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RESEARCH ARTICLE

Newly emerged bumblebees are highly susceptible to gut parasite infection

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Abstract

One factor that can affect infection susceptibility is host age, the effects of which vary in a range of ways. For example, susceptibility may increase with age, due to senescence or decrease with age as a result of maturation of the immune system. If certain ages are more susceptible to infection, populations with contrasting demographics, such as same-age cohorts versus a mixture of ages, will exhibit differing disease prevalence. We use the bumblebee, *Bombus terrestris*, and its interaction with the gut trypanosome *Crithidia sp.* as a model system to investigate age-related susceptibility in a social insect. *Crithidia sp.* are widespread and prevalent parasites of bumblebees that are spread between colonies via faeces on flowers when foraging, and within colonies via contact with infected bees and contaminated surfaces and resources. In the field, *Bombus* spp. live for approximately three weeks. Here, we inoculated bumblebees at 0, 7, 14 and 21 days of age and measured their infection after one week. We also measured the level of gene expression of two antimicrobial peptides important in the defence against *Crithidia bombi* in bumblebees. We found that younger bumblebees are more susceptible to infection by *Crithidia sp.* than their older siblings. Specifically, individuals inoculated on their first day of emergence had infection intensities seven days later that were four-fold higher than bees inoculated at 21 days of age. In contrast, the gene expression of two AMPs known to protect against the trypanosome, abaecin and defensin, did not significantly vary with age. These results suggest that age does affect susceptibility to *Crithidia sp.* infection in *B. terrestris.* The higher susceptibility of callows may have implications for the susceptibility of colonies at different stages of their lifecycle, due to the contrasting age demography of workers in the colony.

Keywords Bumblebee · Epidemiology · Age · Demography · Parasite · Immune system.

Introduction

Susceptibility to parasite infection can vary with age, as has been observed across a wide variety of animal taxa, including invertebrates (reviewed by Ben-Ami 2019). Infection susceptibility can vary with age in a variety of ways, for example susceptibility may increase, decrease or follow cyclic patterns with age. One factor that can increase susceptibility to infection with age is senescence, which can be defined as the increased rate of mortality with age due to a decline in an organism's functioning (Kirkwood and Holliday 1979; Stanley 2012). Senescence can impact infection susceptibility by a fall in the efficacy of the immune system, known as immunosenescence, which occurs in many organisms (reviewed by Peters et al. 2019), including well-studied groups like humans (e.g. Beharka et al. 2001; Goldstein 2012) and birds (Haussmann et al. 2005). Although less studied, there is also increasing evidence that immunosenescence occurs in invertebrates (reviewed by Stanley 2012). For example, older crickets, *Gryllus assimilis*, have increased susceptibility to infection and lower haemocyte counts (Park et al., 2011). Furthermore, susceptibility to infection in red flour beetles (Khan et al., 2016) and honeybees (Amdam et al., 2005; Roberts and Hughes, 2014) has been shown to rise with age.

Alternatively, young organisms may be more susceptible to parasite infection and susceptibility to infection may decrease with age. This is observed for some infections in humans, for example children are more likely to develop an infection when exposed to both malaria (Baird 1998) and cholera (Deen et al., 2008). In some cases,

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higher susceptibility to infection in young organisms can be explained by the development of the immune system. For example, newborn babies are more susceptible to some infections because adaptive components of the immune system, such as the complement, have not yet fully established (McGreal et al. 2012; Kollmann et al., 2017). On the other hand, susceptibility may not follow a linear pattern with age, rather hosts may exhibit periodic increases in infection susceptibility. This can be seen in some invertebrates that are more susceptible to infection following moulting (e.g. Corteel et al. 2009).

The effect of age on host susceptibility to infection may lead to population-level effects. Host populations often consist of a wide variety of different ages, the structure of which is spatially and temporally dynamic. If certain ages are more susceptible to infection, populations with contrasting demographics, such as same-age cohorts versus a mixture of ages, will exhibit differing disease prevalence (Ben-Ami 2019). Thus, the relationship between age and disease susceptibility is critical for understanding the dynamics of parasite transmission in a host population, which can help predict the trajectory and impact of a disease (e.g. Woolhouse and Hargrove 1998).

