# The relative contributions of host density and genetic diversity on prevalence of a multi-host parasite in bumblebees

SUSANN PARSCHE[1] and H. MICHAEL G. LATTORFF[1,2,3,*,]

[1]*Institute of Biology, Molecular Ecology, Martin-Luther University Halle-Wittenberg, Hoher Weg 4, 06099 Halle (Saale), Germany*
[2]*International Centre of Insect Physiology and Ecology (icipe), PO Box 30772-00100 Nairobi, Kenya*
[3]*German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Deutscher Platz 5e, 04103 Leipzig, Germany*

The role of population and demographic factors in influencing the transmission and establishment parasites infecting multiple hosts is poorly understood. We assessed the effects of these factors on parasite prevalence in a model system – the intestinal protozoan *Crithidia bombi* (Trypanosomatidae) infecting a range of bumblebee species (*Bombus* spp.). We used microsatellite markers and sibship reconstruction to infer genetic diversity and the density of host populations to infer their relative contributions to parasite prevalence. We established the prevalence, type (single- vs. multiple-strain infection) and intensity of *C. bombi* infections in workers and males of three common bumblebee species (*B. terrestris*, *B. lapidarius* and *B. pascuorum*) at 30 locations across Germany. We found evidence that colony density promoted prevalence, while increased genetic diversity lowered prevalence in *B. terrestris*. The effect size for genetic diversity was much larger than for colony density. Thus, genetic factors affected the prevalence, while demographic and life-history traits (e.g. population density and seasonal cycle of development) were additional factors shaping the spread and establishment of a multi-host parasite. *Bombus lapidarius* possessed characteristics that indicate it might act as a reservoir species spreading disease to other species.

ADDITIONAL KEYWORDS: *Bombus* – colony density – *Crithidia* – host–parasite interaction – host diversity – intensity of infection – multiple infections –microsatellite.

## INTRODUCTION

Most pathogens infect a range of host species and appear to be generalists rather than specialists (reviewed by Rigaud *et al.*, 2010). In recent years this issue has gained attention, as emerging infectious diseases (EIDs), threatening human as well as wild populations, often reside in reservoir host species (Woolhouse & Gowtage-Sequeria, 2005). However, classical models of host–parasite interactions have focused on single host species interacting with single parasite species. Multi-host–parasite interactions have been studied less extensively, both theoretically and empirically (Rigaud *et al.*, 2010). In light of the evolution of virulence and transmission of pathogens and the resistance and tolerance of hosts, studies of multi-host parasite systems are crucial in providing a basic understanding of the spread and persistence of diseases in order to adjust management options in these systems (Roche & Guégan, 2011).

Besides virulence, transmission to new hosts is one of the crucial factors in the life cycle of pathogens. Generally, it is assumed that the transmission of directly transmitted pathogens is density-dependent (McCallum *et al.*, 2001). Density-dependent transmission is a function of the probability of encounters of new susceptible hosts (Altizer *et al.*, 2006). Additionally, the per-contact probability of transmission, plus the removal of pathogens due to recovery of infected hosts, and the decay of infective particles within the environment will affect transmission and ultimately the prevalence of pathogens within host populations (Altizer *et al.*, 2006).

*Corresponding author. E-mail: mlattorff@icipe.org

Likewise, the genetic factors of host species might influence the transmission, establishment and reproductive rate of pathogens (King & Lively, 2012). Genetic diversity, at various levels of biological organization of the host population, from individuals, to populations and communities, influences the spread of diseases (King & Lively, 2012; Johnson *et al.*, 2013). Genetic diversity within a host population might increase the variance for susceptibility, which overall should lower the prevalence of pathogens. Clearly, multi-host parasites are confronted by various levels of genetic factors as genetic differences occur among and within host species, which should lead to more complex patterns of adaptation.

As pointed out by King & Lively (2012), the relative contributions of density-dependent and genetic effects are virtually unknown. To study these effects simultaneously, we used a well-known study system, the intestinal parasite *Crithidia bombi* infecting a wide range of bumblebee (*Bombus* spp.) host species (Sadd & Barribeau, 2013).

Bumblebees play a pivotal ecological and economic role in the effective pollination of crops (Garibaldi *et al.*, 2013) and thus provide a key ecosystem service that is vital to humans (Klein *et al.*, 2007). Bumblebee colonies are headed by a single mated queen initially raising several generations of workers before producing sexuals (males and gynes, which are unmated queens) (Sladen, 1912). Social insects such as bumblebees are threatened by spread of disease due to the high intra-colonial density of closely related individuals frequently interacting with each other (Schmid-Hempel, 1998, 2001) while interspecific interactions can occur during foraging (Durrer & Schmid-Hempel 1994). Parasites and notably EIDs (Meeus *et al.*, 2011, Fürst *et al.*, 2014) have contributed to a global decline of pollinators in recent decades (Biesmeijer *et al.*, 2006; Potts *et al.*, 2010; Cameron *et al.*, 2011).

