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Protein-poor diet reduces host-specific immune gene expression in *Bombus terrestris*

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Parasites infect hosts non-randomly as genotypes of hosts vary in susceptibility to the same genotypes of parasites, but this specificity may be modulated by environmental factors such as nutrition. Nutrition plays an important role for any physiological investment. As immune responses are costly, resource limitation should negatively affect immunity through trade-offs with other physiological requirements. Consequently, nutritional limitation should diminish immune capacity in general, but does it also dampen differences among hosts? We investigated the effect of short-term pollen deprivation on the immune responses of our model host *Bombus terrestris* when infected with the highly prevalent natural parasite *Crithidia bombi*. Bumblebees deprived of pollen, their protein source, show reduced immune responses to infection. They failed to upregulate a number of genes, including antimicrobial peptides, in response to infection. In particular, they also showed less specific immune expression patterns across individuals and colonies. These findings provide evidence for how immune responses on the individual-level vary with important elements of the environment and illustrate how nutrition can functionally alter not only general resistance, but also alter the pattern of specific host–parasite interactions.

1. Introduction

Variation exists within populations of hosts and parasites that determine the outcome of infection. Hosts can vary in their susceptibility and parasites can vary in infectivity or virulence. Such differences can often be traced to different host genotypes varying in their susceptibility to different genotypes of a parasite. This genotype-by-genotype (G × G) interaction is important not only for the ecological dynamics of host–parasite systems, but, for example, also for the maintenance of genetic diversity within populations. However, these host–parasite interactions are not only determined by the genotypes, but are also subjected to environmental conditions, such as resource availability, that can dramatically alter infection outcome (G × G × environment interactions) [1].

In fact, it is well known that nutrition and immunology are intricately linked [2]. Not only can poor nutritional conditions lead to poor overall health, but various elements of host nutrition can also alter specific components of immunity [3]. At the same time, specific immune responses to parasites are thought to be evolutionarily determined by a variety of costs and trade-offs [4–8], most of these depending on resource availability and nutrition [2,9–11]. Specific nutritional requirements of parasites, in particular, may generate variability in host–parasite interactions in relation to food availability and food quality. These links between parasitism, host nutrition and immune defences have been investigated in various vertebrate hosts [12–14], as well as in some invertebrate hosts [3,15–18].

These studies collectively show that the details of these relationships are complex. For instance, high protein diets allow caterpillars to mount strong lysozyme responses, but at the cost of reduced phenoloxidase (PO) activity [3]. This distinct immune shift could have broad consequences if lysozyme responses are a poor

match to current parasitological conditions or if parasite pressure suddenly changes. In other cases, trade-offs in the functional allocation of specific proteins seem to occur. Apolipophorin III, for instance, is not only a lipid transport protein, but also plays a role in the recognition of parasites [19]. In crickets, energetically expensive activities such as flight reduces the amount of available apolipophorins in the haemolymph [20], and experimentally reducing apolipophorin reduces immune function. Similarly, if crickets were given a high fat diet they had higher lipid concentrations in their haemolymph but were more susceptible to parasites, then a result of intensive lipid transport that binds apolipophorin and makes it unavailable for defence [21]. How investments into one of several arms of the immune response affects the defence repertoire is therefore not always clear. However, the example of apolipophorin illustrates a possible mechanism to explain why reduced caloric intake with infection, known as anorexia, occurs so commonly across taxa. Many immunologically important genes also play a role in metabolism and vice versa (e.g. apolipophorin III [3] and vitellogenin [22,23]).

The buff-tailed bumblebee *Bombus terrestris* L. and its gut parasite *Crithidia bombi* [24] are an established model system for host–parasite interactions and coevolution [25]. *B. terrestris* is a common pollinator in central Europe and lives in colonies headed by single queens. The monogamous haplodiploid mating system provides a very uniform genetic background within colonies, with sister workers being closely related ($r = 3/4$); colonies, therefore, serve as our different genotypic backgrounds in the tests. *C. bombi* is a trypanosome parasite that lives in the bumblebee gut, and is spread among bees via oral uptake of infective cells spread through faeces [26]. Infection with *C. bombi* is fairly benign in workers, affecting fitness directly only under stressful conditions such as starvation [27] but strongly reduces queen success after hibernation [28]. The success of an infection is dependent on both host genotypic background and parasite genotype. In this system, such genotype-by-genotype (G × G) interactions have been shown for many elements, such as infection success and intensity [25], or expression of antimicrobial peptide (AMP) genes [29,30].

The nature of G × G interactions is not static in this system. Host genotypes vary in their immune response to this parasite [29–31], and different parasite genotypes induce different responses in the host [29,30,32]. Furthermore, resource limitation dramatically shifts the pattern of specificity [33]. This plasticity suggests that changes in gene expression may play a role in dictating host–parasite specificity. Here, we explore how nutrition alters both gene expression magnitude and variance upon exposure to this highly prevalent parasite. Adult bees consume nectar for energy and pollen for protein. At the same time, when deprived of pollen, worker bees support fewer *C. bombi* in their guts, and the temporal pattern of parasitaemia is disrupted (Huck & Schmid-Hempel 1997, unpublished data; [34]). Some aspects of disease resistance in this system also appear to be energy-limited. If *B. terrestris* workers are given an immune challenge, then they die sooner than control bees under energy-limited conditions [35], whereas giving workers an immune challenge results in a small, but significant increase in energy consumption [36]. Similarly, workers prevented from energetically expensive foraging have stronger immune responses than those allowed to forage [37,38]. Differences in food quality also affect host survival and, moreover, the phenotypic outcome of G × G interactions [33], although that study did not analyse gene expression.

Generally, infection with *C. bombi* increases expression of genes encoding AMPs and immune pathway signalling genes shortly after infection [31,32,39], and the putative recognition molecule haemomucin after infection establishment [40]. Wounding and bacterial infection of *B. terrestris* workers also increases AMP expression and decreases expression of signalling and other effector genes [41]. Furthermore, immune gene regulation is affected by the social context in which a bee is kept [42] (see [31] for a more thorough review of *B. terrestris* immune expression studies). To assess how nutrition alters immunological gene expression, we tested how short-term protein limitation influences both standing and infection-induced gene expression of *B. terrestris*. We targeted the expression of 26 known important immune and metabolic genes across various pathways against a battery of the four most stable of the six included housekeeping genes. Taking a targeted approach allows greater replication and reduced type I error relative to whole transcriptome sequencing. By assessing PO activity in the haemolymph of our samples at the same time, we also performed a first attempt at linking immune gene expression and immune phenotype in this system.

2. Results

(a) First-level effects on gene expression

Reducing short-term protein access exerted a predictably strong influence on the overall gene expression patterns in *B. terrestris* (table 1). On an individual gene level, the expression of the signalling molecules hopscotch ($F_{1,129} = 9.926$, $p = 0.002$), the AMP defensin ($F_{1,129} = 10.00$, $p = 0.002$), the iron transportation molecule transferrin ($F_{1,129} = 24.57$, $p < 0.001$), the enzyme punch ($F_{1,129} = 10.10$, $p = 0.002$) and the lipid transporter apolipophorin III ($F_{1,129} = 7.268$, $p = 0.008$) differed according to treatment (figure 1*b* and the electronic supplementary material, table S4). Aside from hopscotch, which was upregulated, these were significantly downregulated under protein limitation. The full set of results can be found in the electronic supplementary material. Results from the individual colonies are shown in the electronic supplementary material, figure S1.

