers), as well as the presence and number of sexuals (drones and daughter queens), was recorded. Sexuals were prevented from leaving the field nest box after emergence by

restricting the nest entrance diameter to a small hole that still permitted workers to enter and leave the nest freely. Fitness was calculated as the number of males plus twice the number of females, a commonly used fitness measure for bumblebee colonies (Baer and Schmid-Hempel 1999, 2005). All sexuals were removed from the colony and frozen in liquid nitrogen. Moreover, provided the colony size was larger than five workers, 10 % of the workers were randomly collected (once per week) and frozen for microsatellite analyses of *C. bombi* and *N. bombi* infections. Finally, to control for any effect of the experimental treatments on the foraging behaviour of workers, we observed the number of individuals flying in and out of the nest during 30-min sessions (2–3 sessions per nest, with at least 1 week between each, dependent on the weather conditions). Nests that have been provided with additional food supplies might accordingly reduce their foraging activity, thus artificially lowering the probability of encountering parasites. Were this to be the case, this effect would confound any subsequent statistical analysis. Similarly, nests whose individuals have been bacterially challenged might increase their foraging activity to compensate for the additional energetic cost caused by the activation of their immune system, thus artificially increasing the probability of encountering parasites.

Bacterial culture for immune challenge

The immune challenge consisted of a mixture of the Gram-positive *A. globiformis* (strain DSM 20124) and the Gram-negative *E. coli* (DSM 498). This ensured a general immune response by activating both major insect immune defence mechanisms, the Toll-pathway primarily directed against Gram-positive bacteria (*A. globiformis*) and the Imd-pathway primarily directed against Gram-negative bacteria (*E. coli*) (Hoffmann 2003). Bacteria were cultured in liquid medium (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl in 1,000 mL distilled water, pH 7) at 30 and 37 °C for 24 h and overnight, respectively. One mL of culture was washed three times by centrifuging for 10 min at 3,000 rpm, followed by removal of the supernatant and replacement with insect Ringer (previously autoclaved and filtered though a 0.2-μm filter). Bacteria were finally re-suspended in 1 mL insect Ringer and kept on ice for cell counting (counting each single cell, also in cell aggregates). Both bacteria where then mixed in order to obtain a final concentration of 10^8 bacterial cells mL^{-1} (0.5×10^8 cells mL^{-1} each) and stored in 2 mL aliquots at −80 °C. Before use, bacteria were heat-killed by incubating thawed aliquots at 90 °C for 15 min. Plating out samples on LB agar plates and incubating them at 30 °C for 48 h showed no growth. The effectiveness of the immune challenge was tested with a zone-of-inhibition assay as described in Moret (2001).

Genotyping of *C. bombi* infection and detection of *N. bombi*

Worker guts were extracted and stored at −80 °C. As these samples were to be used in another project looking at bumblebee bacterial gut flora (Koch et al., submitted), DNA was extracted following a modified version of the QIAGEN protocol for purification of total DNA from animal tissues (DNAeasy® 96 protocol) including a pre-treatment for Gram-positive bacteria. A stock solution of lysis buffer was prepared (20 mM Tris–Cl pH 8, 2 mM sodium EDTA, 1.2 % Triton X-100), to which lysozyme was added before use (20 mg mL^{-1}). Each gut sample was macerated in 180 μL lysis buffer, transferred in the 96-well plates of the DNeasy® kit, quickly centrifuged at 3,000 rpm, and let to incubate 30 min at 37 °C, shaking. Then, 200 μL AL buffer (without ethanol) was added, followed by 25 μL proteinase K, the solution mixed by inverting the plate, and quickly centrifuged down. Samples were let to incubate at 56 °C for 30 min (or until lysate was clear), shaking. Finally, 220 μL ethanol (100 %) was added, the plate vigorously shacked up and down for at least 15 s, and the content centrifuged down. The rest of the extraction followed the normal QIAGEN DNAeasy® 96 protocol for extraction from animal tissues.