The trypanosome *C. bombi* (Lipa & Triggiani, 1988) infects numerous bumblebee species (Shykoff & Schmid-Hempel, 1991) and appears to be widespread in natural populations infecting adults of both sexes and all castes (Shykoff & Schmid-Hempel, 1991). The coincidence of a huge number of *C. bombi* genotypes within populations, colonies and individuals has been revealed by microsatellite analyses (Schmid-Hempel & Reber Funk, 2004; Erler *et al.*, 2012; Popp *et al.*, 2012). The parasite is transmitted directly, both within colonies through contact with contaminated surfaces or infected nestmates (Schmid-Hempel & Schmid-Hempel, 1993; Otterstatter & Thomson, 2007) and among colonies (intra- and interspecific) via shared use of flowers during foraging (Durrer & Schmid-Hempel, 1994), although foraging workers might avoid contaminated flowers (Fouks & Lattorff, 2011, 2013).

As horizontal parasite transmission among colonies is dependent on the availability of new susceptible hosts and the probability of encounters, intra-colonial transmission might be a function of colony size, which clearly differs between species (Goulson, 2010; Erler *et al.*, 2012). Host species differ with respect to their preference for floral resources (Goulson & Darvill, 2004), and hence the probability of interspecific transmission differs among host species. Ruiz-González *et al.* (2012) underlined this by providing evidence of inconsistent and asymmetrical inter- and intraspecific transmission by different host species, resulting in a higher prevalence in some of the common host species (Gillespie, 2010; Ruiz-González *et al.*, 2012). Seasonal colony growth (Schmid-Hempel, 1998) and high local colony densities should increase the potential for transmission and thus enhance the risk of infection.

Organisms showing high degrees of seasonality in their growth, activity patterns or reproduction have their life cycle aligned with indicators of seasonality, often temperature. Bumblebee colonies are annual and the growth rate of a colony shows a strong correlation with season. In early spring the queen founds the colony and then produces more individuals towards early summer; these are new susceptible individuals for different diseases. Additionally, growth of colonies and hence the whole population increases the contact rate between individuals, enhancing the transmission of pathogens. These processes are strongly correlated to seasonal variations, especially temperature.

Genetic diversity affects disease spread in bumblebees at various levels of biological organization. Within-colony genetic diversity is low in natural colonies and experimentally manipulated colonies with increased genetic diversity appear to be more resistant to infections with *C. bombi* (Baer & Schmid-Hempel, 1999). Similar effects have been demonstrated at the population and species levels (Cameron *et al.*, 2011; Whitehorn *et al.*, 2011; Jones & Brown, 2014), potentially due to substantial amounts of standing genetic variation affecting *C. bombi* infections (Wilfert *et al.*, 2007). Genetic effects at the individual or colony level might be an expression of differences in gut microbiota, which have been shown to greatly influence interactions with *C. bombi* (Koch & Schmid-Hempel, 2012).

The clearest genetic difference is found between sexes due to the haplodiploid sex-determination system in Hymenoptera (Cook, 1993). Haploid males might face a higher infection risk due to the lack of allelic diversity and the inability to compensate for deleterious alleles, also known as the haploid-susceptibility hypothesis (O'Donnell & Beshers, 2004).

In order to disentangle the relative contributions of various genetic, density-dependent and environmental effects, we conducted an extensive field study to

simultaneously identify potential factors contributing to the epidemiology of *C. bombi*. We measured epidemiological characters such as prevalence (proportion of infected bumblebees), type (single- vs. multiple-strain infection) and intensity (mean number of parasite cells per host) of infection and tested for the influence of genetic, density-dependent and seasonal factors.

We predicted that higher genetic diversity would reduce prevalence, whereas density-dependent effects would increase prevalence, measured at different biological scales.

## MATERIAL AND METHODS

### SAMPLING

Workers (*N* = 1847) and males (*N* = 422) of three bumblebee species [*Bombus terrestris* (*N* = 861), *B. lapidarius* (*N* = 1030), *B. pascuorum* (*N* = 378)] were collected during foraging flights, in semi-natural and agricultural habitats (hedgerows, urban parks, grassland, field margins, fallow land) along a west–east transect with ten sites (max. distance: 311 km) across Germany, each with three locations (Supporting Information, Table S1). The mean distance between locations was 5.2 ± 2.4 km (mean ± SD), exceeding the expected foraging ranges of different bumblebee species (Goulson, 2010). Each location was sampled in a random order three times a year (June, July and August 2010). Time of day was randomized to reduce biased data. Individuals were stored at –20 °C prior to DNA extraction. After initial species identification in the field, individuals were analysed for sex and species identity following the taxonomic key of Mauss (1994).

### DNA ANALYSIS

DNA was extracted from a single leg per individual following a modified Chelex protocol (Walsh *et al.*, 1991; Erler & Lattorff, 2010). Workers were genotyped at eight highly variable microsatellite loci (B11, B96, B124, B126: Estoup *et al.*, 1995, 1996; and BTMS0043, BTMS0045, BTMS0057, SSR0154_56i12: Stolle *et al.*, 2009; 2011; for *B. pascuorum*: SSR155 instead of BTMS0045: Stolle *et al.*, 2009). Loci were amplified in multiplex PCRs with each reaction containing 1 μL template DNA, 5 μL PCR Master Mix (Promega, Madison, WI, USA), 0.4–0.75 μM per primer pair and in a final volume of 10 μL. The thermal profile of the PCR followed the protocol of Erler & Lattorff (2010).