Infection significantly affected the overall, multivariate gene expression patterns (table 1) and almost exclusively caused upregulation of genes (figure 1*a*). *B. terrestris* workers responded to *Crithidia* exposure with significantly higher expression levels in the receptor molecule BGRP2 ($F_{1,129} = 15.41$, $p < 0.001$), the signalling molecules pelle ($F_{1,129} = 12.57$, $p < 0.001$) and hopscotch ($F_{1,129} = 7.674$, $p = 0.006$), the antimicrobial effectors hymenoptaecin ($F_{1,129} = 10.89$, $p = 0.001$) and TEPA ($F_{1,129} = 8.499$, $p = 0.004$) and the peroxidase jafrac ($F_{1,129} = 6.107$, $p = 0.015$; figure 1*a* and the electronic supplementary material, table S4).

Parental collection site and colony affected gene expression patterns both here (table 1 the colony within site effect and electronic supplementary material, figures S1 and S2) and in previous studies [31]. Remarkably, effects of infection and starvation also varied widely among colonies (electronic supplementary material, figure S1).

(b) Interaction of factors

We found a strong interaction between protein limitation and infection (table 1 and figure 2). The antimicrobial effectors abaecin, hymenoptaecin and TEPA, and the signalling

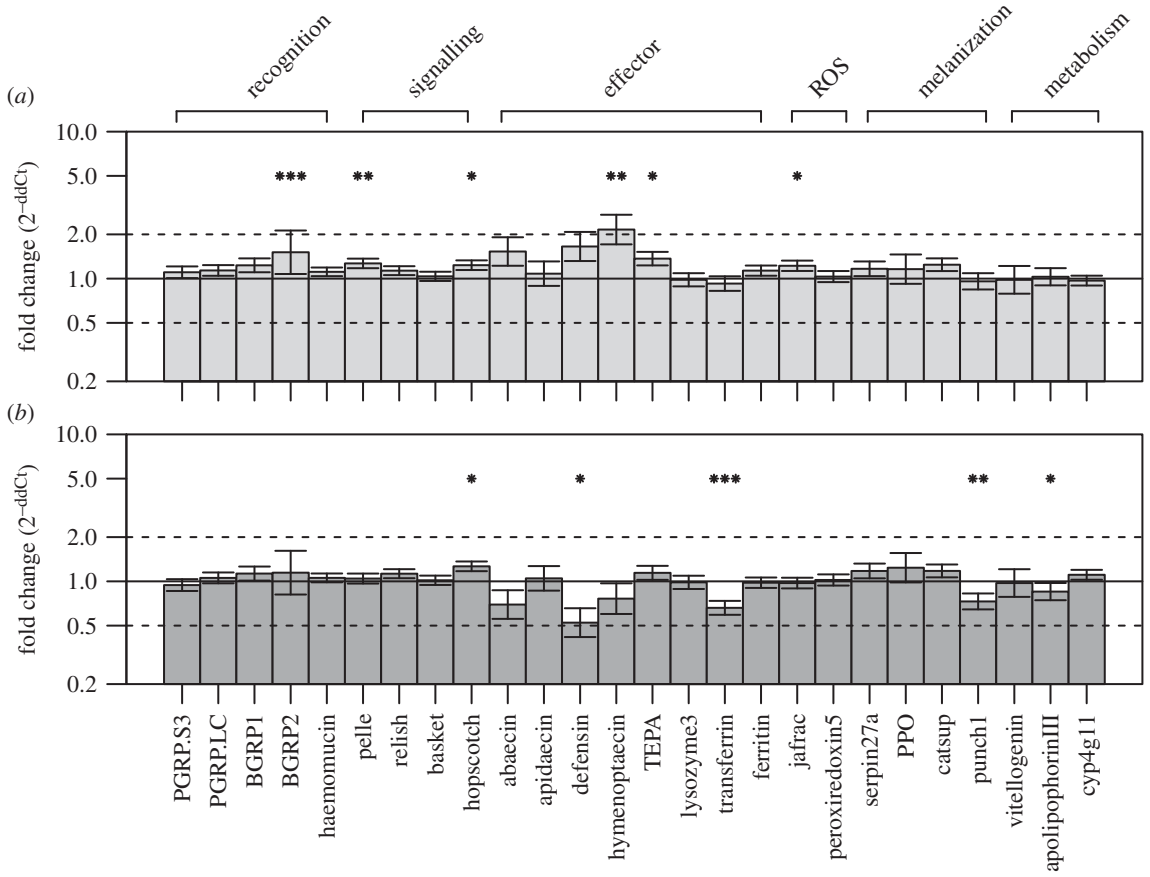

Figure 1. Gene expression changes upon infection (*a*) and protein limitation (*b*) across all colonies. The solid line marks no change between treatment groups (fold-change = 1.0). Error bars are standard error calculated by averaging dCt values within sample groups, transformed to fold-change errors with error propagation. Dashed lines mark the values 2 and 0.5, corresponding to doubled and halved gene expression, respectively. Asterisks mark Bonferroni-corrected significance of effects as detectable in univariate outputs of the MANOVA on the full dataset. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Table 1. MANOVA results: MANOVA was carried out on full dataset of dCt values after Yeo–Johnson transformation for each gene. Transformation values can be found in electronic supplementary material, table S3. The full MANOVA results including univariate effects and the used R code can be found in electronic supplementary material, table S4. As colonies are nested within sites, the site: colony interaction describes the colony effect. Effects significant at $p \leq 0.05$ are highlighted in italics.

multivariate effects						
factor	**d.f.**	**Pillai's trace**	***F*-value**	**num d.f.**	**den d.f.**	***p*-value**
infection status	1	0.400	2.667	26	104	*<0.001*
starvation status	1	0.662	7.820	26	104	*<0.001*
collection site	1	0.905	37.96	26	104	*<0.001*
infection × starvation	1	0.501	4.018	26	104	*<0.001*
infection × site	1	0.269	1.472	26	104	0.088
starvation × site	1	0.319	1.875	26	104	*0.014*
site : colony	4	2.675	8.307	104	428	*<0.001*
infection × starvation × site	1	0.226	1.166	26	104	0.287
residuals	129					

molecule relish were all upregulated upon infection in bees supplied with pollen, but the same genes were not differentially expressed in infected bees when they were deprived of pollen. By contrast, the receptor molecules, haemomucin and vitellogenin, a key protein in many processes including metabolism, were both downregulated under starvation in non-infected bees, but expression levels remained constant under starvation when bees were infected with *C. bombi* (figure 2 shows all significant starvation × infection treatment interactions). There was also an interaction effect between original collection site and starvation treatment in the multivariate analysis but not in the expression of any individual gene (electronic supplementary material, table S4).