We use the bumblebee, *Bombus terrestris*, and its interaction with the gut trypanosome *Crithidia sp.* as a model system to investigate age-related susceptibility in a social insect. *Bombus terrestris* are annual, eusocial insects and colonies consist of one singly-mated queen and her offspring (Schmid-Hempel and Schmid-Hempel 2000). Workers help raise their sisters through brood care, foraging and guarding the nest and therefore, their fitness depends upon the success of the colony. In the field, *Bombus* spp. workers live for approximately three weeks (Brian 1952; Rodd et al. 1980; Cartar 1992). *Crithidia bombi*, a highly prevalent parasite of bumblebees (Shykoff and Schmid-Hempel 1991a; Rutrecht and Brown 2008; Gillespie 2010; Popp et al. 2012), is transmitted between colonies via faeces on flowers (Durrer and Schmid-Hempel 1994; Graystock et al. 2015; Adler et al. 2018; Figueroa et al. 2019; Pinilla-Gallego et al. 2022) and within colonies through contact with infected individuals and contaminated nest material (Schmid-Hempel and Schmid-Hempel 1993; Otterstatter and Thomson 2007; Sah et al. 2021).

There is some evidence that populations with a higher mean age exhibit increased *C. bombi* prevalence (Whitehorn et al. 2011). However, age was not directly manipulated in this study, rather wing wear was used as a proxy for age. This is problematic as wing wear is confounded by activity levels, with individuals partaking in more foraging exhibiting increased wing wear (Foster and Cartar 2011). However, if older individuals are indeed more susceptible to *C. bombi*, this may be explained by immunosenescence. Doums et al. (2002) found a reduction in melanisation and encapsulation of a foreign object in older bumblebee workers and phenoloxidase activity has been shown to decline with age (Whitehorn et al. 2011). While initial work suggested that *C. bombi* might elicit phenoloxidase activity (Brown et al. 2003), since then studies have shown that anti-microbial peptide (AMP) genes expression is upregulated in response to *C. bombi* infection (Riddell et al. 2011; Brunner et al. 2013). These genes are involved in the protection against *C. bombi* as when AMPs were knocked out bumblebees exhibited higher susceptibily to *C. bombi* infection (Deshwal and Mallon 2014). Whether the expression of these immune genes declines with age remains unknown. Alternatively, younger individuals may be more susceptible to *C. bombi* infection because they have not been previously exposed to pathogens or non-pathogenic organisms, such as environmental bacteria. Although bumblebees only have an innate immune system, there is evidence that bumblebees possess species-specific responses upon secondary exposure to a pathogen (known as immune priming). For example, Sadd and Schmid-Hempel (2006) found that *B. terrestris* were less susceptible to secondary infection from a bacteria when they had previously been exposed to it compared to another species. As of yet, there is no evidence of immune priming against *C. bombi* specifically and consequently, it is not clear whether immune priming plays a role in the susceptibility of *B. terrestris* to *C. bombi*.

However, susceptibility in this system is not solely determined by the host immune system. In fact, changes to the gut microbiota through an individual's life may also affect host susceptibility to *C. bombi* infection. A host's microbiome is established through contact with faeces in the colony (Koch and Schmid-Hempel 2011) and its constitution is an important predictor of *C. bombi* infection intensity (Koch and Schmid-Hempel 2012), with certain species, such as *Lactobacillus* spp. and *Gilliamella* spp., conferring reduced susceptibility. Callows emerge without a gut microbiome (Hakim et al. 2009; Kapheim et al. 2021; Hammer et al. 2023) and acquire the microbiome through contact with their colony (Koch and Schmid-Hempel 2011). The gut microbiome takes time to establish and increase in diversity, for example, the relative abundance of *Gilliamella* spp. increases with age (Hammer et al. 2023). Consequently, younger bees could be more susceptible to *C. bombi* infection compared to older bees, reversing the expectation from immunosenescence alone.