The infections were typed with microsatellites Cri4, Cri2F10, Cri4G9, Cri16 and Cri1B6 (see Schmid-Hempel and Funk 2004). Two multiplex PCRs were run: (1) with Cri4, Cri2F10 and Cri4G9 [5 μL eluted DNA, 1× reaction-buffer, 0.3 μL of each primer (10 μM), 0.75 μL of dNTPs of 2.5 mM each, 0.075 μL GoTaq© polymerase (5 U/μL) for a final volume of 15 μL; thermal profile: initial denaturation of 5 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s elongation at 48 °C and 30 s at 72 °C, and a final extension of 7 min at 72 °C]; and (2) with Cri16 and Cri1B6 (exact same conditions, except for 53 °C as elongation temperature). Additional PCRs were run for single primers whenever the multiplex reaction did not amplify correctly. Apart from the annealing temperature (Cri4 at 47 °C; Cri2F10: 48 °C; Cri4G9: 52 °C; Cri16: 59 °C; Cri1B6: 53 °C), the reaction conditions remained the same. All forward primers were labelled with fluorescent dyes, so that samples could be genotyped in a MegaBACE™ DNA capillary sequencer.

In addition, samples were also analysed for the presence of the microsporidian *N. bombi* using the specific primer pair 18f (CACCAGGTTGATTCTGCC) and 1537r (TTATGATCCTGCTAATGGTTC) (Baker et al. 1995).

Statistical analysis

The effects of the two experimental treatments on colony lifespan (measured as the time from placement in the field

until the death of the queen) were analysed with a two-way ANOVA. In order to normalise the residuals, time to the death of the queen was transformed as the square root of the variable plus 0.5. We analysed the reproductive fitness (number of males plus twice the number of queens produced) by bootstrapping (within each combination of the two experimental treatments) 10,000 times with replacement and calculating the 95 % confidence interval (CI). A group (A) was considered as significantly different from another one (B) at a 5 % threshold A's mean was not included in B's 95 % CI.

The last colony that was immune-challenged but not food-supplemented died after only 6 weeks in the field, and the last worker in this colony was sampled after 4 weeks of treatment (5 weeks since placement in the field). We therefore limited all the following analyses to these 4 weeks of the experiment. The probability of getting infected by *C. bombi* and *N. bombi*, and the effectiveness of the immune challenge were analysed in a GLMM (generalised linear mixed model) using *lmer* from the *lme4* package (Bates et al. 2008) in R2.14.0 (R Development Core Team 2011) with a binomial error structure (logit link), and with both experimental treatments, as well as time (in weeks) since the placement of the colony in the field, as fixed factors. Colony identity was treated as a random factor. To look at colony size and average allelic diversity of infections per bumblebee worker over the five microsatellite loci, we implemented linear mixed effect models with *lme* from the *nlme* package (Pinheiro et al. 2006), with both experimental treatments and time as fixed factors, and colony identity as random. For colony size, time had to be included in the random part of the model as a repeated within-colony factor. The average allelic diversity was transformed using a Box–Cox transformation ($\lambda = -0.7$). Lastly, the average foraging activity (corrected for colony size and log-transformed) of each colony was analysed with a GLM with normal error structure.

Results

Colony size and fitness

As expected, the addition of food had a significant effect on colony growth and colony lifespan (Tables 1 and 2; Fig. 1). Colonies supplemented with food were on average bigger and lived longer than colonies that were not. However, no significant effect on these variables was detected for either factor immune challenge or for the interaction between addition of food supply and immune challenge.

Food-supplemented colonies also had a higher fitness (Table 1; Fig. 2), measured as the sum of males plus twice the number of queens produced, compared to colonies that were not supplemented. Again, immune challenge did not have any effect on colony fitness.

Infection probability and allelic diversity

Neither additional food supply nor the immune challenge had an effect on the probability of becoming naturally infected by *C. bombi* or *N. bombi*. Colonies were more infected by *C. bombi* as time went by (Table 2), but no such trend was detected for *N. bombi*. Considering the

Table 1 Summary of the number of colonies at the beginning of the experiment (*n*), date of the death of the last colony (*Last colony*, in weeks since placement in the field), lifespan of the colony (date of death of the queen, in weeks since placement in the field), size (average per week), production of sexuals, fitness (2 × queens + males), *Crithidia* infection prevalence and allelic diversity (over 5 microsatellite loci), and *Nosema* infection prevalence

	Nt	Fs	Ch	Fs × Ch
n	10	10	10	10
Last colony	8	17	6	17
Lifespan	4.83 ± 0.95[a]	8.9 ± 1.02	4.67 ± 0.33	9.22 ± 1.24
Size	15.58 ± 4.33	20.91 ± 3.15	9.47 ± 2.36	19.22 ± 2.75
Males	2.67 ± 1.86	8.1 ± 3.06	0.5 ± 0.5	11.33 ± 4.56
Queens	0 ± 0	1.4 ± 0.86	0 ± 0	0.11 ± 0.11
Fitness	2.67 ± 1.86	10.9 ± 4.02	0.5 ± 0.5	11.56 ± 4.65
Crithidia infection prevalence	0.42 ± 0.13	0.35 ± 0.12	0.24 ± 0.11	0.45 ± 0.09
Crithidia infection diversity	2.25 ± 0.15	1.98 ± 0.12	2.36 ± 0.35	2.31 ± 0.14
Nosema infection prevalence	0.29 ± 0.14	0.25 ± 0.091	0.14 ± 0.09	0.28 ± 0.077