Novel primers for the unambiguous discrimination of *B. terrestris* and *B. lucorum*, which show high levels of morphological resemblance (Wolf *et al.*, 2010), were designed (Appendix S1). Forward primers were labelled with different fluorescent dyes (Metabion International AG, Martinsried, Germany)

and included in the multiplex PCR. The amplified fragments were visualized using an automated DNA capillary sequencer (MegaBACE 1000, GE Healthcare, Munich, Germany) according to the manufacturer's instructions and using a standard protocol (Erler & Lattorff, 2010). Allele sizes were scored with the software MegaBACE Fragment Profiler v1.2 after visual inspection of processed raw data.

To genotype *C. bombi*, the gut of each bee was removed and DNA extraction was done according to the aforementioned Chelex protocol (Walsh *et al.*, 1991; Erler & Lattorff, 2010). Four polymorphic microsatellite loci were genotyped (Cri 4, Cri 1.B6, Cri 4.G9 and Cri 2.F10; Schmid-Hempel & Reber Funk, 2004). All loci were amplified in one multiplex PCR following the protocol of Popp & Lattorff (2011). The final volume of 10 μL contained 1 μL template DNA, 5 μL PCR Master Mix (Promega), 0.3 μM (Cri 1.B6, Cri 4.G9), 0.6 μM (Cri 4, Cri 2.F10) per primer pair and 2.2 μL double distilled $H_2O$. PCR products were run on a MegaBACE 1000 system (GE Healthcare) and fragments were sized using Fragment Profiler v1.2. As *C. bombi* is a diploid organism (Schmid-Hempel & Reber Funk, 2004), more than two peaks per locus indicate an infection of the individual host with more than one strain (i.e. multiple infection). Due to the strong relationship between peak height and the mean number of *C. bombi* cells per host (Fouks & Lattorff 2014), we estimated the intensity of infection on the basis of Cri 1.B6 and Cri 4.G9 peak heights following the 'microsatellite method' of Fouks & Lattorff (2014). Briefly, peak heights of two microsatellite markers are used as an indicator of the amount of the initial template DNA along with a standard curve based on purified *C. bombi*. Such methods have been used in other contexts and proven to be reliable as positive correlations between increasing DNA amount and corresponding peak heights have been shown (Moritz *et al.*, 2003; Schulte *et al.*, 2011).

### KINSHIP RECONSTRUCTION AND POPULATION GENETICS

As the colony represents the genetically relevant unit in social insects, it is crucial to identify kinship relationships of the sampled foraging bumblebees, thus enabling estimation of both colony density and population genetic metrics. COLONY v2.0.5.0 (Wang, 2004; Jones & Wang, 2010) was used to assign workers to matrilines according to their individual genotypes and the overall allele frequencies in the sample. COLONY is thought to be the most accurate software in assigning colonies, but reconstruction of multi-locus queen genotypes is as poor as in all other available software packages (Lepais *et al.*, 2010). Two replicate COLONY runs per location and species, each with a different random number

seed, were conducted using the full-likelihood method. Locations with fewer than ten genotyped workers per species were excluded (final sample size: *B. terrestris*, 17; *B. lapidarius*, 20; *B. pascuorum*, 14; Tables S1 and S4). Error rates for allelic dropouts and other genotyping errors were set to 0.05 for all loci. Hence, we calculated all population genetic parameters on the basis of real genotypes of the sampled workers using one randomly selected representative per reconstructed colony. The number of alleles ($A_N$) as well as the observed and expected heterozygosities ($H_O$, $H_E$) were obtained using the Excel Microsatellite Toolkit (Park, 2001). To account for finite sample sizes, the non-sampling error (NSE) was calculated using the mark-recapture software Capwire (Miller *et al.*, 2005). Capwire allows for multiple sampling of an individual (or full-sib) and proved to be useful for estimating the number of colonies (e.g. Goulson *et al.*, 2010). We ran the likelihood ratio test (LRT) to identify the best model (Table S5), and either the Even Capture Model (ECM) or the Two Innate Rate Model (TIRM) per location and species (Miller *et al.*, 2005). The ECM assumes an equal probability, while the TIRM is based on a heterogeneous probability for capture of individuals. The ECM predominantly provided the better fit to our data. Therefore, those estimates were used for further analyses. All results, including the associated colony density estimates, are shown in Table S5.

### STATISTICAL ANALYSIS

Generalized Linear Mixed Models (GLMMs) based on individual data were used to test for the effects of species identity, sex, sampling period (SP) and their interactions on the prevalence and the type (single- vs. multiple-strain infection) of *C. bombi* infection (Table S2). As both response variables are binary, modelling was done with a binomial error distribution and the logit link function. For the main analyses (using workers only), colony ID was treated as a random effect and nested within location and within site. Testing for sex-related differences was done excluding the first sampling period, because production of males started later. Sex, species ID, sampling period and their interactions were included as fixed factors. Colony ID was not used as a random effect because males were not assigned to colonies.