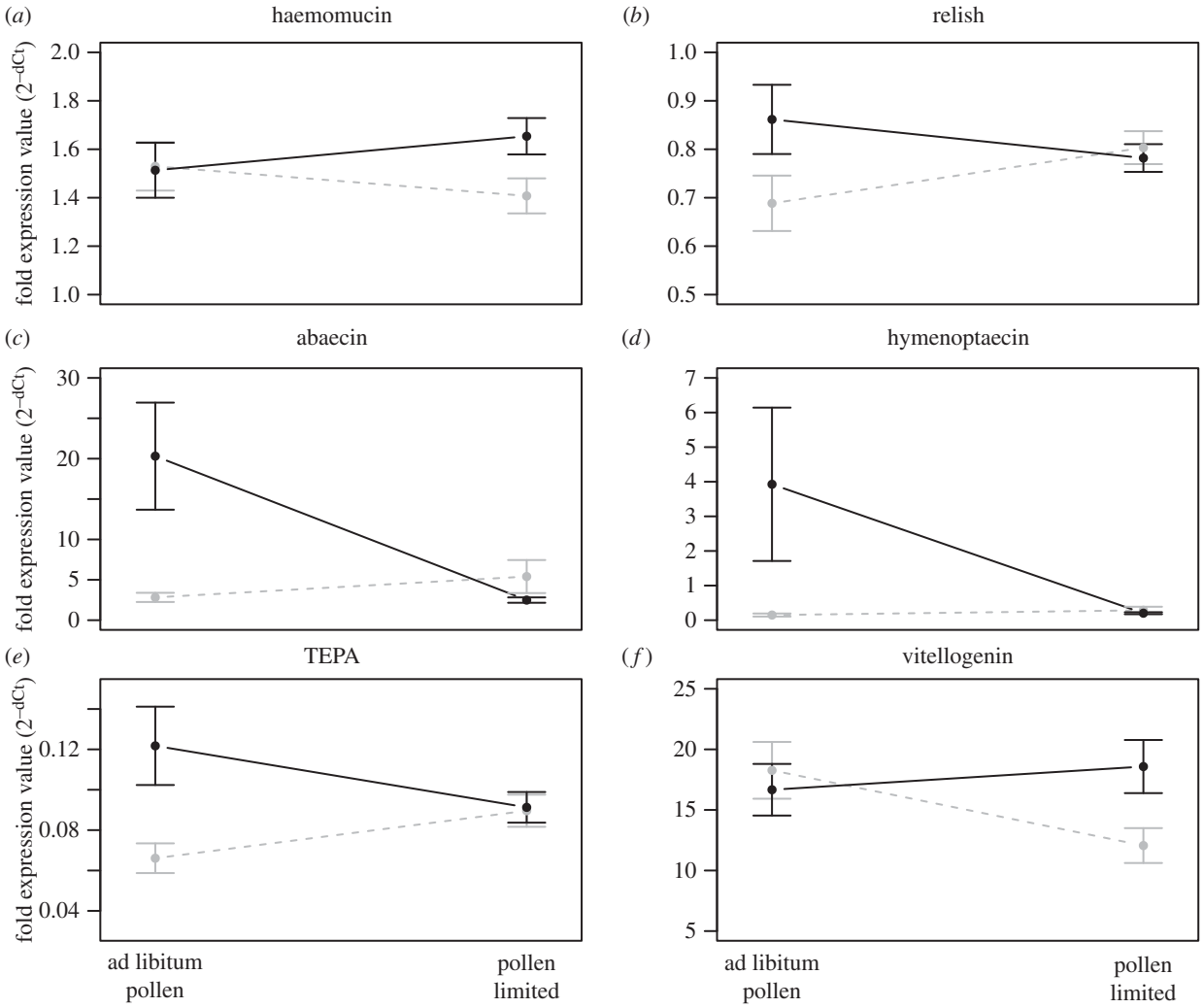

Figure 2. Interaction plots showing modulation of the infection effect by protein limitation. The significant effects are restricted to the six genes shown here. Circles mark the mean fold expression values (2^{-dCt}) and error bars show standard error. Infected samples are depicted in filled circled and solid lines and control samples in open circled and dashed lines.

Variation in expression was strongly influenced by starvation (figure 3). Starvation treatment significantly decreased variation in gene expression (permutation test for homogeneity of multivariate dispersion of all genes within starved versus non-starved samples: $F_{1,51} = 9.19$, $p = 0.002$, figure 3*a*,*b*), whereas infection slightly but non-significantly increased variation in gene expression ($F_{1,51} = 1.47$, $p = 0.24$, figure 3*c*,*d*).

(c) Phenoloxidase activity

PO activity, a key measure of immunity in invertebrates, was significantly negatively correlated with the expression of the AMPs abaecin, apidaecin and the anti-bacterial transferrin (figure 4). In our ANOVA analysis, ancestor collection site and colony were the only factors with a significant effect on PO activity. Yet, the strongest effect on PO activity resulted from the interaction of site and starvation treatment (starvation × site: $F_{128} = 15.30$, $p < 0.001$, table 2 and the electronic supplementary material, figure S3).

3. Discussion

Defence levels and the associated immune responses are likely to be moderated by a variety of factors, including sex [43], nutrition [2,3,44] and the presence of a microbiota [45,46]. We found that poor nutrition condition—here, pollen deprivation—influences the expressed immune response in workers of *B. terrestris* to *C. bombi* at several levels. Pollen deprivation strongly, and mostly negatively, influenced the expression of immune and metabolic genes. Perhaps not surprisingly, pollen-deprived bees fail to induce strong immune expression relative to bees with pollen (figure 1). This suggests that not only the macroscopically observable outcome of an infection [33], but also the assumed underlying expression of the immune-related genes are resource-limited. At the same time, we also find that variation in expression levels is reduced under starvation conditions but slightly increased during infection. Hence, limited access to resources such as pollen not only distorts the genotypic interaction effects at the observed level of infection outcome [33], but also shows corresponding expression signatures at the level of the immune-related genes. In particular, pollen limitation reduces the variability of responses across hosts and this may be responsible for reducing the strength of the host genotype effect in G × G interactions such as in [30,33].

The homogenization of expression upon pollen limitation raises an interesting, if speculative, idea. At the population level, genetic diversity has a protective effect against infection [47] and disease spread [48] as any given parasite will only be able to infect a subset of the population. If stressful conditions,

