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Strain filtering and transmission of a mixed infection in a social insect

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Abstract

Mixed-genotype infections have attracted considerable attention as drivers of pathogen evolution. However, experimental approaches often overlook essential features of natural host–parasite interactions, such as host heterogeneity, or the effects of between-host selection during transmission. Here, following inoculation of a mixed infection, we analyse the success of different strains of a trypanosome parasite throughout the colony cycle of its bumblebee host. We find that most colonies efficiently filter the circulating infection before it reaches the new queens, the only offspring that carry infections to the next season. A few colonies with a poor filtering ability thus contributed disproportionately to the parasite population in the next season. High strain diversity but not high infection intensity within colony was associated with an increased probability of transmission of the infection to new queens. Interestingly, the representation of the different strains changed dramatically over time, so that long-term parasite success could not be predicted from short-term observations. These findings highlight the shaping of within-colony parasite diversity through filtering as a crucial determinant of year-to-year pathogen transmission and emphasize the importance of host ecology and heterogeneity for disease dynamics.

Introduction

Mixed-genotype infections have theoretically been shown to be of great importance in host–parasite systems, having major consequences for the epidemiology of diseases, for the evolution of virulence and for the emergence of resistance to drugs (May & Nowak, 1995; Gandon *et al.*, 2001; Alizon *et al.*, 2009). Empirically, mixed-genotype infections are also frequently found in a variety of host–parasite systems (Babiker *et al.*, 1999; Lord *et al.*, 1999; Read & Taylor, 2001; López-Villavicencio *et al.*, 2007). Experimental studies on mixed infections have so far focused on the outcome of competition between pairs of parasites genotypes within isolated hosts, especially to test predictions about the evolution of virulence (Brown *et al.*, 2002; Gower & Webster, 2005; Bell *et al.*, 2006; Ben-Ami *et al.*, 2008). But focusing on the short-term within-host competitive success of parasites does not necessarily reflect their success in natural host populations. In particular, parasites need not only establish and grow within a host but they must also be transmitted to new hosts. To obtain a grasp on disease spread in the context of mixed-genotype infections, experimental studies are therefore needed that take into account the entire life cycle of parasites, which includes between-host selection during transmission as well as within-host selection by interactions with other parasites or the host's immune system.

Another important feature of natural infections is host genetic and ecological heterogeneity, which has been shown to affect the outcome of competition between co-infecting parasite genotypes (Hodgson *et al.*, 2004; de Roode *et al.*, 2004). Because many host–parasite systems exhibit genotype × genotype specificity (Carius *et al.*, 2001; Schulenburg & Ewbank, 2004; Lambrechts *et al.*, 2006; Little *et al.*, 2006; Salvaudon *et al.*, 2007), mixed infections in different host genetic backgrounds should result in distinct infection profiles, representing the range of susceptibilities to the different parasite genotypes. Little is known, however, about the

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natural variation in host ability to fight mixed-genotype infections, and how this might affect the dynamics of the host–parasite interaction. Finally, not only hosts but also parasites show genotypic and phenotypic diversity. The outcome of within-host competition will therefore vary according to the identity of the parasite genotypes involved. Specifically, relatedness between co-infecting parasite genotypes is theoretically predicted to affect the outcome of multiple infections; for example, kin selection should decrease competition between parasite genotypes sharing a host if those parasites are related (Frank, 1992), and this prediction has received some empirical support (Puustinen *et al.*, 2004; Jäger & Schjørring, 2006; Koskella *et al.*, 2006; López-Villavicencio *et al.*, 2007).

Experimental approaches that consider a timeframe representative of the studied host–parasite interaction, and that include both host and parasite heterogeneity, are required to further our understanding of the natural dynamics of multiple infections. Here, we use such a semi-natural approach to analyse the success of various strains of an intestinal trypanosome parasite, *Crithidia bombi*, co-inoculated into colonies of a social insect, the bumblebee *Bombus terrestris*. Social insects present interesting and convenient systems to study the transmission of infectious diseases within dense groups of frequently interacting host individuals (Schmid-Hempel, 1998). *B. terrestris* is naturally infected by a wide range of strains of *C. bombi*, which are transmitted via faeces deposited in the nest (within-colony transmission) and via flowers during foraging activity (between-colony transmission) (Durrer & Schmid-Hempel, 1994). The parasite has been shown to exert strong selection on the host, as it increases worker mortality under stressful conditions, slows down colony growth and drastically reduces the colony founding success of infected queens after diapause (Brown *et al.*, 2000; Schmid-Hempel, 2001; Brown *et al.*, 2003).

In Central Europe, mated bumblebee queens emerge in spring from hibernation and initiate colonies. Yearly field surveys revealed that between 5% and 10% of these queens exiting hibernation are infected with one or several strains of *C. bombi* (P. Schmid-Hempel, unpublished data). Towards the end of the reproductive season, a fraction of the established colonies produce new queens that subsequently enter hibernation after mating with a single male. As *C. bombi* cannot survive outside its host for a prolonged period (Schmid-Hempel *et al.*, 1999), an important consequence of this life cycle of bumblebees is that the parasite can only persist from 1 year to the next by infecting the new queens at the end of the colony cycle, because these are the only hosts that survive the winter. There should thus be strong selection on the parasite to be able to infect queens.