The *dredge* function implemented in the R package MuMIn 1.9.5 (Bartoń, 2013) was used to identify the best subset of fixed effects based on the full model. The best models, ranked by Aikake's Information Criterion (AIC), are provided in Table S3. The final model was compared to the null model (without fixed effects) using standard maximum likelihood (ML) for parameter estimation and subsequently fitted with REML (restricted maximum likelihood) via Laplace approximation (Bolker *et al.*, 2009). Goodness-of-fit ($R^2$) of the final model was calculated using *r.squaredGLMM* (MuMIn 1.9.5). Marginal $R^2$ ($R^2_{GLMM(m)}$: variance explained by fixed effects) as well as conditional $R^2$ ($R^2_{GLMM(c)}$: variance explained by both fixed and random effects) are provided (Nakagawa & Schielzeth, 2013). In case of significant fixed effects, Tukey's honest significant difference (HSD) post-hoc tests were used to test for significant differences between factors simultaneously correcting for multiple comparisons.

For the analysis of intensity of the *C. bombi* infection, Linear Mixed Models (LMMs) were used. The random effects structure and the model selection procedure remained the same as described above, but $R^2$ was calculated using *r.squaredLR*. All analyses were performed using R 2.15.3 (R Core Team, 2013) and the packages lme4 (v0.999999-2, function *lmer*; Bates *et al.*, 2013), MuMIn 1.9.5 (Bartoń, 2013) and multcomp (Hothorn *et al.*, 2008).

Finally, multiple regression analyses were used to understand the relationship between colony density and genetic diversity as predictors of the prevalence and infection intensity of *C. bombi*, respectively. Analyses were performed using data for *B. terrestris* and *B. lapidarius*. The colony density data were derived using the non-sampling error obtained from the ECM method in Capwire (Miller *et al.*, 2005) and the species-specific flight ranges of workers reported by Knight *et al.* (2005). Expected heterozygosity ($H_E$) served as a proxy for genetic diversity. Using linear models, F-tests were carried out to assess the significance of regression coefficients (R packages MASS 7.3-23, Venables & Ripley, 2002; and car 2.0-16, Fox & Weisberg, 2011).

## RESULTS

### INFECTION WITH *CRITHIDIA BOMBI*

In total, 2269 individuals of *B. terrestris* [$N$ = 861, infected 21.95%, 95% confidence interval (CI) 19.23–24.87%], *B. lapidarius* ($N$ = 1030, infected 16.99%, 95% CI 14.75–19.43%) and *B. pascuorum* ($N$ = 378, infected 10.85%, 95% CI 7.9–14.43%) were included in the analyses (Table S1). In total, 405 bumblebees were infected (single/multiple infection: $N$ = 266/139).

Here we present the results of the final models (i.e. the minimal adequate model) compared to null models using LRTs and an overview of the contribution of each term (Table 1) including results of Tukey's HSD post-hoc tests. A summary of the entire model selection procedure and associated statistics is given in Table S3.

### SPECIES IDENTITY AND PREVALENCE OF *C. BOMBI*

Species, sampling period and their interaction strongly influenced the prevalence of *C. bombi* (LRT: $\chi^2$ = 105.25, d.f. = 12, $P$ < 0.0001, $R^2_{GLMM(m/c)}$ = 0.16/0.34;

**Table 1.** Results of (Generalized) Linear Mixed Models of *Crithidia bombi* prevalence, type [single vs. multiple strain(s)] and intensity of infection

| Analysis | Predictor variables* | Prevalence† | | | Type†‡ | | | Intensity§ | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | d.f. | $\chi^2$ | $P$ | d.f. | $\chi^2$ | $P$ | d.f. | $\chi^2$ | $P$ |
| (a) Females only | species: SP | 8 | 19.58 | **0.0006** | 7 | 15.67 | **0.0004** | – | – | – |
| | species | 2 | 14.62 | **0.0007** | 2 | 1.46 | 0.483 | – | – | – |
| | SP | 2 | 55.63 | **<0.0001** | 1 | 1.62 | 0.203 | – | – | – |
| (b) Comparison of sexes | sex: SP | 5 | 5.03 | **0.025** | – | – | – | – | – | – |
| | species: SP | – | – | – | – | – | – | 7 | 5.31 | 0.070 |
| | sex | 1 | 2.63 | 0.105 | – | – | – | – | – | – |
| | species | – | – | – | – | – | – | 2 | 8.73 | **0.013** |
| | SP | 1 | 29.95 | **<0.0001** | – | – | – | 1 | 2.01 | 0.157 |

SP, sampling period; significant results are highlighted in bold type. *P*-values were calculated from likelihood ratio tests following stepwise term removal from final models: *fixed effects, †GLMMs, ‡LMMs.
§To ensure model convergence the third sampling period was excluded.
A dash indicates that terms were not included in the final model/the final model did not contain an interaction term.

Fig. 1, Table 1). All pairwise comparisons of species revealed significant differences, with *B. terrestris* showing the largest proportion of infected individuals (overall mean: 21.9%) compared to *B. lapidarius* (16.9%; Tukey's test: $z = 2.968$, $P = 0.008$) and *B. pascuorum* (10.8%; Tukey's test: $z = 5.100$, $P < 0.001$). *Crithidia bombi* was also significantly more prevalent in *B. lapidarius* than in *B. pascuorum* (Tukey's test: $z = 3.375$, $P = 0.002$). Significantly more individuals were infected in June (overall mean: 29.9%) than in July (15.4%; Tukey's test: $z = 5.904$, $P < 0.001$, Fig. 1A) and August (3.9%; Tukey's test: $z = 3.045$, $P = 0.005$; Fig. 1A). Five out of 18 possible interaction terms were significant (Fig. 1).