As *B. terrestris* colonies grow, they are characterised by contrasting population demographics. At the beginning of the lifecycle, there is a higher number of younger workers compared to the end, when the colony consists of a larger population of older workers. Elucidating the relationship between age and *C. bombi* susceptibility may therefore shed

light on the spread and impact of *C. bombi* infection at different points in the colony lifecycle. In addition, if certain ages are more susceptible to infection, this relationship could highlight disease reservoirs in a population. Here, we investigated the susceptibility of *B. terrestris* workers between 0 and 21 days old to *Crithidia sp.* infection. We inoculated workers with a standardised inoculum, allowed the infection to develop for one week and measured the infection intensity through faecal sampling. We also used qPCR to measure the impact of infection on AMP expression, as these genes are involved in host defence against *C. bombi* (Deshwal and Mallon 2014; Marxer et al. 2016). If age does affect infection susceptibility, we hypothesise that infection intensity will be highest in the youngest and oldest individuals. In addition, we predict that AMP expression will be lowest in the youngest and oldest individuals. This is because in the youngest individuals the immune system may not have fully established and in older individuals the immune system may be less effective due to immunosenescence.

Methods

Experimental organisms

Bumblebees

Five commercial colonies of *Bombus terrestris audax*, with approximately 50 workers, were purchased from Agralan (UK). All bees were housed under red light, 25°C and 50% humidity throughout the experiment. Faecal samples of 15 bees per colony were screened for *Crithidia sp., Vairimorpha* (formerly *Nosema*) spp. and *Apicystis bombi* using a phase contrast microscope (Nikon Eclipse 50i) at X400 magnification (Rutrecht and Brown 2009). None of the samples contained these parasites. Colonies were provided with honeybee collected pollen (Agralan, UK) and sterile sugar solution (50% concentration) *ad-libitum.*

Crithidia sp. and inoculation protocol

One additional colony (Agralan, UK) was used as *Crithidia sp.* stock for inoculations. This colony was infected with *Crithidia sp.* originating from post-hibernation spring queens collected at Windsor Great Park (Surrey, UK) in March 2021. While we were unable to screen our parasite with molecular tools to confirm its species identity, we believe that it was most likely *Crithidia bombi* because our previous molecular screening of *Crithidia sp.* infections from this bumblebee population has only ever found *C. bombi.* However, to be conservative we refer to our parasite source as *Crithidia sp.* throughout. On the day of inoculation, faecal samples from 15 to 20 workers were purified using a modified triangulation protocol developed by Cole (1970). Bees were starved for two hours and then fed a standardised inoculum of 12,000 cells mixed with sterile 50% sugar solution in a 30 µl droplet. This dose is field-realistic (Schmid-Hempel and Schmid-Hempel 1993) and has a high chance of leading to infection (Ruiz-González and Brown 2006). Bees from all treatment groups were inoculated using nicot cages (Becky's bees, UK), which are cylindrical containers adapted from hair rollers to house bees (see Fig. S1). Bees were placed individually in a nicot cage. They drank the inoculum from a 2 ml syringe connected to the nicot cage through a hole in the base secured with masking tape (adapted from the OECD 247 protocol for ecotoxicity testing). They were left to drink the inoculum for four hours. If the entire droplet was not consumed after four hours the individual was discarded from the experiment. Following inoculation, bees were housed individually in transparent plastic cages (12 × 7 × 5.5 cm) for one week. 50% sterile sugar solution and honeybee collected pollen (Agralan, UK) were supplied *ad-libitum*, since restricted access to pollen reduces longevity (Smeets and Duchateau 2003).

Experimental design

Treatment groups

To test the impacts of worker age on susceptibility to infection, we inoculated workers at four different ages: 0 days, 7 days, 14 days and 21 days old. These ages were chosen to represent a range of ages over the average worker life in the field, which is approximately three weeks for *Bombus* spp. (Brian 1952; Rodd et al. 1980) and to allow the inoculation to develop into a full infection, which takes 7–10 days (Schmid-Hempel and Schmid-Hempel 1993; Imhoof and Schmid-Hempel 1998; Logan et al. 2005; Otterstatter and Thomson 2006). Within each age group there were two treatment groups. To assess the impact of age on infection all treatment groups were inoculated with *Crithidia sp.* One group was screened for infection through faecal sampling and in the second group, AMP expression was measured using qPCR to test the effect of age on the immune response, resulting in a 4 × 2 factorial design.