Field data have shown that colonies of the host are typically exposed to numerous, genetically distinct strains throughout the season and frequently become multiply infected (Schmid-Hempel & Reber Funk, 2004). In fact, nearly half of all individuals are infected by more than one strain (R. Salathé & P. Schmid-Hempel, pers. comm.). Infection success and transmission depends on the origin of both the host and the parasite, indicating the existence of genotype × genotype interactions (Mallon *et al.*, 2003; Schmid-Hempel, 2001). The pattern is, however, not as simple as this notion suggests. In fact, some colonies appear to be resistant to most strains of the parasite, whereas others are susceptible to almost any strain (Schmid-Hempel & Schmid-Hempel, 1993; Schmid-Hempel *et al.*, 1999; Mallon *et al.*, 2003; Schmid-Hempel & Reber Funk, 2004). Rather than a one-to-one pattern of genotypic interaction, hosts thus vary in how many strains (and which strains) they are susceptible to. Hence, from any mixed-genotype infection, only a (varying) subset of strains will be transmitted further. We henceforth refer to the way that hosts decrease the genetic diversity of an infection by specifically eliminating some parasite genotypes as 'filtering'.

In the experiment presented here, with each colony representing a host unit, we address the following questions relevant for understanding the dynamics and consequences of mixed-genotype infections: (i) How does filtering vary across host colonies? (ii) Does infective dose affect filtering? (iii) Does filtering ability relate to colony fitness? (iv) Which subset of the strains is transmitted to the next generation of hosts? (v) How do the different strains distribute themselves over hosts? (vi) Do parasite strains associate in mixed infections according to their genetic relatedness?

Material and methods

Insects and parasites

Twenty-five laboratory colonies of *B. terrestris* were started from queens collected in the spring of 2008 from a single population in Northern Switzerland. Queens and workers from all colonies were carefully checked before the start of the experiment to ensure that they were free of parasites. The five parasite strains used for the experimental infections were obtained from faeces of naturally infected queens collected in spring 2008 in the same population. Single infective cells were isolated using a fluorescence-activated cell-sorter and maintained clonally in liquid medium at 27 °C and 3% CO_2 (R. Salathé & P. Schmid-Hempel, unpublished data). The strains were chosen to have distinct multilocus genotypes, so as to be readily differentiated by genetic markers in a mixture. All colonies and individually isolated bees were kept at 28 ± 2 °C under constant red light illumination, with pollen and sugar water (ApiInvert®, Südzucker, Ochsenfurt, Germany) provided *ad libitum*. Colonies were kept in circular perlite observation nests (Pomeroy & Plowright, 1980) with a diameter of 23 ± 2 cm.

Infection protocol and colony sampling

For experimental infections, parasite cells were counted and adjusted to 40 cells μL^{-1} (low infective dose; total of 200 cells μL^{-1}) or to 200 cells μL^{-1} (high infective dose; total of 1000 cells μL^{-1}) per strain. For each dose, the infective cells of the five strains were then mixed together and administered as a cocktail in 50% sugar water. As soon as the colonies reached a sufficient size (ca. 20 workers), workers were taken out of each colony, starved for 4–5 h and presented with 10 μL of this infection cocktail. Workers from 15 colonies received a low infective dose (2000 cells in total), whereas those from the remaining 10 colonies received a high infective dose (10 000 cells). We visually checked that each worker ingested the infection cocktail. Bees that did not ingest the infection cocktail after ca. 45 min were excluded from the experiment. To start the infection within the colonies, five of the 13 exposed workers were immediately put back into their colony of origin. The eight remaining workers were kept individually for 7 days with *ad libitum* sugar water and pollen before being frozen. The former group simulates the start of a natural epidemic as workers bring back parasite strains from foraging trips at the beginning of the colony cycle (Imhoof & Schmid-Hempel, 1999). The latter group of workers was used to establish a colony 'filtering profile', as described elsewhere. We left the infection to propagate in the 25 colonies without intervening, except for the removal of 20% of the workers (once) and all males (regularly) to mimic the natural loss of individuals from the colony. All newly produced daughter queens were marked using professional beekeeper marking pens on the day of adult emergence, left in their natal colony for 5 days and subsequently kept individually for seven additional days before being frozen. Five days are thought to be the time that new queens spend in their natal colonies before leaving on a mating flight (Alford, 1975). The experiment ended when the colonies stopped producing queens; the entire experiment lasted for about 3 months.

Genotyping of infections

All individually kept workers and, on average, 27% of all produced queens of a colony (ranging from a subset of 16% for colonies with many queens, to 100% for colonies with very few queens) were dissected, and their hind gut homogenized in 100 μL (workers) or 200 μL (queens) of Ringer solution. DNA was extracted from these individual gut preparations using a QIAGEN DNeasy 96 Tissue kit. The infection status of queens (infected vs. noninfected) was assessed by the amplification of a portion of the *C. bombi* 18sRNA gene using primers CB-SSUrRNA-F2 and CB-SSUrRNA-B4 (Schmid-Hempel & Tognazzo, 2010) and visualization of the amplification product on a 1.5% agarose gel. The gut extracts of all individually kept workers and of the queens found to be infected with the previous method were then genotyped at the *C. bombi* microsatellite markers Cri4, Cri1B6, Cri4G9 and Cri2F10 (Schmid-Hempel & Reber Funk, 2004). Because the five parasite strains used for the infections had distinct multilocus genotypes based on these four loci, genotyping the gut DNA extracts enabled us to identify the strains present in the infection. The four loci were amplified in one multiplex polymerase chain reaction (PCR) containing 2 μL eluted DNA, 0.2 μM primers Cri4 and Cri4G9, 0.25 μM of primers Cri2F10 and Cri1B6, 150 μM of each dNTP, 0.5 U Amersham Taq Polymerase for a final volume of 10 μL, and according to the following thermal profile: initial denaturation of 5 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C, and a final extension of 7 min at 72 °C. Primers were labelled with fluorescent dyes, and amplification products were analysed on a MegaBACE sequencer (both from GE Healthcare, Glattburgg, Switzerland). Alleles were scored with MegaBACE Fragment Profiler software v1.2.