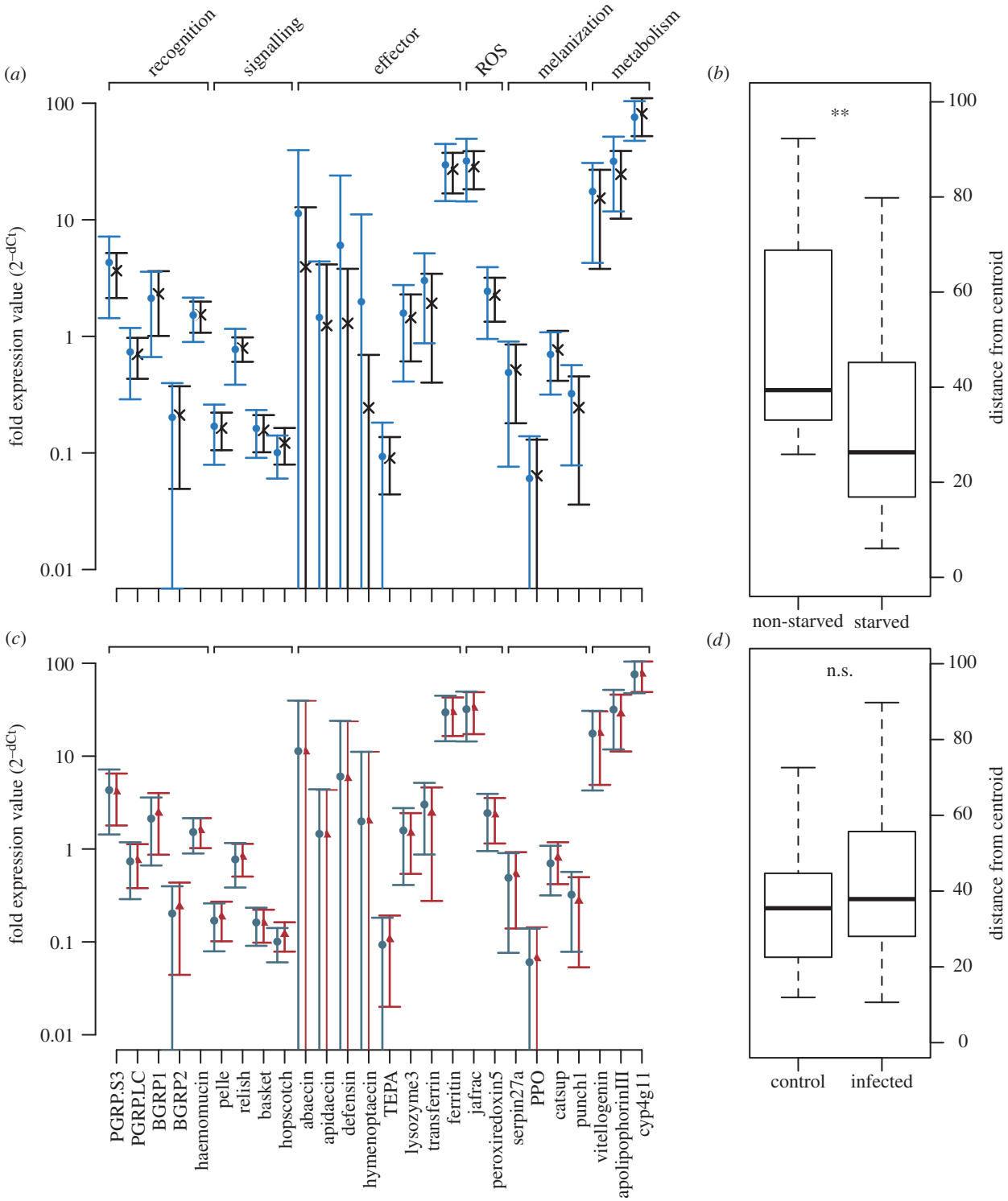

Figure 3. Mean fold expression value ± standard error for each gene in starved (black 'x's) versus fed (blue circles) bees (*a*) and in infected (red triangles) versus naive control (grey circles) bees (*c*). (*b*,*d*) Boxplots of the eigen distances from samples to the median centroid of that group's PCA cloud based on the two most explanatory axes. The variation in expression of immune genes is significantly lower in starved bees than in fed bees (permutation test for homogeneity of multivariate dispersions on the group variances, $F_{1,51} = 9.19$, $p = 0.002$). The variation in expression of immune genes is lower in the control bees than in infected bees (permutation test $F_{1,51} = 1.47$, $p = 0.24$).

such as resource limitation, diminish the population-wide variance of the immune expression phenotype, this may render a population phenotypically depauperate and thus, functionally, more ('genetically') uniform. We would expect the population to become more prone to parasite invasion and spread. It is indeed known that diminished host condition under stress can support epidemic outbreaks, particularly by opportunistic pathogens [49].

Parasite exposure increased expression of immune effector and mediator genes (hymenoptaecin, TEP A and jafrac) and of some receptor and signalling molecules (BGRP2, hopscotch and pelle). These results suggest that the immune response of *B. terrestris* to *C. bombi* involves the toll and JAK/STAT immune pathways. Furthermore, genes that differed in expression upon infection according to feeding regime can be clustered according to function. Infected bees increased their

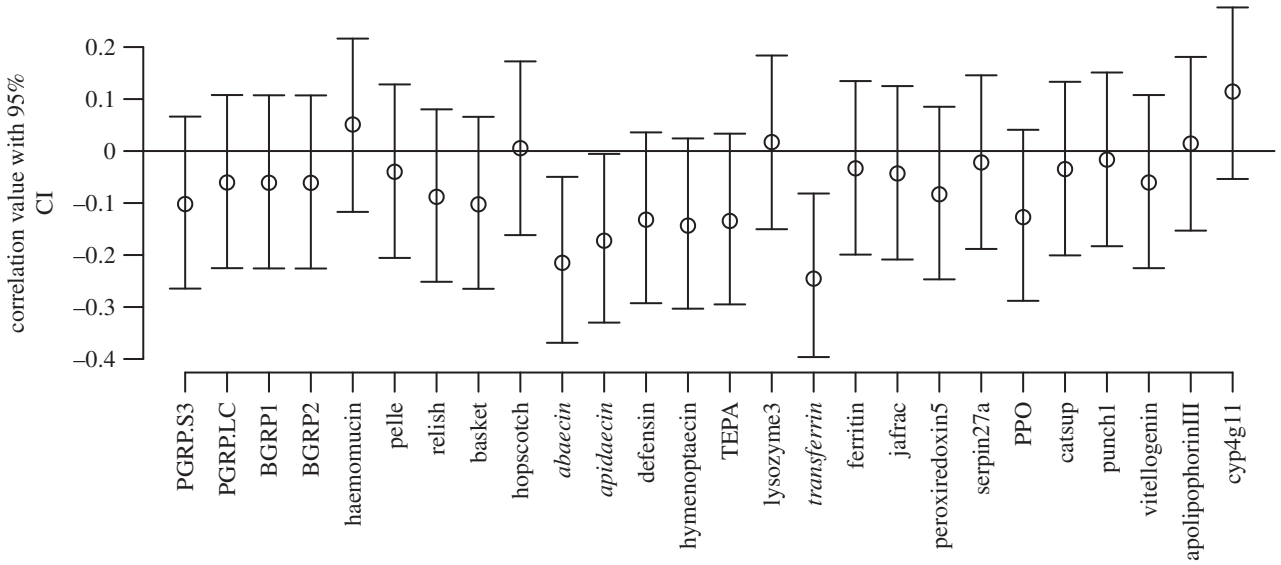

Figure 4. Correlations between phenoloxidase (PO) activity in haemolymph samples and the expression of various genes in whole abdomens measured at the same time point. Circles mark correlation values and bars show their respective 95% CIs. Expression patterns of abaecin, apidaecin and transferrin are significantly correlated with PO activity at the same time point (highlighted in italics).