Nt no treatment, *Fs* additional food supply (60 mL 50 % Apiinvert sugar water per week), *Ch* a weekly immune challenge of all workers with 2 µL of a mixed bacterial solution of *Arthrobacter globiformis* and *Escherichia coli* in insect Ringer injected in the abdomen between the second and third tergites with a pulled glass micro-capillary needle, *FsCh* both additional food supply and immune challenge

[a] Mean ± SE

Table 2 Final statistical models (all main effects, and interactions with *P* values <0.1) for the mean colony size, colony lifespan (measured as the time in weeks of the death of the queen after colony placement in the field), infection prevalence of the colony by *C. bombi* and *N. bombi*, and *C. bombi* infection diversity (average allelic diversity per bee over 5 microsatellite markers)

Colony lifespan[a]	*df*	SS	MS	*F*	*P*
Fs	1	4.49	4.49	15.49	**<0.001**
Ch	1	0.0085	0.0085	0.029	0.87
Fs × Ch	1	0.0011	0.0011	0.0037	0.95
Residuals	27	7.82	0.29		

Colony size[b]	*df*	Estimate	SE	*t*	*P*
Intercept	90	4.9	0.47	10.32	**<0.001**
Fs	28	−1.058	0.51	−2.054	**0.049**
Ch	28	0.24	0.5	0.48	0.64
Time	90	−0.55	0.15	−3.61	**<0.001**
Fs × time	90	0.86	0.17	5.17	**<0.001**
Ch × time	90	−0.27	0.16	−1.69	0.094

Crithidia infection prevalence[c]		Estimate	SE	*z*	*P*
Intercept		−3.33	1.45	−2.29	**0.022**
Fs		1.89	1.77	1.073	0.28
Ch		0.76	2.19	0.35	0.73
Time		1.18	0.48	2.45	**0.014**
Fs × Ch		−4.51	2.88	−1.57	0.12
Fs × time		−0.97	0.58	−1.68	0.093
Ch × time		−0.61	0.86	−0.71	0.48
Fs × Ch × time		2.21	1.045	2.12	**0.034**

Crithidia infection diversity[b]	*df*	Estimate	SE	*t*	*P*
Intercept	28	−0.69	0.1	−6.61	**<0.001**
Fs	21	−0.35	0.13	−2.8	**0.0108**
Ch	28	0.031	0.052	0.6	0.5542
Time	28	−0.061	0.034	−1.83	0.078
Fs × time	28	0.1	0.041	2.48	**0.019**

Nosema infection prevalence[c]		Estimate	SE	*z*	*P*
Intercept		−2.0057	0.68	−2.96	**0.0031**
Fs		0.16	0.58	0.27	0.79
Ch		−0.33	0.54	−0.62	0.54
Time		0.37	0.19	1.96	0.05

Significant effects in bold

Fs additional food supply (60 mL 50 % Apiinvert sugar water per week), *Ch* a weekly immune challenge of all workers with 2 µL of a mixed bacterial solution of *Arthrobacter globiformis* and *Escherichia coli* in insect Ringer injected in the abdomen between the second and third tergites with a pulled glass micro-capillary needle

[a] ANOVA

[b] Linear mixed effect model with *lme* from the *nlme* package

[c] GLMM with binomial error structure (logit link) using *lmer* from the *lme4* package in R2.14.0

diversity of alleles in the infecting population of *C. bombi* (as measured over all 5 microsatellite loci), food-supplied colonies harboured significantly fewer diverse infections, but neither immune challenge nor time in the season had any significant effect on the average allelic diversity per individual bumblebee worker (Table 2).