All samples were genotyped and the alleles scored independently twice. Only fully concordant genotypes were included in the analyses. Previous work shows that the methods used here are sensitive enough to detect very low concentrations of different *C. bombi* strains in mixed infections (Schmid-Hempel *et al.*, 1999).

Quantification of infections

We developed a quantitative real-time PCR (qPCR) assay based on SYBR Green chemistry to measure the total infection intensity of *C. bombi* in an individual. For this purpose, the primers 'CriRTF2' (GGCCACCCACGGGAATAT) and 'CriRTR2' (CAAAGCTTTCGCGTGAAGAAA) were designed with the computer program Primer3 (Rozen & Skaletsky, 2000) and used to amplify a 56-bp fragment of the *C. bombi* 18sRNA gene. The qPCRs were performed on a ABI 7500 Real-time PCR System (Applied Biosystems, Rotkreuz, Switzerland) in 20 μL reaction volumes containing 2 μL eluted DNA, 0.3 μM of each primer and 1× Power SYBR Green Universal MasterMix (Applied Biosystems), according to the following thermal profile: 10 min preincubation at 95 °C followed by 40 cycles of amplification with 15 s denaturation at 95 °C and 1 min simultaneous annealing and extension at 60 °C. Melting curves and DNA agarose gels were used to confirm the specificity of the reaction. Amplification efficiency of the primers and reproducibility of qPCR were assessed with a dilution series of DNA extracted from a known number of cells from a pure cell culture. The dilution series reflected the range of intensities occurring in infected animals. Negative controls of double-distilled H_2O and parasite-free bumblebee DNA were included in every run. Because qPCR depends strongly on sample quality, we

corrected the qPCR reads for the variation in total DNA content introduced during extraction (Refardt & Ebert, 2006). Relative infection intensity was determined by measuring parasite DNA by qPCR and to incorporate variation in extraction efficiency across samples and by correcting the qPCR value by the total DNA (parasite and host DNA) present in the sample as determined by optical density measurements performed at 260 nm on a SpectraMax M2^e microplate reader (Molecular Devices, Sunnyvale, CA, USA). This approach is appropriate because parasitic DNA typically represents a negligible proportion of the total DNA content after extractions, as indicated by the fact that samples taken from uninfected bees show total DNA concentrations that were undistinguishable from those of infected bees (Y. Ulrich, unpublished data).

Analysis and statistics

We statistically analysed the distribution of the parasite strains across workers using a randomization approach. To generate an expected distribution (under the assumption of no interaction between strains), we considered that there are 32 possible infection genotypes (e.g. none, A, BD, ACE, ABCDE, where letters denote the five parasite strains) and six possible *n*-strains infections ($n = 0$–5 strains). Taking into account the observed prevalence of each strain, we re-sampled the observed presence/absence data for each strain 1000 times to produce an expected distribution of strain combinations. We tested whether the observed frequency of each infection genotype and *n*-strains infection differed significantly from the expectation by generating *P*-values at the 5% level and applying a sequential Holm–Bonferroni correction for multiple comparisons (Holm, 1979). Noninfected individuals were excluded from the latter analysis because their presence would inflate the number of significant comparisons by shifting the expected distribution of genotypes towards less diverse infections.

Finally, we tested for a correlation between the genetic relatedness of pairs of strains and their association, i.e. whether closely related strains tended to be found together more or less often than expected by chance. The association between two strains was calculated as the overall standardized difference between the expected and observed frequency of all genotypes containing these two strains, so that a positive association value indicates that the two strains are found in the same host more often than expected by chance. Multilocus genetic relatedness between pairs of strains was computed using a standardized Euclidian distance metric and assuming a stepwise mutational model (Goldstein *et al.*, 1995; Ostrowski *et al.*, 2008). This measure is calculated as follows: given strain A with genoytpe a_1 a_2 (allele lengths) and strain B with genotype b_1 b_2 (allele lengths) at locus *i*, the genetic distance between strains A and B at this locus, dAB, and the standardized Euclidian genetic distance between the two strains across all loci, *D*, respectively are:

$$\mathrm{dAB}_i - \frac{|b_1 - a_1| + |b_2 - a_2|}{2},$$

$$D = \sqrt{\sum_{i=1}^{n} \left(\frac{\mathrm{dAB}_i}{n_i}\right)^2}$$

where *n* is the number of alleles at locus *i*. All statistical analyses were performed in R2.8.1 (R Development Core Team, 2008). In particular, G-tests were performed to analyse differences in strain representation between specific groups of individuals (all workers vs. infected queens, workers from nontransmitting colonies vs. workers from transmitting colonies, workers from transmitting colonies vs. infected queens), with a William's adjustment to account for small sizes of expected values (Sokal & Rohlf, 1995). Unless otherwise stated, all values are ± SE.