Table 2. ANOVA results from PO activity. The ANOVA was carried out after Yeo–Johnson transformation of the dataset with $\lambda = 0.477$. Site effects dominate the PO activity but are also modulated by starvation. The interaction effect of starvation and ancestor collection site are visualized in electronic supplementary material, figure S3. Effects significant at $p \leq 0.05$ are highlighted in italics.

factor	d.f.	*F*-value	*p*-value
starvation	1	1.166	0.282
infection	1	1.089	0.299
site	1	7.168	*0.008*
starvation × infection	1	2.470	0.119
starvation × site	1	15.30	*<0.001*
infection × site	1	0.085	0.772
site : colony	2	4.793	*0.010*
starvation × infection × site	1	0.078	0.780
residuals	128		

expression of genes encoding AMPs and the effector molecule TEP A but only when (protein-rich) pollen was available, tentatively indicating that the production of these effectors is resource-limited. The signalling molecule relish is expressed upon both infection and starvation, indicating an involvement of the Imd pathway in both responses. And finally, haemomucin and vitellogenin, both involved in a variety of metabolic and immune system-related functions, are both downregulated upon starvation but maintained when infected. Previous studies also suggest an important role of nutrition (energy) for the maintenance of an immune response in *B. terrestris* [35].

We could confirm that bees that originated from different populations differed in their gene expression (electronic supplementary material, figures S1 and S2). These populations differ in parasite pressure, infection prevalence is significantly higher in queens caught in Aesch than in Neunforn [31]. Note that we found population differences despite using granddaughters of field-caught queens. In an earlier study, daughters of the field-collected queens also differed in their immune gene expression [31], which could be due to maternal transfer of immunological memory [50]. However, the persistence of this effect into the second laboratory generation—as shown here—is more suggestive of a site-specific adaptation of the bees' gene expression patterns. This possible sign of local adaptation is especially noteworthy as the European *B. terrestris* population is panmictic [51,52]. The direction of the site effect differed among genes (electronic supplementary material, figure S3), whereas in the previous study [31], bees from Neunforn showed higher immune gene expression levels than bees from Aesch for all significantly differently expressed genes.

We also assessed the relationship between immunological expression and a phenotypic measure of one major form of insect immunity, the PO response. However, we did not detect a correlation between prophenoloxidase (PPO) expression and PO enzyme activity. This may be due to an inherent delay between gene transcription of PPO and the eventual activation of PO. However, the expression of the AMPs abaecin and apidaecin and the iron-binding protein transferrin was negatively correlated with PO expression suggesting a trade-off between different parts of the insect immune system. Similarly, previous phenotypic assays of immune responses found that bees mounting a strong PO response had reduced anti-bacterial activity [53]. The strength of PO response has been linked to genetic diversity of the population and reduced infection with *C. bombi* in island populations of the bumblebee *Bombus muscorum* [47]. We did not test the genetic diversity of our populations and any such differences may explain some of the differences we see between our two populations. However, given that all mainland European *B. terrestris* appear to be part of a single panmictic population [51,52], and the lack of any geographical barriers to gene flow between these populations, suggests that these two populations should not differ greatly in diversity.

In summary, we find that the response to infection depends strongly on the availability of pollen and we also identify immunological traits that seem to be most resource-limited, such as the expression of AMPs. Moreover, we find that differences in gene expression among host genotypic backgrounds

are reduced under limited pollen supply. These results illustrate the importance of the ecological context in determining host responses to parasitism and a population-wide pattern of interaction with parasites.

4. Material and methods

(a) Bee breeding and treatment

We collected *B. terrestris* queens in Northern Switzerland (Aesch BL; (47°27′52″ N, 7°34′38″ E) and Neunforn TG (47°35′40″ N, 8°47′32″ E), these locations are approximately 90 km distant, as the crow flies) in spring 2011 and then mated their drone and gyne (daughter queen) offspring. Gynes and males for each mating pair were taken from different colonies but from within the same original collection sites. Each pairing and the resulting colony had different parents to any other colony in the experiment. We hibernated inseminated queens at 4°C for 45–82 days before they were allowed to establish colonies in the laboratory.

Six colonies were used for this experiment, three with ancestors from Aesch (colonies 2, 4 and 6) and three with ancestors from Neunforn (colonies 1, 5 and 9). Queens of these two collection sites differ in infection prevalence with our model parasite *C. bombi* [31].

Colonies were kept at 28 ± 2°C under standard constant red light conditions. Pollen and 50% sugar water (Apiinvert) were provided to the colonies ad libitum. Four groups of six workers were taken from every colony. Workers were between 1 and 6 days old when they were placed into the feeding treatments. Until 24 h prior to their placement, all bees were provided with sugar water and pollen ad libitum. In the treatment phase, half of the worker groups from each colony were randomly selected and given access to pollen, whereas the other half had no access to pollen. Within each of these feeding regimes, half of the groups were exposed to the parasite *C. bombi* and half were given a sham inoculum. For infection, bees were fed 10 000 *C. bombi* cells, with the same dose for each of the four clonal strains (2500 cells), delivered in 10 µl of 50% sugar water. Bees were denied both sugar water and pollen for 2 h before being presented with the inoculum or a sham inoculum of clean sugar water. All bees imbibed this meal. We used clonal *C. bombi* strains that were isolated from spring queens in 2008 (two strains from Neunforn, one from Aesch) and 2009 (one strain from Aesch). Each *C. bombi* strain originated from a single infective cell and was cultured at 27°C and 3% CO_2 in liquid medium [54].

Eighteen hours after the infection or control treatment, bees were anaesthetized on ice, and 5 µl of haemolymph was extracted from each bee and stored in sodium cacodylate/$CaCl_2$ buffer for PO activity measurements [55]. Afterwards, the abdomens of bees were snap-frozen in liquid nitrogen. We used the 18 h post-infection time point based on the patterns of gene expression reported in earlier studies [31,32,39] and phenotypic immune assays [56]. Two bees died before sampling, one each from the infected but non-starved groups from colonies nos. 4 and 5, leaving a total of 142 samples.

(b) Genetic analyses

We homogenized the bee abdomens with 0.5 g zirconium beads once at −2 to −4°C using an Omni bead Ruptor 24 homogenizer (Omni International) and then again in lysis buffer (RLT plus from the RNeasy plus mini kit) at room temperature. Next, we extracted RNA from these samples, using the RNeasy plus mini kit (Qiagen) in 12 randomized extraction groups of 11–12 samples each, according to the manufacturer's recommended protocol. To assess RNA quantity and purity, all samples were checked using a NanoDrop 8000 (ThermoScientific). Samples with low 260/280 or 260/230 nm ratios were purified on RNeasy columns again to eliminate contamination. We then reverse-transcribed 0.7 µg of RNA from each sample with the Quantitect reverse transcription kit (Qiagen). We included control reactions without the reverse transcriptase (noRT controls) for each sample and checked these technical controls by qPCRs on an ABI 7500 fast real-time PCR system with at least two of the reference genes to ensure absence of genomic DNA. All noRT control samples amplified at least 10 Ct cycles later than the reverse-transcribed samples. This can be interpreted as any genomic contamination being below 0.1% of the signal detected in our actual samples. All 142 samples passed these quality tests.