Species, sampling period and their interaction had a significant effect on the type of infection (LRT: $\chi^2 = 19.37$, d.f. = 9, $P = 0.002$, $R^2_{GLMM(m/c)} = 0.09/0.26$; Table 1). The occurrence of multiple-strain infections was higher in *B. terrestris* than in *B. lapidarius* (Tukey's test: $z = 2.698$, $P = 0.016$) and was marginally higher than in *B. pascuorum* ($z = 2.030$, $P = 0.093$). Additionally, more multiple-strain infections were found in June than in July ($z = 3.372$, $P = 0.0007$).

The intensity of infection differed between sampling periods (LRT: $\chi^2 = 6.28$, d.f. = 7, $P = 0.043$, $R^2_{LR} = 0.08$) with fewer infected individuals in July than in June (Tukey's test: $z = -2.382$, $P = 0.040$).

TESTING THE HAPLOID-SUSCEPTIBILITY HYPOTHESIS

Sex, sampling period and their interaction influenced prevalence (LRT: $\chi^2 = 43.39$, d.f. = 6, $P < 0.0001$, $R^2_{GLMM(m)/(c)} = 0.11/0.27$; Table 1). More females than males were infected (Tukey's test: $z = 2.359$, $P = 0.018$; means: 14.5% and 5.9%, respectively; Fig. 1bB). The prevalence of *C. bombi* was considerably higher in July

than in August (Tukey's test: $z = 4.720$, $P < 0.0001$; Fig. 1B). One out of four possible interaction terms was significant (Fig. 1).

No sex-specific differences in the distribution of single vs. multiple infections were found. The final model including species was marginally, but not significantly, better than the null model (LRT: $\chi^2 = 4.62$, d.f. = 5, $P = 0.099$, $R^2_{GLMM(m)/(c)} = 0.04$). Species, sampling period and their interaction, but not sex, affected the intensity of infection (LRT: $\chi^2 = 14.88$, d.f. = 9, $P = 0.011$, $R^2_{LR} = 0.09$; Table 1).

COLONY DENSITY AND GENETIC DIVERSITY AFFECTING PREVALENCE

Locations with at least ten genotyped workers were included in the population analyses (see Table S4 for results at the location level). Based on 1642 genotyped workers of *B. terrestris* ($N = 605$), *B. lapidarius* ($N = 750$) and *B. pascuorum* ($N = 287$), 396, 362, and 123 colonies were reconstructed, respectively. All microsatellites were highly polymorphic in *B. terrestris* and *B. lapidarius* (except for BTMS0043 in *B. lapidarius* which was excluded) with an average of $9.33 \pm 2.80$ and $8.06 \pm 2.87$ alleles over all loci (means ± SD over all locations; Table 2), respectively. In *B. pascuorum*, an average of $4.01 \pm 1.95$ alleles was found. Observed and expected heterozygosities ($H_O$, $H_E$; overall means ± SD) were higher in *B. terrestris* and *B. lapidarius* ($H_O$: $0.75 \pm 0.04$ and $0.69 \pm 0.05$; $H_E$: $0.82 \pm 0.04$ and $0.79 \pm 0.05$, respectively; Table 2, Table S4) compared to *B. pascuorum* ($H_O$: $0.57 \pm 0.08$; $H_E$: $0.62 \pm 0.10$; Table 2). The data sets for *B. terrestris* and *B. lapidarius* were similar in terms of sample size and the overall distribution of infected individuals per location (Tables S1 and S4). To enhance the power
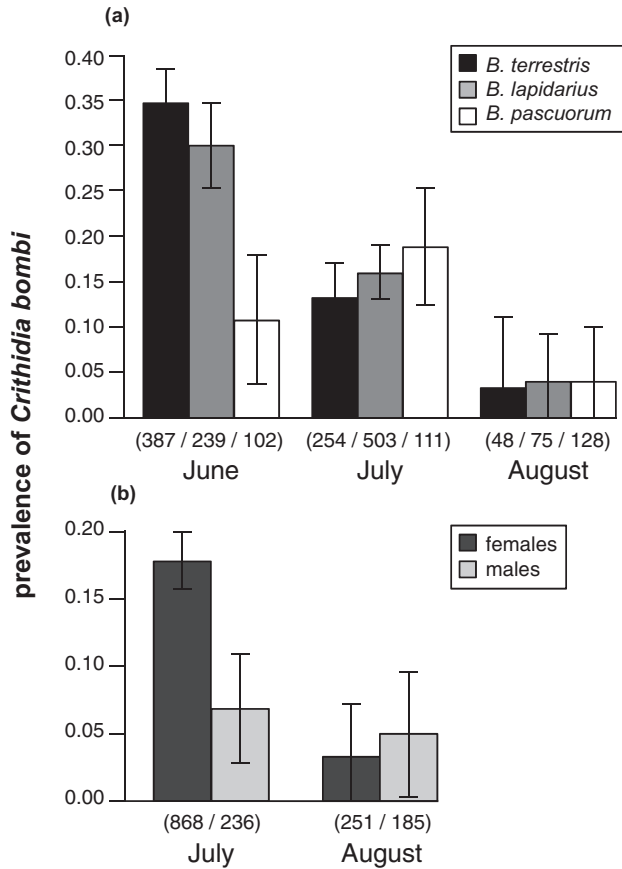
**Figure 1.** Results of Tukey's HSD post-hoc tests for the prevalence of *Crithidia bombi*: A, main analysis (females only); B, comparison of sexes. Means ± SE are shown (*N* = 30 locations; individual sample sizes are given in parentheses). Te, *B. terrestris*; La, *B. lapidarius*; Pas, *B. pascuorum*; f, females; m, males. *P*-values of interaction terms (significant values in bold) including direction of effect – A: TeJune > TeJuly *P* < **0.01**, TeJune > TeAug *P* = **0.044**, LaJune > LaJuly *P* = **0.031**, TeJune > LaJune *P* = 0.057, TeJune > PasJune *P* < **0.01**, LaJune > PasJune *P* = **0.015**; B: fJuly > fAug *P* < **0.001**, fJuly > mJuly P = 0.079.