Results

Validation of the qPCR assay

The qPCR assay developed for this study proved to be specific, efficient and sensitive. Negative controls of bumblebee DNA yielded either no amplification or residual amplification equivalent to a maximum of six parasitic cells on average. The average qPCR efficiency was 99.6%, with 100% indicating a doubling of products at every amplification cycle. We could detect parasite DNA in samples that were diluted down to the equivalent of three parasite cells, but samples with an amplification level lower than that of the negative controls were considered noninfected, which was confirmed by the absence of amplification at parasite microsatellite loci in all of these cases.

Colony filtering profiles

Of the 200 experimentally exposed workers that were kept individually to yield a colony filtering profile, five either died or escaped during the 7 days of isolation and so could not be analysed. Infective strains could be detected and unambiguously identified in 139 of the 195 genotyped gut extracts. Additionally, 16 samples had ambiguous microsatellite genotypes but significant infection intensities as revealed by qPCR, resulting in an overall infection rate of 79%. A total of 40 individuals showed no signs of infection. In all but eight individual workers, one or more strains from the initial infection cocktail disappeared or were reduced to undetectable levels, confirming the occurrence of strain filtering within individual hosts. As expected, colonies differed significantly in their 'filtering ability' as given by the

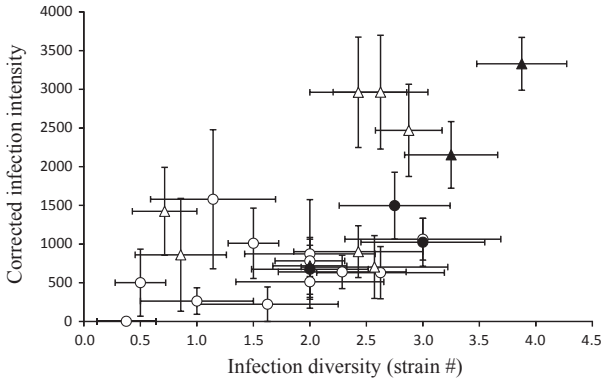

Fig. 1 Relationship between colony mean infection diversity (number of strains per bee; *x*-axis) and intensity (corrected parasite infection intensity per bee; *y*-axis) in workers from 25 colonies (Spearman's $\rho = 0.58$, $P = 0.002$; bars indicate ± 1 SE; circles: low dose; triangles: high dose; solid symbols: colonies that produced infected queens).

number of strains retained in the infection of workers from each colony (Kruskal–Wallis test on the number of strains per colony: $\chi^2_{24} = 63.34$, $P < 0.0001$).

Mean colony infection intensity (the fraction of parasitic DNA in host, see Material and methods) was higher in colonies whose workers received a high infective dose compared to those that received a low dose (Wilcoxon–Mann–Whitney test, $W_1 = 123$, $P = 0.007$). Furthermore, colony mean infection intensity was positively correlated with colony mean infection diversity, i.e. the average number of strains per bee (Spearman's $\rho = 0.58$, $P = 0.002$) (Fig. 1), even though the diversity of infections *per se* did not differ between the two dose treatments (Wilcoxon–Mann–Whitney test: $W_1 = 99.5$, $P = 0.18$). Separate analyses for the low-dose (2000 cells, Spearman's $\rho = 0.54$, $P = 0.037$) and the high-dose treatment (10 000 cells, Spearman's $\rho = 0.58$, $P = 0.08$) produced qualitatively similar results, i.e. a positive correlation of infection intensity and diversity. Because dose had an altogether weak or absent effect on filtering and transmission, all subsequent results are shown for the pooled data only.

A few infection types showed frequencies that differed significantly from expectation; for example, the infection genotypes B, D, ABCDE and ABE and single infections were more common than would be expected if the parasite strains were distributed randomly across hosts (Table 1). A Mantel test did not detect any correlation between the relatedness and association of pairs of strains ($P = 0.19$).

Table 1 Expected and observed distribution of infection types in isolated workers. Shown are the expected (exp) and observed (obs) frequencies of the 32 infection genotypes and six degrees of strain diversity possible with five strains (from None indicating no infection to multiple infections with all strains). Pairs of values in bold represent significantly differences at the 5% level after sequential Holm–Bonferroni correction. Noninfected individuals were not included in the analyses.

Genotype	Exp	Obs	# Strains	Exp	Obs
None	1.9	0	0	1.9	0
A	6.2	7	1	**15.3**	**26**
B	**0.7**	**4**			
C	1.4	2			
D	**5.1**	**11**			
E	1.9	2			
AB	2.2	2	2	43.1	37
AC	4.8	1			
AD	17.1	18			
AE	6.2	6			
BC	0.5	1			
BD	1.7	1			
BE	0.6	2			
CD	3.8	4			
CE	1.4	1			
DE	4.9	1			
ABC	1.6	0	3	50.2	42
ABD	5.8	2			
ABE	**2.1**	**5**			
ACD	12.7	16			
ACE	4.6	2			
ADE	16.4	14			
BCD	1.3	1			
BCE	0.5	0			
BDE	1.6	0			
CDE	3.5	2			
ABCD	4.2	1	4	24.5	26
ABCE	1.5	3			
BCDE	1.2	0			
ABDE	5.4	5			
ACDE	12.1	17			
ABCDE	**4.0**	**8**	5	**4.0**	**8**