Expression was measured on two Fluidigm 96.96 dynamic array IFCs on the BioMark system using EvaGreen DNA binding dye (Biotium) according to the advanced development protocol 14 (PN 100–1208 B). Treatment groups were distributed across the two chips in the same way, and several calibration samples were included on both chips to ensure comparability of data across chips. Our tested gene set included putative immune genes (PGRP-S3, PGRP-LC, BGRP1, BGRP2, haemomucin, pelle, relish, basket, hopscotch, abaecin, apidaecin, defensin, hymenoptaecin, TEPA, lysozyme3, transferrin, ferritin, jafrac, peroxiredoxin5, serpin27a, prophenoloxidase (PPO), catsup, punch), some metabolism related genes (vitellogenin, apolipophorin III, cytochrome P450) and a suite of six reference genes (AK, PLA2, ACTB, RPL13, ef1α, ITPR). The immune gene set was selected to span the receptor, signalling and effector levels of all four insect innate immune pathways (toll, IMD, JAK/STAT, JNK) and other immune functions such as melanization reactions and reactive oxygen species regulation. We preferentially included genes where interesting transcriptional responses upon infection have already been found [29,31,39–41,57–59]. The reference gene choice was based on previous studies [60] and our own expression stability tests [31]. Putative gene functions, primer sequences and NCBI accession numbers are summarized in the electronic supplementary material, table S1.

We used published primers for haemomucin and relish [40], vitellogenin [61], ACTB and RPL13 [60] and for ITPR [41]. All other primers were designed in Primer3 [62] or Quantprime [63], based on the GenBank sequences (electronic supplementary material, table S1). We used the following primer design settings in both programs: primers 20 ± 2 bp long, melting temperature of 60 ± 1°C with a maximum of 0.5°C difference in annealing temperature between forward and reverse primers. We tested all primers for reliability and amplification efficiency (1.9–2.1) in qPCRs with several samples and an annealing temperature of 60°C. All primers used in this study met these quality criteria.

The three technical triplicates for each sample/gene combination were measured on the same Fluidigm 96.96 chip and averaged for the raw expression value (Ct). The geometrical mean of Ct values from AK, PLA2, ITPR and ef1α was then used for normalization of target gene expression data [69], yielding the dCt values. (Details of reference gene analysis and data normalization in the electronic supplementary material, table S2.)

(c) Phenoloxidase activity assays

PO activity was measured using a spectrophotometric assay as described in [55]. This enzyme is involved in melanization reactions of invertebrates upon pathogen exposure [70] and depends on the activation of its inactive form proPO by a series of reactions mediated by several serine proteases which themselves are activated by bacterial antigens [64]. We used a SpectraMAX-340PC (Molecular Devices) with SOFTMAX PRO 3.1 software for the spectrophotometric measurements of L-DOPA conversion to L-dopaquinone to assess PO activity in the bumblebee haemolymph samples. The reaction curve slope in the linear phase of reaction (V_{max} value) was taken as the enzyme activity value for further analyses.

(d) Statistical analyses

We used R v. 2.13.1 [65] for the statistical analysis of exponentially transformed dCt values as recommended by Yuan *et al*. [66]. We used MANOVA to analyse gene expression, because the expression of any gene is not independent of other genes, and because MANOVA accounts for multiple testing it is thus robust to type I error. In order to improve normality and homoscedasticity of data distribution within groups, we Yeo–Johnson transformed the data for each gene (λ-values in the electronic supplementary material, table S3). For most of the genes, we also excluded one sample of the non-infected, non-starved group of colony no. 6 as it disrupted normal distributions severely both before and after Yeo–Johnson transformation. These transformed dCt values were then used as response variables in a MANOVA, taking infection status, starvation status and parental collection site as fixed factors and colony identity as a nested factor within site (table 1 and the electronic supplementary material, S4). To accommodate the nested factor 'colony', a type I sums of squares MANOVA was conducted. We included colony as a fixed effect, nested within collection site, because random effects are not permitted in MANOVA in R, and because including it as an explicit fixed effect indicates the strength of host genotype effects. Host genotype strongly influences infection outcome and gene expression in this system. As the dataset was almost balanced, the order of factors in the model did not influence the outcome of the analyses, neither for multivariate nor for univariate models. The full MANOVA output can be found in the electronic supplementary material, table S4.

To visualize treatment effects, we display fold-change values of gene expression upon treatment calculated as $2^{-\mathrm{ddCt}}$ [67]. The ddCt represents the difference between the gene of interest, and the composite housekeeping value and how the expression of the gene of interest varies according to experimental condition. For visualization of significant interaction effects between starvation and infection treatments, we show means and standard errors of fold expression values ($2^{-\mathrm{dCt}}$) within treatment groups. These are not to be confused with fold-change values that can be interpreted directly; for fold expression values, the scale is arbitrary and has no inherent biological meaning as it is based only on the expression of the target gene relative to reference genes.

We analysed variation in gene expression across treatment groups by testing for differences in variance of fold expression values ($2^{-\mathrm{dCt}}$) with permutation test for homogeneity of multivariate dispersion (vegan package [68]).

PO activity was analysed with an ANOVA after Yeo–Johnson transformation. We also assessed the relationship between PO activity and the expression of individual genes by calculating correlation values and the respective 95% confidence intervals in the base R package.