of analyses we excluded the smaller data set for *B. pascuorum* from subsequent analyses.

In *B. terrestris* (overall effects: $F_{2,14}$ = 11.93, *P* = 0.0009, $R^2_{adjusted}$ = 0.58; Table 3) high colony density was associated with a high prevalence of infection (*t* = 3.843, *P* = 0.002; Fig. 2A). Higher genetic diversity was related to a lower prevalence (*t* = −4.502, *P* = 0.0005; Fig. 2C). In *B. lapidarius* (overall effects: $F_{2,17}$ = 1.49, *P* = 0.253, $R^2_{adjusted}$ = 0.05; Table 3), neither colony density (*t* = 0.522, *P* = 0.608; Fig. 2B) nor genetic diversity (*t* = 0.975, *P* = 0.343; Fig. 2D) was significantly associated with prevalence. However, the association between genetic diversity and prevalence showed opposite directions for the two species and these effects were significantly different (*P* = 0.002).

For intensity of infection we found no significant effects (overall effects – *B. terrestris*: $F_{2,13}$ = 2.45, *P* = 0.125, $R^2_{adjusted}$ = 0.16; *B. lapidarius*: $F_{2,17}$ = 0.59, *P* = 0.566, $R^2_{adjusted}$ = −0.05; Table 3).

## DISCUSSION

We found pronounced differences of *C. bombi* infections in natural bumblebee populations, with host species, sampling period and sex emerging as significant predictors of disease dynamics. Colony density was positively associated with infection prevalence, whereas genetic diversity was negatively related to prevalence in *B. terrestris*, while for *B. lapidarius* no association was found.

### SPECIES DIFFERENCES IN PREVALENCE

Species-specific differences in *C. bombi* prevalence have been reported previously (Shykoff & Schmid-Hempel, 1991) with higher prevalence in the more common species (Gillespie, 2010; Ruiz-González *et al.*, 2012), which is likely to be due to their higher probability of encountering parasites (Ebert 2008).

**Table 2.** Summary of sampling data and derived genetic parameters per species, based on the female (i.e. worker) genotypes

| Species (number of locations) | Σ Genotyped workers | Colonies observed (NSE*) | Colony density† (km²) | $H_O$ | $H_E$ | $A_N$ |
|---|---|---|---|---|---|---|
| *B. terrestris* (17) | 605 | 396 (442) | 27.38 ± 16.23 | 0.75 ± 0.04 [a] | 0.82 ± 0.04 [a] | 9.44 ± 2.84 |
| *B. lapidarius* (20) | 750 | 362 (125) | 40.45 ± 13.84 | 0.69 ± 0.05 [a] | 0.79 ± 0.05 [a, b] | 8.10 ± 2.92 |
| *B. pascuorum* (19) | 293 | 140 (30) | 14.18 ± 5.64 | 0.62 ± 0.10 [b] | 0.56 ± 0.09 [b] | 3.78 ± 1.68 |

Only locations with at least ten genotyped workers are included. $H_O$/$H_E$ = observed/expected heterozygosity, $A_N$ = number of alleles over all loci. Means ± SD are shown. Differences in $H_O$ and $H_E$ were calculated using the Mann–Whitney U-test over loci and different letters indicate a significant difference at the 0.05 level.
*Non-sampling error = number of non-detected colonies (over all locations) based on the ECM method implemented in Capwire (Miller *et al.* 2005).
†Estimated colony density (km²) derived from the NSE and species-specific flight ranges of workers (*B. terrestris*: 758 m, *B. lapidarius*: 450 m, *B. pascuorum*: 449 m; Knight *et al.* 2005).