Transmission of the infection to daughter queens

A total of 976 daughter queens were produced by 21 of the 25 colonies, starting 20 days post-infection in the earliest reproducing colony. Nineteen daughter queens from six colonies were found to be infected among the subset of 263 daughter queens that were screened for *C. bombi* (prevalence 7.2%). Whereas workers harboured up to five strains, queens were all singly (13 individuals) or doubly (six individuals) infected. Although all strains present in workers were also present in queens, the representation of the different strains contrasted sharply between the two groups (G-test with Williams' correction: $G_4 = 21.1$, Bonferroni-corrected $P = 0.0009$, all workers vs. daughter queens in Fig. 2). This difference is not because of a different 'filtering profile' of the colonies that transmitted the infection to their queen offspring, because the representation of strains in workers from those six colonies was undistinguishable from

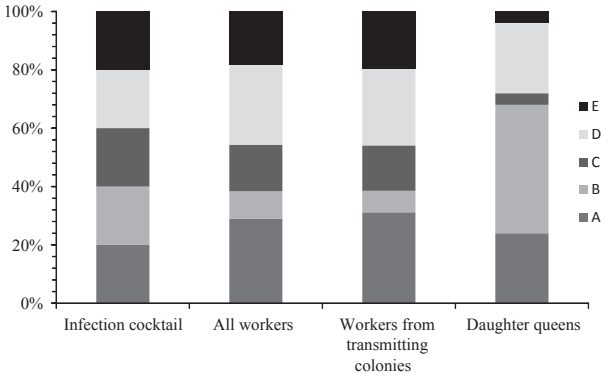

Fig. 2 Proportions of different strains (shades of grey, letters A–E) present in infected individuals – at the time of infection (infection cocktail), 7 days post-infection in individual workers (n = 139 infected of 194 exposed) (all workers), 7 days post-infection in individual workers from the six colonies that produced infected queens (n = 47) (workers from transmitting colonies) and in the infected daughter queens (n = 19) (daughter queens).

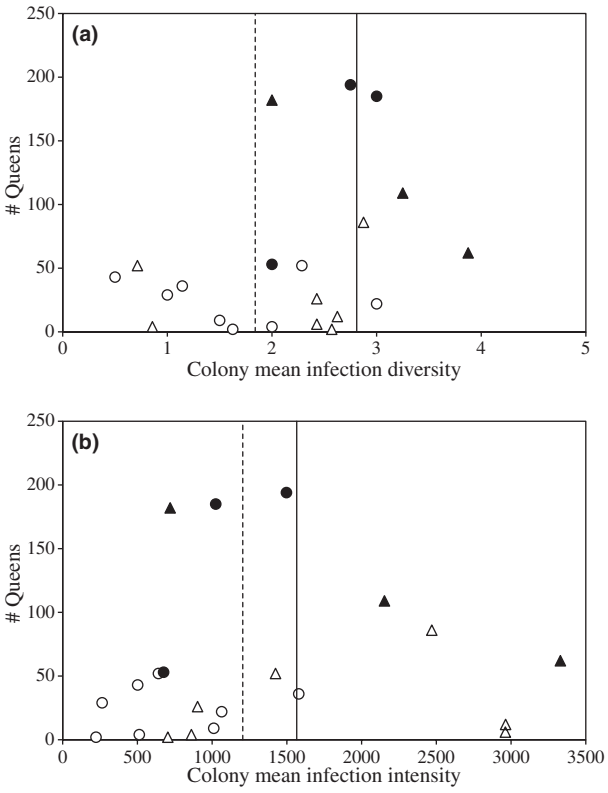

Fig. 3 Total number of queens produced by each colony (y-axis), as a function of (a) the colony average infection diversity (number of strains) and (b) the colony average infection intensity (fraction of parasite DNA, corrected by sample DNA concentration), as measured in workers 7 days post-infection. The six colonies that produced infected queens (solid symbols) had significantly more diverse infections than the 15 colonies that produced uninfected queens (open symbols) in (a), but showed undistinguishable infection intensities in (b). Vertical lines are mean infection diversity and intensity for infected (solid line) and uninfected (dashed line) colonies, respectively; triangles represent high-dose treatment, circles low-dose treatment. Colonies that did not produce queens are not shown here.

that of workers from nontransmitting colonies (G-test with Williams' correction: $G_4 = 1.4$, Bonferroni-corrected $P > 0.99$). Instead, the change in strain representation occurred between workers and queens from the same colonies (G-test with Williams' correction: $G_4 = 21.0$, Bonferroni-corrected $P = 0.0009$, workers from transmitting colonies vs. daughter queens in Fig. 2).

Interestingly, the six colonies that produced infected queens had significantly more diverse infections (mean = 2.81 ± 0.30 strains) than the 15 colonies that produced uninfected queens (mean = 1.84 ± 0.22 strains; Wilcoxon–Mann–Whitney test: $W_1 = 17.5$, $P = 0.0352$), but undistinguishable infection intensities (colonies with uninfected queens: mean = 1205 ± 235; colonies with infected queens: mean = 1566 ± 419; Wilcoxon–Mann–Whitney: $W_1 = 32$, $P = 0.34$) (Fig. 3). Therefore, although colony infection intensity and diversity were correlated (Fig. 1), infection diversity was a good predictor of whether a colony will transmit an infection to its queen offspring (binomial logistic regression: $\chi^2_{1,19} = 6.13$, $P = 0.013$), whereas infection intensity was not (binomial logistic regression: $\chi^2_{1,19} = 0.65$, $P = 0.42$). The colonies that produced infected queens also produced more queens in total (131 ± 26 queens compared to 26 ± 6 queens; Wilcoxon–Mann–Whitney test: $W_1 = 2$, $P = 0.0009$). Simply, distinguishing colonies that either produced daughter queens (n = 21) and those that did not (n = 4, not shown in Fig. 3) revealed no differences in strain diversity or infection intensity.