Obtaining bumblebee samples

Colonies were checked three times per day for callow workers. Callow workers were removed within 24 h of emergence. They were identified through greyish legs, white stripes, ruffled fur, sluggish and clumsy behaviour, curved wings, little wing movement and low levels of aggression

or resistance when handled (HWG pers. obs.; O'Donnell et al. 2000; see Fig. S2).

Callows were marked with a coloured spot that corresponded to their colony of origin and were randomly assigned to one of the eight treatment groups: 0 day faecal screening, 7 day faecal screening, 14 day faecal screening, 21 day faecal screening, 0 day qPCR, 7 day qPCR, 14 day qPCR and 21 day qPCR. These groups contained 2–10 individuals from multiple colonies and were housed together until inoculation. Individuals were inoculated when they reached the age assigned to their group. Multi-colony groups were housed in wooden boxes (21 × 12 × 10 cm) containing cat litter to prevent faeces accumulating. They were provided with honeybee collected pollen (Agralan, UK) and sterile sugar solution (50% concentration) *ad-libitum*. Individuals assigned to the 0 day faecal screening and 0 day qPCR treatments were inoculated immediately and therefore, were not housed in groups. The date that callows were removed from their colony was recorded, since colony age may affect the immune response of workers (Moret and Schmid-Hempel 2009).

Measuring the infection outcome

Measuring infection intensity

Seven days post-inoculation faecal samples were taken from those bees assigned to the faecal sampling treatment groups. Infection intensity was measured using an improved Neubauer chamber haemocytometer and phase contrast microscope at X400 magnification. A digital calliper (Mitutoyo) was used to measure the thorax width at the wing intersection. Each individual was measured three times and the mean calculated. This measure was used as a proxy for bee size, since size affects infection intensity of *C. bombi* (Otterstatter and Thomson 2006).

Measuring the immune response

Individuals assigned to qPCR groups were snap frozen 18 h post-inoculation in liquid nitrogen and stored at -80°C. Eighteen hours was chosen due to practical constraints and previous work showing that AMP expression remains elevated 18 h after inoculation (Riddell et al. 2011; Brunner et al. 2013). The gene expression of the AMPs abaecin and defensin were chosen because previous work has shown that they are upregulated following *C. bombi* inoculation (Barribeau and Schmid-Hempel 2013; Brunner et al. 2013; Riddell et al. 2014) and are involved in the immune response against *C. bombi* (Deshwal and Mallon 2014).

Total RNA extraction

Whole abdomens were ground in liquid nitrogen and approximately half was used to extract Total RNA using 0.5 ml TRIzol reagent (Invitrogen) following the manufacturers protocol. Total RNA was further purified twice using 2 M lithium chloride and finally dissolved in 50 uL of nuclease-free water. RNA quality was checked spectrophotometically using nanodrop (Thermofisher Scientific) and RNA gel electrophoresis. Throughout RNA extraction, all equipment, gels and water were sterile and autoclaved to reduce the risk of RNA denaturation or contamination.

Primer design

Primers for qPCR were designed using the PrimerQuest tool and checked using Geneious ver.8.1.9. based on the mRNA sequences of *B. terrestris* which were (RPL13: FN391387.1., Arginine kinase: AF492888, RPS18: XM_048411652.1, RPS6: XM_012314237.3, abaecin: XM_003394653.4, defensin: FJ161700.1). Primer sequences are given in Table 1.

qPCR

RNA samples were treated with RQ1 RNase-Free DNase (Promega). First strand cDNA was synthesised from 500ng total RNA in a volume of 20µL using random pentadecamer primers and SuperScript III Reverse Transcriptase (Invitrogen). No genomic DNA contamination in the prepared cDNA was confirmed by PCR using primers for RPS6 which span an intron. The qPCR reaction in 10µL contained cDNA prepared from 2.5ng total RNA, 400 nM of forward