**Table 3.** Results of multiple regressions on the prevalence and intensity of *Crithidia bombi* infection

| Species | C | Estimate ± SE | $t$-value | $R^2$ | $P$ | $R^2$ | adj_$R^2$ | $F$ | d.f. | $P$ |
|---|---|---|---|---|---|---|---|---|---|---|
| **Prevalence** | | | | | | | | | | |
| Te | cd | 38.72 ± 10.07 | 3.843 | 0.22 | **0.002** | 0.63 | 0.58 | 11.93 | 2, 14 | **0.0009** |
| | $H_E$ | −342.93 ± 76.17 | −4.502 | 0.41 | **0.0005** | | | | | |
| La | cd | 9.34 ± 17.88 | 0.522 | 0.05 | 0.608 | 0.15 | 0.05 | 1.49 | 2, 17 | 0.253 |
| | $H_E$ | 43.96 ± 45.10 | 0.975 | −0.12 | 0.343 | | | | | |
| **Intensity** | | | | | | | | | | |
| Te | cd | 2.65 ± 1.70 | 1.559 | 0.07 | 0.143 | 0.27 | 0.16 | 2.45 | 2, 13 | 0.125 |
| | $H_E$ | −23.21 ± 11.11 | −2.089 | 0.20 | 0.057 | | | | | |
| La | cd | 1.50 ± 3.09 | 0.484 | −0.01 | 0.634 | 0.06 | −0.05 | 0.59 | 2, 17 | 0.566 |
| | $H_E$ | −8.30 ± 7.81 | −1.063 | 0.07 | 0.303 | | | | | |

Te = *B. terrestris* ($N$ = 17 locations), La = *B. lapidarius* ($N$ = 20 locations), C = coefficient, cd = colony density ($\log_{10}$), $H_E$ = expected heterozygosity, $R^2$/adj_$R^2$ = coefficient/adjusted coefficient of determination. Significant results are highlighted in bold type.
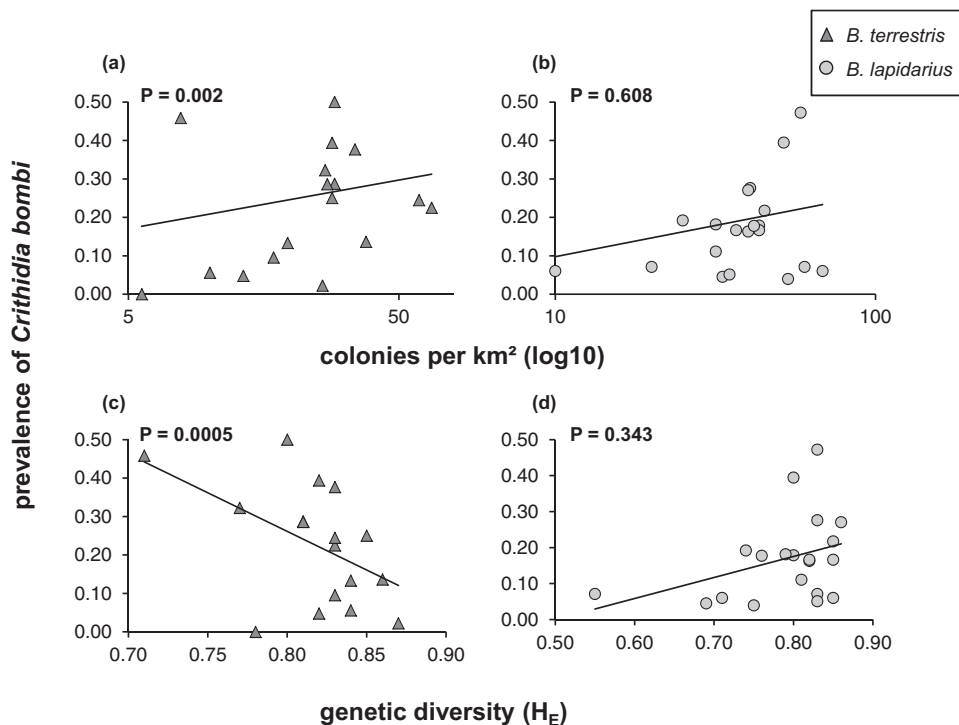


**Figure 2.** Prevalence of *Crithidia bombi* in *Bombus terrestris* and *Bombus lapidarius* ($N$ = 17/20 locations) in relation to (A, B) colony density (*x*-axes are $\log_{10}$-transformed) and (C, D) genetic diversity ($H_E$). Regression lines with associated *P*-values are derived from multiple regressions (Table 3).

Host species-specific conditions of parasite growth rate and transmission efficacy (Ruiz-González *et al.*, 2012) might influence parasite distribution. Salathé & Schmid-Hempel (2011) tested whether ecological factors (resource overlap) or genetic factors (host species) are good predictors of host–parasite associations in the *Bombus/Crithidia* system. In regions of high prevalence of the parasite, the two factors contributed equally, whereas in regions of low prevalence ecological factors were more important (Salathé & Schmid-Hempel, 2011). This indicates that ecological factors play a major role, but that genetic factors additionally can have a substantial impact.

Differences among host species might reflect the differences in host susceptibility, but also host competence (Johnson *et al.* 2013). The study of Ruiz-González *et al.* (2012) indicated that host species are a major driver, and that the probability of self-infection

was highest for *B. lapidarius*. This species plays a key role as it served as a major infection source for the other host species (Ruiz-González *et al.*, 2012). Furthermore, it has been shown that *B. lapidarius* has a three- to four-fold smaller effective population size, reducing the ability to effectively adapt to environmental challenges such as pathogens (Lattorff *et al.*, 2016). *Bombus lapidarius* was abundant throughout our sampling locations and exhibits a high level of homogeneity in parasite prevalence across locations, although its range of colony density and genetic diversity is larger than for *B. terrestris*. This corroborates the role of *B. lapidarius* as a disease reservoir.