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References

1. Lazzaro BP, Little TJ. 2009 Immunity in a variable world. *Phil. Trans. R. Soc. B* **364**, 15–26. (doi:10.1098/Rstb.2008.0141)
2. Ponton F, Wilson K, Cotter SC, Raubenheimer D, Simpson SJ. 2011 Nutritional immunology: a multi-dimensional approach. *PLoS Pathog.* **7**, e1002223. (doi:10.1371/journal.ppat.1002223)
3. Cotter SC, Simpson SJ, Raubenheimer D, Wilson K. 2011 Macronutrient balance mediates trade-offs between immune function and life history traits. *Funct. Ecol.* **25**, 186–198. (doi:10.1111/j.1365-2435.2010.01766.x)
4. Schmid-Hempel P. 2011 *Evolutionary parasitology: the integrated study of infections, immunology, ecology, and genetics*. Oxford, UK: Oxford University Press.
5. Sheldon BC, Verhulst S. 1996 Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**, 317–321. (doi:10.1016/0169-5347(96)10039-2)
6. Rolff J, Siva-Jothy MT. 2003 Invertebrate ecological immunology. *Science* **301**, 472–475. (doi:10.1126/science.1080623)
7. Schmid-Hempel P, Ebert D. 2003 On the evolutionary ecology of specific immune defence. *Trends Ecol. Evol.* **18**, 27–32. (doi:10.1016/S0169-5347(02)00013-7)
8. Schmid-Hempel P. 2003 Variation in immune defence as a question of evolutionary ecology. *Proc. R. Soc. Lond. B* **270**, 357–366. (doi:10.1098/rspb.2002.2265)
9. Smith V. 2007 Host resource supplies influence the dynamics and outcome of infectious disease. *Integr. Comp. Biol.* **47**, 310–316. (doi:10.1093/Icb/Icm006).
10. Scrimshaw NS, SanGiovanni JP. 1997 Synergism of nutrition, infection, and immunity: an overview. *Am. J. Clin. Nutr.* **66**, 464S–477S.
11. Simpson SJ, Raubenheimer D. 2012 *The nature of nutrition: a unifying framework from animal adaptation to human obesity*. Princeton, NJ: Princeton University Press.
12. Connors VA, Nickol BB. 1991 Effects of *Plagiorhynchus cylindraceus* (Acanthocephala) on the energy metabolism of adult starlings, *Sturnus vulgaris*. *Parasitology* **103**, 395–402. (doi:10.1017/S0031182000059916)
13. Murray DL, Keith LB, Cary JR. 1998 Do parasitism and nutritional status interact to affect production in snowshoe hares? *Ecology* **79**, 1209–1222. (doi:10.1890/0012-9658(1998)079[1209:DPANSI]2.0.CO;2)
14. Booth DT, Clayton DH, Block BA. 1993 Experimental demonstration of the energetic cost of parasitism in free-ranging hosts. *Proc. R. Soc. Lond.* **253**, 125–129. (doi:10.1098/rspb.1993.0091)
15. Shik JZ, Kaspari M, Yanoviak SP. 2011 Preliminary assessment of metabolic costs of the nematode *Myrmeconema neotropicum* on its host, the tropical ant *Cephalotes atratus*. *J. Parasitol.* **97**, 958–959. (doi:10.1645/GE-2735.1)
16. Koella JC, Sorense FL. 2002 Effect of adult nutrition on the melanization immune response of the malaria vector *Anopheles stephensi*. *Med. Vet. Entomol.* **16**, 316–320. (doi:10.1046/j.1365-2915.2002.00381.x)
17. Triggs A, Knell RJ. 2012 Interactions between environmental variables determine immunity in the Indian meal moth *Plodia interpunctella*. *J. Anim. Ecol.* **81**, 386–394. (doi:10.1111/j.1365-2656.2011.01920.x)
18. Lee KP, Simpson SJ, Wilson K. 2008 Dietary protein-quality influences melanization and immune function in an insect. *Funct. Ecol.* **22**, 1052–1061. (doi:10.1111/J.1365-2435.2008.01459.X)
19. Weers PMM, Ryan RO. 2006 Apolipophorin III: role model apolipoprotein. *Insect Biochem. Mol. Biol.* **36**, 231–240. (doi:10.1016/j.ibmb.2006.01.001)
20. Adamo SA, Roberts JL, Easy RH, Ross NW. 2008 Competition between immune function and lipid transport for the protein apolipophorin III leads to stress-induced immunosuppression in crickets. *J. Exp. Biol.* **211**, 531–538. (doi:10.1242/Jeb.013136)
21. Adamo SA, Bartlett A, Le J, Spencer N, Sullivan K. 2010 Illness-induced anorexia may reduce trade-offs between digestion and immune function. *Anim. Behav.* **79**, 3–10. (doi:10.1016/j.anbehav.2009.10.012)
22. Seehuus SC, Norberg K, Gimsa U, Krekling T, Amdam GV. 2006 Reproductive protein protects

functionally sterile honey bee workers from oxidative stress. *Proc. Natl Acad. Sci. USA* **103**, 962–967. (doi:10.1073/pnas.0502681103)