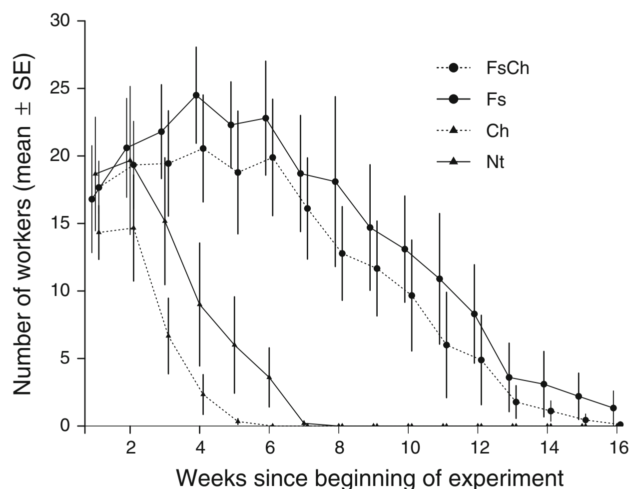

Fig. 1 Growth cycle of *Bombus terrestris* colonies (mean number of workers ± SE). The four treatment groups are no treatment (*Nt*), food supplemented only (*Fs*), immune challenged (*Ch*), food supplemented and immune challenged (*FsCh*)

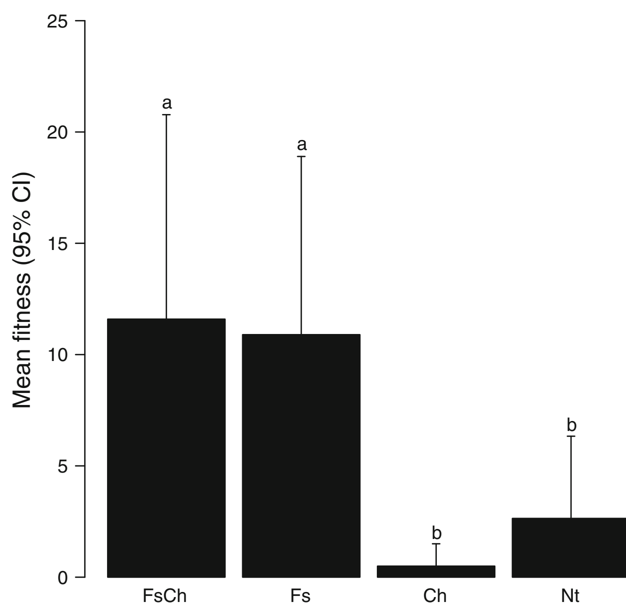

Fig. 2 Fitness of the four experimental treatments, measured as twice the number of queens produced, plus the number of males (mean ± 95 % CI). The plot results from bootstrapping the data (see "Materials and methods"). *Small letters* indicate statistically different groups. No treatment (*Nt*), food supplemented only (*Fs*), immune challenged (*Ch*), food supplemented and immune challenged (*FsCh*). Sample sizes are 10 colonies for FsCh, Fs and Nt, and 9 for Ch

Immune challenge significantly increased the probability for workers to show antimicrobial activity (generalised linear mixed model, $z = 2.94$, $P = 0.0033$). There was also a significant effect of time ($z = 2.51$, $P = 0.012$) and the interaction between time and additional food supply (generalised linear mixed model, $z = -1.98$, $P = 0.048$). Food supplementation and all other interaction terms were not significant. Furthermore, additional food supply (generalised linear model, $t = -1.3$, $P = 0.2$) or immune challenge ($t = 1.52$, $P = 0.14$) had no influence on the colony's foraging activity.

Discussion

Our field experiment was designed to confirm in the field the earlier laboratory findings on the cost of an immune challenge in the bumblebee *B. terrestris*, using, in a full-factorial design, additional food supply (sugar water) and immune challenge by bacteria as experimental treatments. We found that all analysed fitness traits, i.e. colony size, colony life span and the production of sexuals, were positively correlated with the quantity of food available but were not influenced by the immune status of the colony. Moreover, the interaction between the nutritional and immune status was not significant, which would have been indicative of a condition-dependent effect. This contrasts to some degree with Brown et al. (2000) and Moret and Schmid-Hempel (2004) who respectively showed—even though under harsher conditions than the ones in the present experiment—an increase in mortality of starved *Crithidia*-infected individual bees, and a reduction of the colony fitness under chronic thermal stress when the workers' immune system was stimulated with bacterial surface molecules (LPS).

Over the duration of the experiment, colonies became more infected by *Crithidia*, but not significantly so by the microsporidian parasite *N. bombi*. Food supplemented colonies had overall genetically less diverse *Crithidia* infections, genetic diversity that, however, increased over time in food-supplemented colonies, while decreasing in the ones that were not (Fs × Time term in Table 2). Finally, additional food had no influence on infection prevalence by *Crithidia* or *N. bombi*, and immune challenge affected neither the infection prevalence or genetic diversity (Mallon et al. 2003), nor infection by *N. bombi*.