Discussion

As expected, we found considerable natural variation in the extent to which different hosts filtered parasite strains from the circulating infection, here mimicked by starting an infection in the colony by introducing a cocktail of five strains. Notably, we found a positive relationship between colony infection intensity and strain diversity, corroborating results from an earlier study (Schmid-Hempel *et al.*, 1999). This is indicative of an accumulation of infections (and presumably effects) of different strains, in line with the co-infection model of May & Nowak (1995). In this model, each strain is assumed to transmit at a rate unaffected by the presence of other strains in the same host. The result also suggests limited resource competition between strains in this system, leaving host identity as the main determinant of the number of strains able to coexist. This result incidentally mirrors recent work on *Trypanosoma brucei*, where the salivary glands of tsetse flies containing a mixed infection appeared to have higher parasite

densities than singly infected glands (Peacock *et al.*, 2007). Overall, initial infective dose played only a minor role in the outcome of infection for host colonies. High dose resulted in increased colony mean infection intensities but did not affect the probability of transmission to the next generation of hosts (half of the colonies that produced infected daughter queens had received a low dose).

Looking at the composition of the infecting parasite population in isolated workers, we found an excess, relative to expected frequencies, of some infection genotypes, mostly at the extremes of the diversity range (i.e. infections with either a low or high number of strains were more common than expected by chance). A number of studies suggest that in mixed infections, relatedness between co-infecting parasite genotypes reduces competition, so that multiple infections involving related strains are found more frequently (Jäger & Schjørring, 2006; Koskella *et al.*, 2006; López-Villavicencio *et al.*, 2007). Here, we did not find any evidence for an effect of genetic relatedness on the probability of association between parasite strains, although the analysis might have been confounded by the small number of markers used.

A screening of colony offspring for infections by *C. bombi* revealed that 7.2% of the daughter queens became infected within their natal colony. This approximately matches the infection rates found in the field among emerging spring queens in our study areas, which is in the order of 5–10%. We furthermore found that in the overall experiment, none of the five infecting strains was lost from the population as a whole. This maintenance of strains is consistent with the very high genetic diversity observed in the field (Schmid-Hempel & Reber Funk, 2004).

The contrast between strain representation in workers and daughter queens was striking. On one hand, this could have simply resulted from the experimental setting: although workers were simultaneously exposed to equal amounts of each strain, queens were infected 'naturally' in their natal colony. However, this difference would not necessarily explain the differences in the relative proportions of infecting strains. On the other, the difference could hint at processes capable of modulating parasite dynamics in this system, which deserves particular attention. This could involve (i) Parasitic strains that have a short-term competitive advantage in the form of a high establishment success might suffer from a long-term disadvantage as they are less often transmitted to the daughter queens and thus to the next host generation. This would constitute a trade-off between within-host growth and transmission to the next hosts. No such trade-off, however, was found in earlier studies (Schmid-Hempel & Schmid-Hempel, 1993; Schmid-Hempel *et al.*, 1999). (ii) Social insects are a textbook example of phenotypic plasticity, whereby a single genetic background gives rise to different categories of individuals that can vastly differ in morphology, fecundity and longevity. Daughter queens and workers from a colony, although they are full-sisters, are known to differ considerably in parasite resistance (Chan *et al.*, 2006; Gräff *et al.*, 2007; Schmid *et al.*, 2008). It is possible that not only their general resistance but also their relative susceptibility to different strains differs from the workers, although data to support this claim is lacking. Nevertheless, it is reasonable to assume that because daughter queens are costly and valuable, the colony is expected to protect them against parasites. This might occur for example, by immune-priming (Sadd *et al.*, 2005; Sadd & Schmid-Hempel, 2006; Sadd & Schmid-Hempel, 2007) against the most prevalent strains, although it is not yet known in bumblebees how fine-tuned the immune-priming against *C. bombi*, if any, might be.

Our results particularly show that the relevant transmission success (to daughter queens) of different strains cannot be inferred from their short-term success, i.e. from their representation in the workers. In a similar way, Peacock *et al.* (2007) found no correlation between the composition of the *T. brucei* population in successively infected compartments of its host, the tsetse fly. Additionally, in an experiment on the transmission of two co-infecting clones of *P. chabaudi* from mice to mosquitoes, Taylor *et al.* (1997) found that numerical dominance during the early stages of the infection was poorly correlated with transmission probability. Here, we have one additional insight: although colony infection intensity and strain diversity were positively correlated, the probability of infecting daughter queens was positively associated with strain diversity but not with infection intensity (Fig. 3). In other words, a high number of circulating strains at the beginning of the colony cycle made it more likely that at least one of them would infect the next generation of hosts, irrespective of both the prevalence of the different strains and the total number of infective cells present at that early stage. Furthermore, the colonies that harboured diverse infections (as assessed early in the season when all colonies had approximately the same size) and produced infected queens also produced more queens in total. This positive association between infection diversity and reproductive output has been found in other host–parasite systems (Marzal *et al.*, 2008; Ben-Ami *et al.*, 2008) and is compatible with an immunity-reproduction trade-off scenario, where hosts filter the circulating infection at the cost of their reproductive output.