Table 1 Primer sequences used in the qPCR. RPL13, Arginine kinase and RPS18 were used as reference genes. Abaecin and defensin AMPs were chosen because they are upregulated following *C. bombi* inoculation and are involved in the immune response against *C. bombi*

Gene	Forward primer	Reverse primer	Annealing temperature (°C)
RPL13	GGTGATGCTACTGAAGAAGAAATG	AGAAATGACACGGGCCTTAG	60
Arginine kinase	TCTAGCACTTTGTCTGGCTTAG	AGTGGTCGTCGATCAGTTTC	60
RPS18	AAGGTGTTGGTCGTCGTTAC	CATTCTCCAGCACGCTTATCT	64
RPS6	ATGTCGTTCGTATCTCGGGC	CGCTCACCATCACGTCTAGG	66
Abaecin	GAAGGAACAAGTTGTGGAGAGA	GGTCGTGGCGGATTATATGG	64
Defensin	GCTCTTCTCTTTGTGGCTGTA	TCGCAGGTCACTCTTCTTTG	60

and reverse primers and 4µL iTaq™ Universal SYBR® Green Supermix (BioRad). qPCR was performed using a CFX 96 thermal cycler (Bio-Rad), using the following programme: 95 °C, 15 min, then 49 cycles of 95 °C, 15s, 60/64/66°C (Table 1), 30s and 72 °C, 30s. Melt curve analysis was conducted to check for primer dimer amplification. The mean Cq value of technical replicates was calculated, and gene expression was normalised against the mean of the three reference genes, RPS18, AK and RPL13 by subtracting the mean Cq values of the reference genes from each target gene Cq value to produce ΔCq. Standardised gene expression was calculated using $2^{-\Delta Cq}$. Two best stably expressed reference genes (AK and RPL13) were selected out of 4 tested reference genes (reference gene expression across treatments is shown in Fig. S3).

Data analysis

Statistical analyses were conducted in R version 4.1.0 (R Core Team, 2022). Figures were produced using the package 'ggplot2' (Wickham 2016).

Throughout analyses age was analysed as a categorical variable as we had no *a priori* assumption that age would have a linear effect. All model assumptions were tested using the 'DHARMa' package (Hartig 2022). To test how age affected infection intensity (number of *Crithidia sp.* cells per µl) the glmmTMB package (Brooks et al. 2017) was used to conduct a mixed effects model with a negative binomial error distribution. The model included age as a fixed effect and colony as a random effect. Bee size, and the date the individual was removed from their colony of origin were covariates and included as fixed effects. When plotting the results, the variance within each age group appeared to change with age and therefore, we tested whether there were significant differences in the coefficient of variation of each level of age using the asymptotic test in the 'cvequality' package (Marwick and Krishnamoorthy 2019).

The sample sizes for each treatment group in the qPCR were low (Table 2). Therefore, a power analysis was conducted using the package 'pwr' and function 'pwr.f2.test' (Champely et al. 2017) to identify the sample size required to detect a difference in infection intensity with age if the model explained either 30% or 85% of the variation in infection intensity with 85% power. These values were chosen to obtain sample size estimates for a wide range of age effects on infection intensity. To test how age affected abaecin and defensin gene expression, separate general linear models with a Gamma error distribution were used including standardised AMP expression as the response variable, and treatment and colony as fixed factors. Colony was not included as a random effect because there were not a sufficient number of samples of each colony within each age treatment group (Gelman and Hill 2006; Arnqvist 2020). Similarly, the model did not include the date callow was removed from the colony since on some days only one or two individuals were collected per age treatment group per day. A Gamma error distribution was used due to a high level of overdispersion when Gaussian and quasi error distributions were used. Tukey post-hoc tests were conducted using the package 'emmeans' (Lenth 2022) if fixed factors were significant.