It would nevertheless be premature to claim that the field evidence contradicts the laboratory-based evidence, as several plausible factors may explain this outcome. Firstly, the type of immune challenge used in this experiment may not have triggered an immune response in the bumblebees. This is, however, not very likely as zone-of-inhibition assays of collected samples showed that bumblebees from immune-challenged colonies had a greater probability of showing antimicrobial activity. Moreover, this method has already proved to be effective in a laboratory study by Sadd and Schmid-Hempel (2007). Secondly, the effect of the wounding itself (applied to both treatment groups) may have been so important that it masked the effect of the

injection of heat-killed bacteria. Whereas pricking may be relatively benign (compared to the injection of other immune elicitors such as LPS or bacteria) in a laboratory (Lemaitre et al. 1997), its effect may be amplified when rearing conditions are not well under control, as is the case in a field experiment or in an agricultural landscape, where subsequent infections of the wound may be more common. Thirdly, the various pressures of not just one but diverse pathogens in the wild on the immune system of the bumblebees may simply have cancelled the effect of the immune challenge. Indeed, bumblebees are host to many different parasites such as viruses, fungi and bacteria (Schmid-Hempel 1998, 2001; Goulson 2010). Finally, food supplementation may have been the overriding effect and, hence, no statistical signal of immune challenge was seen.

No less puzzling is the lack of influence of the nutritional status of the colony on the probability of being infected by *C. bombi*. However, while not showing signs of increased resistance to infection, colonies that were provided with additional food may have gained increased tolerance (Råberg et al. 2007; de Roode and Altizer 2010). Hence, although not being able to prevent the infection itself, these colonies may have suffered less from it than food-limited colonies (the controls). Our finding that an additional supply of food resulted in colonies being bigger, living longer and having a higher reproductive output is, of course, not very surprising, but also in line with this hypothesis. Additionally, Ulrich et al. (2010) found a positive correlation between infection genetic diversity and infection intensity. Hence, the fact that colonies we supplemented with food had genetically less diverse infection (i.e. had a lower infection intensity) could also be indicative of their higher tolerance. One might argue that the strong effect of additional food supply on colony success may be more likely due to a scarcity of floral resources in the agricultural landscape where this study was conducted, as suggested by a corresponding field experiment of Pelletier and McNeil (2003) where colonies supplemented with food (also) had a higher reproductive success. Intensified agricultural practices and the subsequent reduction of bumblebee forage plants have been pointed out at as the main cause of bumblebee decline in Europe (Williams and Osborne 2009; Goulson 2010). Supplying additional sugar water may thus have alleviated this limiting factor for our experimental colonies. Finally, the lack of statistically significant differences can also be due to sample sizes. However, similar sample sizes and designs were able to detect differences in similar experiments in the past, both in the field (Baer and Schmid-Hempel 2003, 2006; Otti and Schmid-Hempel 2008) and in the laboratory (Schmid-Hempel and Schmid-Hempel 1998; Brown et al. 2000; Sadd et al. 2005; Sadd and Schmid-Hempel 2009). In the present study, the effect (if any) might have been too small to be detected. In particular, the sample sizes for the molecular genetic analyses were particularly low (0–2 per colony and week), especially for the non-food-supplemented group. While colonies from the latter group gradually decreased in size (and, hence, the sample size per colony and for the treatment group in general), those from the food-supplemented group increased. Although we did not find a general trend towards increasing genetic diversity of *Crithidia* infection with the number of analysed workers (Spearman rank correlation: $S = 9{,}311.082$, $\rho = 0.3$, $P = 0.053$), such a trend was apparent when only food-supplemented colonies were considered ($S = 2{,}356.48$, $\rho = 0.42$, $P = 0.023$), which could explain the significant interaction term Fs × Time in the analysis of *Crithidia* infection diversity (Table 2).

This experiment therefore emphasises the need for field studies when it comes to potential practical applications, e.g. in the context of pollinator decline (Murray et al. 2009; Williams and Osborne 2009). The results may also suggest that the outcome in a field situation can be complex but not necessarily be based on different underlying processes. Rather, important abiotic and biotic factors and their natural variation usually encountered in the wild may profoundly change the observed outcome as well as the relationships between the different aspects under investigation.

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