The high degree of skew in reproductive output (a few colonies produced most of the daughter queens) means that a small number of host genotypes contribute to a disproportionate part of the host population in the next year. Our results now suggest that these host genotypes are also the major source of infection for other hosts in the next year (assuming a low immigration rate of

infected hosts from neighbouring populations) and can thus be viewed as 'super-spreaders' (Beldomenico & Begon, 2010).

In conclusion, the diversity of the infection circulating within a colony appeared as a crucial determinant of disease dynamics, overshadowing absolute numbers of infective cells (dose, infection intensity). The discrepancy between the short-term success of strains (as it is usually measured in such experiments) and their longer-term transmission underlines the need to consider host ecology and heterogeneity when investigating the spread of infectious diseases.

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References

Alford, D.V. 1975. *Bumblebees*. Davis-Poynter, London.

Alizon, S., Hurford, A., Mideo, N. & van Baalen, M. 2009. Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *J. Evol. Biol.* **22**: 245–259.

Babiker, H.A., Ranford-Cartwright, L.C. & Walliker, D. 1999. Genetic structure and dynamics of *Plasmodium falciparum* infections in the Kilombero region of Tanzania. *Trans. R. Soc. Trop. Med. Hyg.* **93**: 11–14.

Beldomenico, P.M. & Begon, M. 2010. Disease spread, susceptibility and infection intensity: vicious circles? *Trends Ecol. Evol.* **25**: 21–27.

Bell, A., de Roode, J., Sim, D., Read, A. & Koella, J. 2006. Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. *Evolution* **60**: 1358–1371.

Ben-Ami, F., Mouton, L. & Ebert, D. 2008. The effects of multiple infections on the expression and evolution of virulence in a *Daphnia*-endoparasite system. *Evolution* **62**: 1700–1711.

Brown, M., Loosli, R. & Schmid-Hempel, P. 2000. Condition-dependent expression of virulence in a trypanosome infecting bumblebees. *Oikos* **91**: 421.

Brown, S.P., Hochberg, M.E. & Grenfell, B.T. 2002. Does multiple infection select for raised virulence? *Trends Microbiol.* **10**: 401–405.

Brown, M., 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.

Carius, H.J., Little, T.J. & Ebert, D. 2001. Genetic variation in a host–parasite association: potential for coevolution and frequency-dependent selection. *Evolution* **55**: 1136–1145.

Chan, Q.W., Howes, C.G. & Foster, L.J. 2006. Quantitative comparison of caste differences in honeybee hemolymph. *Mol. Cell Proteomics* **5**: 2252–2262.

Durrer, S. & Schmid-Hempel, P. 1994. Shared use of flowers leads to horizontal pathogen transmission. *Proc. R. Soc. B* **258**: 299–302.

Frank, S.A. 1992. A kin selection model for the evolution of virulence. *Proc. R. Soc. B* **250**: 195–197.

Gandon, S., Mackinnon, M., Nee, S. & Read, A. 2001. Imperfect vaccines and the evolution of pathogen virulence. *Nature* **414**: 751–756.

Goldstein, D.B., Linares, A.R., Cavalli-Sforza, L.L. & Feldman, M.W. 1995. An evaluation of genetic distances for use with microsatellite loci. *Genetics* **139**: 463–471.

Gower, C.M. & Webster, J.P. 2005. Intraspecific competition and the evolution of virulence in a parasitic trematode. *Evolution* **59**: 544–553.

Gräff, J., Jemielity, S., Parker, J.D., Parker, K.M. & Keller, L. 2007. Differential gene expression between adult queens and workers in the ant *Lasius niger*. *Mol. Ecol.* **16**: 675–683.

Hodgson, D.J., Hitchman, R.B., Vanbergen, A.J., Hails, R.S., Possee, R.D. & Cory, J.S. 2004. Host ecology determines the relative fitness of virus genotypes in mixed-genotype nucleopolyhedrovirus infections. *J. Evol. Biol.* **17**: 1018–1025.

Holm, S. 1979. A simple sequentially rejective multiple test procedure. *Scand. J. Stat.* **6**: 65–70.

Imhoof, B. & Schmid-Hempel, P. 1999. Colony success of the bumble bee, Bombus terrestris, in relation to infections by two protozoan parasites, *Crithidia bombi* and *Nosema bombi*. *Insectes Soc.* **46**: 233–238.

Jäger, I. & Schjørring, S. 2006. Multiple Infections: relatedness and time between infections affect the establishment and growth of the cestode *Schistocephalus solidus* in its stickleback host. *Evolution* **60**: 616–622.

Koskella, B., Giraud, T. & Hood, M.E. 2006. Pathogen relatedness affects the prevalence of within-host competition. *Am. Nat.* **168**: 121–126.

Lambrechts, L., Fellous, S. & Koella, J. 2006. Coevolutionary interactions between host and parasite genotypes. *Trends Parasitol.* **22**: 12–16.

Little, T., Watt, K. & Ebert, D. 2006. Parasite-host specificity: experimental studies on the basis of parasite adaptation. *Evolution* **60**: 31–38.