Results

Sample for faecal screening

A total of 103 callows from five colonies were collected for use in the faecal screening experiment. They were collected on seven different dates from 29/04/2021–10/05/2021. The final sample sizes for the 0, 7, 14 and 21-day age treatment were 27, 30, 27 and 19 respectively. Sample sizes varied between treatment groups and colonies due to variation in size and production of callows across colonies, the random allocation of callows to age treatment groups and the loss of samples due to reasons shown in Table 2. Sample sizes across treatments per colony are shown in Table S1.

Effect of age on infection intensity

All individuals inoculated became infected. Age significantly affected infection intensity ($X^2_3 = 11.139$, $p = 0.011$). Tukey's HSD test for multiple comparisons found that the estimated mean infection intensity of 0 day old individuals (40,325 cells/µl, 95% C.I. = [22,104, 73,567]) was significantly higher than that of 7 day old individuals (13,910 cells/µl, 95% C.I. = [8,427, 22,961], $p = 0.0486$) and 21-day individuals (9,732 cells/µl, 95% C.I. = [5130, 18,462], $p = 0.009$, Fig. 1). There was no significant difference between 0 and 14 days (14 days: 19,896 cells/µl, 95% C.I. = [11,725, 33,761]; $p = 0.227$), 7 and 14 days ($p = 0.751$), 7 and 21 days ($p = 0.789$) or 14 and 21 days ($p = 0.278$). Size was not a significant predictor of infection intensity ($X^2_1 = 1.067$, $p = 0.301$). There was no significant difference in infection intensity between bees collected on different dates ($X^2_6 = 5.633$, $p = 0.466$). In addition, plots of the intercepts (±95% C.I) for colonies illustrated that there was no significant difference in the susceptibilities of colonies to *Crithidia sp.* infection (Fig. 1; Fig. S4). Comparing the coefficient of variation between levels of age showed that variance did not significantly differ across age groups ($p = 0.733$).

Table 2 Samples collected and lost for the infection experiment and qPCR

Treatment	Total collected from colonies	Failed to drink inoculum	Failed to defecate	Unforeseen circumstances	Died before screening	Final sample
Infection experiment						
0	30	0	0	0	3	27
7	32	0	0	0	2	30
14	42	1	0	0	13	27
21	42	1	1	15	6	19
qPCR						
0	10	0	NA	5	0	5
7	11	0	NA	0	0	11
14	12	1	NA	0	0	11
21	10	0	NA	0	0	10