### SEX-SPECIFIC DIFFERENCES – THE HAPLOID-SUSCEPTIBILITY HYPOTHESIS

We found sex-specific differences, with 14.5% of the workers but only 5.9% of the males being infected with *C. bombi*. These findings contradict the haploid-susceptibility hypothesis, which predicts a larger infection risk for males due to their lack of allelic variability at the individual level (O'Donnell & Beshers, 2004). While parasitism does not always differ between sexes (Ruiz-González & Brown, 2006; Gillespie, 2010), Murray *et al.* (2013) showed that males of *B. terrestris*, commercially used in glasshouses, were more likely to harbour *Crithidia* infections. Nonetheless, our results are in accordance with those of Shykoff & Schmid-Hempel (1991) who observed *C. bombi* prevalences of 39.6% and 26.3% in workers and males, respectively. Ruiz-González & Brown (2006) also found no empirical support for the haploid-susceptibility hypothesis, in fact showing the opposite, i.e. that males were less susceptible and less likely to become infected. The reverse pattern, a higher prevalence in males, was found for another parasite, *Nosema bombi*, a harmful microsporidian, in natural bumblebee populations (Shykoff & Schmid-Hempel, 1991; Gillespie, 2010; Huth-Schwarz *et al.*, 2012). One explanation for such opposing sex-specific prevalences of the two parasites might be the reduced activity of workers infected with *N. bombi* (reviewed by Shykoff & Schmid-Hempel, 1991) causing a male-biased sample (Murray *et al.*, 2013) as males always leave the nest within a few days after eclosion (Sladen, 1912; Goulson, 2010). By contrast, workers infected with *C. bombi* continue foraging, although their flower visitation rate declines with rising infection intensity due to an increasing time needed to handle flowers (Otterstatter *et al.*, 2005). Hence, rather than ploidy, the sex-specific life-history differences of bumblebees (Shykoff & Schmid-Hempel, 1991) combined with the parasites' adaptation to more frequently encountered female hosts (Ruiz-González & Brown, 2006) may explain the prevalence of *C. bombi*.

### COLONY DENSITY AND GENETIC DIVERSITY

We detected a positive association between colony density and *C. bombi* prevalence in *B. terrestris*. A high density of suitable hosts at a given location may facilitate the transmission of *C. bombi*, perhaps due to enhanced contact at flowers during foraging (Durrer & Schmid-Hempel, 1994), although bumblebees are able to avoid flowers contaminated with *C. bombi* (Fouks & Lattorff, 2011, 2013). This agrees with theory predicting a density-dependent process for directly transmitted pathogens (McCallum *et al.*, 2001). Our study indicated that higher genetic diversity was associated with lower *C. bombi* prevalence in *B. terrestris*. This agrees with other studies reporting on similar patterns in *B. muscuorum* infected with *C. bombi* (Whitehorn *et al.*, 2011) and with *Locustacarus buchneri* (Whitehorn *et al.*, 2014). Together these studies provide evidence for positive effects of host genetic heterogeneity resulting in variation in susceptibility to infection (Sherman *et al.* 1988), which is expected to lower prevalence.

The parallel assessment of genetic diversity and colony density allowed us to disentangle their relative contributions in this system. The effect of genetic diversity influencing prevalence was much stronger than for colony density. From this we assume that adaptation of a multi-host parasite towards the different host species identity is a larger challenge than ensuring transmission. This is supported by the fact that host species identity was an important factor in areas of high prevalence, explaining bumblebee infection with *Crithidia* (Salathé & Schmid-Hempel, 2011). It is also in agreement with several studies on different animal systems showing that genetic diversity protects individuals, populations and communities from the spread of disease (King & Lively, 2012; Johnson *et al.*, 2013).

## CONCLUSION

This study provides important insights into key factors – host diversity, host density and host identity – and their relative contributions to disease prevalence of a widespread multi-host parasite in bumblebees. We show that genetic factors, both among and within species, differ, and thus result in decreased parasite prevalence when genetic diversity is high. Density-dependent effects promote parasite transmission, but with a much smaller effect than diversity. For *B. lapidaries*, associations between genetic diversity and parasite prevalence were not detectable and density-dependent effects were low, supporting the role of this species as a disease reservoir.

Controlled experiments would help to disentangle the exact contribution of density- and diversity-mediated

effects (Johnson *et al.*, 2013). The management of multi-host parasites (Salkeld *et al.*, 2013; Streicker *et al.*, 2013) will benefit from detailed knowledge of local species communities including the identification of key hosts dominating interspecific transmission.

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### AUTHOR CONTRIBUTIONS

SP and HMGL designed the study. SP collected data, performed statistical analyses, wrote the initial draft, and revised the manuscript. HMGL designed novel primers and contributed substantially to the data analysis and the final version of the manuscript.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web site.

**Appendix S1.** Molecular discrimination of *B. terrestris* and *B. lucorum*.
**Table S1.** Sampling overview.
**Table S2.** Variables used in the data analyses of *C. bombi* infection.
**Table S3.** Summary of model selection statistics.
**Table S4.** Summary of genotyped individuals per species and location.
**Table S5.** Estimated colony density for *B. terrestris*, *B. lapidarius* and *B. pascuorum* per location