López-Villavicencio, M., Jonot, O., Coantic, A., Hood, M.E., Enjalbert, J. & Giraud, T. 2007. Multiple infections by the anther smut pathogen are frequent and involve related strains. *PLoS Pathog.* **3**: e176.

Lord, C.C., Barnard, B., Day, K., Hargrove, J.W., McNamara, J.J., Paul, R.E.L., Trenholme, K. & Woolhouse, M.E.J. 1999. Aggregation and distribution of strains in microparasites. *Philos. Trans. R Soc. B* **354**: 799–807.

Mallon, E.B., Loosli, R. & Schmid-Hempel, P. 2003. Specific versus nonspecific immune defense in the bumblebee, *Bombus terrestris* L. *Evolution* **57**: 1444–1447.

Marzal, A., Bensch, S., Reviriego, M., Balbontin, J. & De Lope, F. 2008. Effects of malaria double infection in birds: one plus one is not two. *J. Evol. Biol.* **21**: 979–987.

May, R. & Nowak, M. 1995. Coinfection and the evolution of parasite virulence. *Proc. R. Soc. B* **261**: 209–215.

Ostrowski, E., Katoh, M., Shaulsky, G., Queller, D. & Strassmann, J. 2008. Kin discrimination increases with genetic distance in a social amoeba. *PLoS Biol.* **6**: e287.

Peacock, L., Ferris, V., Bailey, M. & Gibson, W. 2007. Dynamics of infection and competition between two strains of

Trypanosoma brucei brucei in the tsetse fly observed using fluorescent markers. *Kinetoplastid Biol. Dis.* **6**: 4.

Pomeroy, N. & Plowright, R.C. 1980. Maintenance of bumble bee colonies in observation hives (Hymenoptera: Apidae). *Can. Entomol.* **111**: 289–294.

Puustinen, S., Koskela, T. & Mutikainen, P. 2004. Relatedness affects competitive performance of a parasitic plant *Cuscuta europaea* in multiple infections. *J. Evol. Biol.* **17**: 897–903.

R Development Core Team 2008. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Read, A.F. & Taylor, L.H. 2001. The ecology of genetically diverse infections. *Science* **292**: 1099–1102.

Refardt, D. & Ebert, D. 2006. Quantitative PCR to detect, discriminate and quantify intracellular parasites in their host: an example from three microsporidians in Daphnia. *Parasitology* **133**: 11–18.

de Roode, J., Culleton, R., Cheesman, S., Carter, R. & Read, A. 2004. Host heterogeneity is a determinant of competitive exclusion or coexistence in genetically diverse malaria infections. *Proc. R. Soc. B* **271**: 1073.

Rozen, S. & Skaletsky, H. 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* **132**: 365–386.

Sadd, B. & Schmid-Hempel, P. 2006. Insect immunity shows specificity in protection upon secondary pathogen exposure. *Curr. Biol.* **16**: 1206–1210.

Sadd, B.M. & Schmid-Hempel, P. 2007. Facultative but persistent trans-generational immunity via the mother's eggs in bumblebees. *Curr. Biol.* **17**: R1046–R1047.

Sadd, B.M., Kleinlogel, Y., Schmid-Hempel, R. & Schmid-Hempel, P. 2005. Trans-generational immune priming in a social insect. *Biol. Lett.* **1**: 386–388.

Salvaudon, L., Heraudet, V. & Shykoff, J.A. 2007. Genotype-specific interactions and the trade-off between host and parasite fitness. *BMC Evol. Biol.* **7**: 189.

Schmid, M.R., Brockmann, A., Pirk, C.W.W., Stanley, D.W. & Tautz, J. 2008. Adult honeybees (*Apis mellifera* L.) abandon hemocytic, but not phenoloxidase-based immunity. *J. Insect Physiol.* **54**: 439–444.

Schmid-Hempel, P. 1998. *Parasites in Social Insects*. Princeton University Press, Princeton, NJ.

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.

Schmid-Hempel, P. & Reber Funk, C. 2004. The distribution of genotypes of the trypanosome parasite, *Crithidia bombi*, in populations of its host, *Bombus terrestris*. *Parasitology* **129**: 147–158.

Schmid-Hempel, P. & Schmid-Hempel, R. 1993. Transmission of a pathogen in *Bombus terrestris*, with a note on division of labour in social insects. *Behav. Ecol. Sociobiol.* **33**: 319–327.

Schmid-Hempel, R. & Tognazzo, M. 2010. Molecular divergence defines two distinct lineages of *Crithidia bombi* (Trypanosomatidae), parasites of bumblebees. *J. Eukaryot. Microbiol.* **57**: 337–345.

Schmid-Hempel, P., Puhr, K., Kruger, N., Reber, C. & Schmid-Hempel, R. 1999. Dynamic and genetic consequences of variation in horizontal transmission for a microparasitic infection. *Evolution* **53**: 426–434.

Schulenburg, H. & Ewbank, J.J. 2004. Diversity and specificity in the interaction between Caenorhabditis elegans and the pathogen Serratia marcescens. *BMC Evol. Biol.* **4**: 49.

Sokal, R.R. & Rohlf, F.J. 1995. *Biometry: The Principles and Practice of Statistics in Biological Research*, 3rd edn. W.H. Freeman, New York.

Taylor, L.H., Walliker, D. & Read, A.F. 1997. Mixed-genotype infections of malaria parasites: within-host dynamics and transmission success of competing clones. *Proc. R. Soc. B* **264**: 927–935.

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