23. Amdam GV, Simoes ZLP, Hagen A, Norberg K, Schroder K, Mikkelsen O, Kirkwood TBL, Omholt SW. 2004 Hormonal control of the yolk precursor vitellogenin regulates immune function and longevity in honeybees. *Exp. Gerontol.* **39**, 767–773. (doi:10.1016/j.exger.2004.02.010)
24. Lipa J, Triggiani O. 1988 *Crithidia bombi* Sp. N. A flagellated parasite of a bumblebee *Bombus terrestris* L. (Hymenoptera, Apidae). Acta Protozool. **27**, 287–290.
25. Schmid-Hempel P. 2001 On the evolutionary ecology of host–parasite interactions: addressing the question with regard to bumblebees and their parasites. *Naturwissenschaften* **88**, 147–158. (doi:10.1007/s001140100222)
26. Schmid-Hempel P, Schmid-Hempel R. 1993 Transmission of a pathogen in *Bombus terrestris*, with a note on division-of-labor in social insects. *Behav. Ecol. Sociobiol.* **33**, 319–327. (doi:10.1007/BF00172930)
27. Brown MJF, Loosli R, Schmid-Hempel P. 2000 Condition-dependent expression of virulence in a trypanosome infecting bumblebees. *Oikos* **91**, 421–427. (doi:10.1034/j.1600-0706.2000.910302.x)
28. Brown MJF, Schmid-Hempel R, Schmid-Hempel P. 2003 Strong context-dependent virulence in a host–parasite system: reconciling genetic evidence with theory. *J. Anim. Ecol.* **72**, 994–1002. (doi:10.1046/j.1365-2656.2003.00770.x)
29. Riddell CE, Adams S, Schmid-Hempel P, Mallon EB. 2009 Differential expression of immune defences is associated with specific host–parasite interactions in insects. *PLoS ONE* **4**. ARTN e7621. (doi:10.1371/journal.pone.0007621)
30. Barribeau SM, Sadd BM, du Plessis L, Schmid-Hempel P. 2014 Gene expression differences underlying genotype-by-genotype specificity in a host–parasite system. *Proc. Natl Acad. Sci. USA* **111**, 3496–3501. (doi:10.1073/pnas.1318628111)
31. Brunner FS, Schmid-Hempel P, Barribeau S. 2013 Immune gene expression in *Bombus terrestris*: signatures of infection despite strong variation among populations, colonies and sister workers. *PLoS ONE* **8**, e68181. (doi:10.1371/journal.pone.0068181)
32. Barribeau SM, Schmid-Hempel P. 2013 Qualitatively different immune response of the bumblebee host, *Bombus terrestris*, to infection by different genotypes of the trypanosome gut parasite, *Crithidia bombi*. *Infect. Genet. Evol.* **20**, 249–256. (doi:10.1016/J.Meegid.2013.09.014)
33. Sadd BM. 2011 Food-environment mediates the outcome of specific interactions between a bumblebee and its trypanosome parasite. *Evolution* **65**, 2995–3001. (doi:10.1111/j.1558-5646.2011.01345.x)
34. Logan A, Ruiz-Gonzalez MX, Brown MJF. 2005 The impact of host starvation on parasite development and population dynamics in an intestinal trypanosome parasite of bumble bees. *Parasitology* **130**, 637–642. (doi:10.1017/S0031182005007304)
35. Moret Y, Schmid-Hempel P. 2000 Survival for immunity: the price of immune system activation for bumblebee workers. *Science* **290**, 1166–1168. (doi:10.1126/science.290.5494.1166)
36. Tyler ER, Adams S, Mallon EB. 2006 An immune response in the bumblebee, *Bombus terrestris* leads to increased food consumption. *BMC Physiol.* **6**, 6. (doi:10.1186/1472-6793-6-6)
37. Konig C, Schmid-Hempel P. 1995 Foraging activity and immunocompetence in workers of the bumble bee, *Bombus terrestris* L. *Proc. R. Soc. Lond. B* **260**, 225–227. (doi:10.1098/Rspb.1995.0084)
38. Doums C, Schmid-Hempel P. 2000 Immunocompetence in workers of a social insect, *Bombus terrestris* L, in relation to foraging activity and parasitic infection. *Can. J. Zool.* **78**, 1060–1066. (doi:10.1139/cjz-78-6-1060)
39. Riddell CE, Sumner S, Adams S, Mallon EB. 2011 Pathways to immunity: temporal dynamics of the bumblebee (*Bombus terrestris*) immune response against a trypanosomal gut parasite. *Insect Mol. Biol.* **20**, 529–540. (doi:10.1111/J.1365-2583.2011.01084.X)
40. Schlüns H, Sadd BM, Schmid-Hempel P, Crozier RH. 2010 Infection with the trypanosome *Crithidia bombi* and expression of immune-related genes in the bumblebee *Bombus terrestris*. *Dev. Comp. Immunol.* **34**, 705–709. (doi:10.1016/J.Dci.2010.02.002)
41. Erler S, Popp M, Lattorff HMG. 2011 Dynamics of immune system gene expression upon bacterial challenge and wounding in a social insect (*Bombus terrestris*). *PLoS ONE* **6**, e18126. (doi:10.1371/journal.pone.0018126)
42. Richter J, Helbing S, Erler S, Lattorff HMG. 2012 Social context-dependent immune gene expression in bumblebees (*Bombus terrestris*). *Behav. Ecol. Sociobiol.* **66**, 791–796. (doi:10.1007/s00265-012-1327-2)
43. McKean KA, Nunney L. 2005 Bateman's principle and immunity: phenotypically plastic reproductive strategies predict changes in immunological sex differences. *Evolution* **59**, 1510–1517. (doi:10.1554/04-657)
44. Alaux C, Ducloz F, Crauser D, Le Conte Y. 2010 Diet effects on honeybee immunocompetence. *Biol. Lett.* **6**, 562–565. (doi:10.1098/rsbl.2009.0986)
45. Koch H, Schmid-Hempel P. 2011 Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proc. Natl Acad. Sci. USA* **108**, 19 288–19 292. (doi:10.1073/pnas.1110474108)
46. Koch H, Schmid-Hempel P. 2012 Gut microbiota instead of host genotype drive the specificity in the interaction of a natural host–parasite system. *Ecol. Lett.* **15**, 1095–1103. (doi:10.1111/j.1461-0248.2012.01831.x)
47. Whitehorn PR, Tinsley MC, Brown MJF, Darvill B, Goulson D. 2011 Genetic diversity, parasite prevalence and immunity in wild bumblebees. *Proc. R. Soc. B* **278**, 1195–1202. (doi:10.1098/Rspb.2010.1550)
48. King KC, Lively CM. 2012 Does genetic diversity limit disease spread in natural host populations? *Heredity* (*Edinb.*) **109**, 199–203. (doi:10.1038/hdy.2012.33)
49. Lafferty KD, Holt RD. 2003 How should environmental stress affect the population dynamics of disease? *Ecol. Lett.* **6**, 654–664. (doi:10.1046/J.1461-0248.2003.00480.X)
50. Sadd BM, Kleinlogel Y, Schmid-Hempel R, Schmid-Hempel P. 2005 Trans-generational immune priming in a social insect. *Biol. Lett.* **1**, 386–388. (doi:10.1098/rsbl.2005.0369)
51. Estoup A, Solignac M, Cornuet JM, Goudet J, Scholl A. 1996 Genetic differentiation of continental and island populations of *Bombus terrestris* (Hymenoptera: Apidae) in Europe. *Mol. Ecol.* **5**, 19–31. (doi:10.1111/j.1365-294X.1996.tb00288.x)
52. Widmer A, Schmid-Hempel P, Estoup A, Scholl A. 1998 Population genetic structure and colonization history of *Bombus terrestris* s.l. (Hymenoptera: Apidae) from the Canary Islands and Madeira. *Heredity* **81**, 563–572. (doi:10.1038/sj.hdy.6884070)
53. Moret Y, Schmid-Hempel P. 2009 Immune responses of bumblebee workers as a function of individual and colony age: senescence versus plastic adjustment of the immune function. *Oikos* **118**, 371–378.
54. Salathé R, Schmid-Hempel R, Schmid-Hempel P. 2012 Probing mixed-genotype infections I: extraction and cloning of infections from hosts of the trypanosomatid *Crithidia bombi*. *PLoS ONE* **7**, e49046. (doi:10.1371/journal.pone.0049046)
55. Moret Y. 2001 *Evolutionary ecology and adaptiveness of immune defenses of the social insect* Bombus terrestris (*L.*) (*Hymenoptera: Apidae*). Zürich, Switzerland: Swiss Federal Institute of Technology.
56. Korner P, Schmid-Hempel P. 2004 *In vivo* dynamics of an immune response in the bumble bee *Bombus terrestris*. *J. Invertebr. Pathol.* **87**, 59–66. (doi:10.1016/j.jip.2004.07.004)
57. Roditi I, Lehane MJ. 2008 Interactions between trypanosomes and tsetse flies. *Curr. Opin. Microbiol.* **11**, 345–351. (doi:10.1016/J.Mib.2008.06.006).
58. Vogel H, Altincicek B, Glockner G, Vilcinskas A. 2011 A comprehensive transcriptome and immune-gene repertoire of the lepidopteran model host *Galleria mellonella*. *BMC Genomics* **12**, 308. (doi:10.1186/1471-2164-12-308)
59. Radyuk SN, Michalak K, Klichko VI, Benes J, Orr WC. 2010 Peroxiredoxin 5 modulates immune response in *Drosophila*. *Biochim. Biophys. Acta. Gen. Subjects* **1800**, 1153–1163. (doi:10.1016/j.bbagen.2010.06.010).
60. Hornakova D, Matouskova P, Kindl J, Valterova I, Pichova I. 2010 Selection of reference genes for real-time polymerase chain reaction analysis in tissues from *Bombus terrestris* and *Bombus lucorum* of different ages. *Anal. Biochem.* **397**, 118–120. (doi:10.1016/J.Ab.2009.09.019)
61. Li JL, Huang JX, Cai WZ, Zhao ZW, Peng WJ, Wu J. 2010 The vitellogenin of the bumblebee, *Bombus*

hypocrita: studies on structural analysis of the cDNA and expression of the mRNA. *J. Comp. Physiol. B* **180**, 161–170. (doi:10.1007/S00360-009-0434-5)

62. Rozen S, Skaletsky H. 2000 Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* **132**, 365–386.
63. Arvidsson S, Kwasniewski M, Riano-Pachon DM, Mueller-Roeber B. 2008 QuantPrime: a flexible tool for reliable high-throughput primer design for quantitative PCR. *BMC Bioinformatics* **9**, 465. (doi:10.1186/1471-2105-9-465)
64. Poulin R, Thomas F. 1999 Phenotypic variability induced by parasites. *Parasitol. Today* **15**, 28–32. (doi:10.1016/S0169-4758(98)01357-X)
65. R Core Team. 2012 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
66. Yuan JS, Reed A, Chen F, Stewart CN. 2006 Statistical analysis of real-time PCR data. *BMC Bioinformatics* **7**, 85. (doi:10.1186/1471-2105-7-85)
67. Schmittgen TD, Livak KJ. 2008 Analyzing real-time PCR data by the comparative C_T method. *Nat. Protoc.* **3**, 1101–1108. (doi:10.1038/Nprot.2008.73)
68. Oksanen J *et al*. 2013 vegan: Community Ecology Package. R package version 2.0-10.
69. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, (doi:10.1186/Gb-2002-3-7-Research0034)
70. Gillespie JP, Kanost MR, Trenczek T. 1997 Biological mediators of insect immunity. *Annu. Rev. Entomol.* **42**, 611–643. (doi:10.1146/annurev.ento.42.1.611)