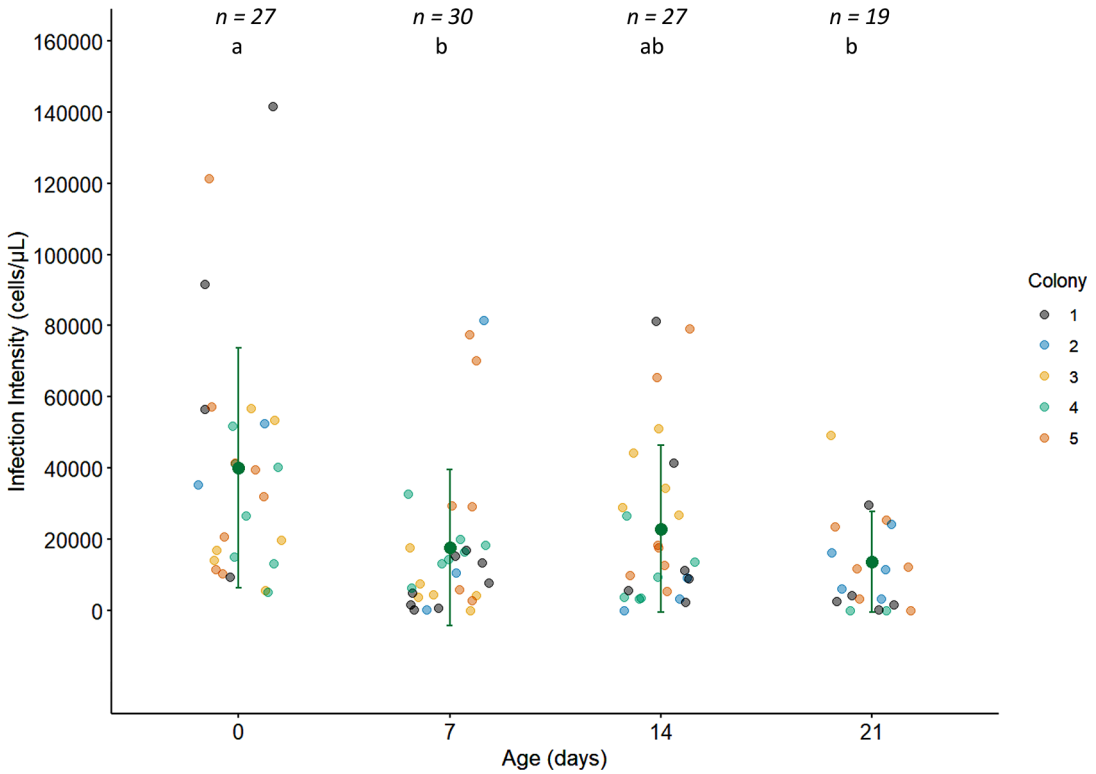

Fig. 1 The mean of the raw data is shown by the large circular datapoint, the error bars show the standard deviations and the smaller circular datapoints show the raw data. Datapoints are coloured with respect to the five colonies bumblebees originated from. 'a' and 'b' denote significant differences after Tukey post hoc tests. Sample sizes are shown above

Effect of age on AMP expression

The final sample sizes used in qPCR for the 0, 7, 14 and 21 day age treatment were 5, 11, 11 and 10 respectively (see Table 2). Sample sizes varied between groups due to variation in size and production of callows across colonies, the random allocation of callows to age treatment groups and the reasons shown in Table 2. Sample sizes across treatments per colony are shown in Table S2. Power analysis showed that if age predicted 30% of the variation in infection intensity, a sample size of 26 per group would be needed to detect this difference, while for 85% a sample size of 6 would be required. Consequently, the following analyses should be interpreted with caution, as our sample sizes are likely not large enough to detect anything but a very large difference in AMP expression.

In two samples (one from 0 days and one from 14 days) abaecin was not amplified (Cq = 0) and these were not included in further analysis because it was not possible to calculate standardised immune gene expression. Age did not affect abaecin expression (standardised using RPL13: $X^2_3 = 78.6$, $p = 0.255$; standardised using AK: $X^2_3 = 84.1$,

$p = 0.263$; Fig. 2a). Abaecin gene expression standardised against AK is shown in Fig. 2a and against RPL13 in Fig. S5a. In contrast, colony significantly affected abaecin expression (standardised using RPL13: $X^2_4 = 32.6$, $p < 0.001$; standardised using AK: $X^2_4 = 115.3$, $p < 0.001$; Fig. 3). Abaecin gene expression standardised against AK across colonies is shown in Fig. 3a and against RPL13 in Fig. S6a. When adjusted for multiple comparisons, however, Tukey-post hoc tests found no pairwise significant differences in abaecin expression between colonies.

There was one large outlier in the defensin gene expression dataset (from the 0 day treatment group; Fig. S5). Figure 2b shows the data without this outlier for clearer visualisation. Age did not significantly affect defensin gene expression (standardised using RPL13: $X^2_3 = 70.1$, $p = 0.0614$; standardised using AK: $X^2_3 = 72.1$; $p = 0.091$ Fig. 2b). Defensin gene expression standardised against AK is shown in Fig. 2a and against RPL13 in Fig. S5b excluding one outlier from 0 day treatment group to aid visualisation. All data are shown in Fig. S6a and S6b. However, like abaecin, defensin expression significantly varied with colony (standardised using RPL13: $X^2_3 = 107.8$, $p < 0.001$; standardised using AK: $X^2_3 = 111.4$, $p < 0.001$; Fig. 3b). Defensin gene expression standardised against AK across colonies is shown in Fig. 3b and against RPL13 in Fig. S6b. When adjusted for multiple comparisons, Tukey-post hoc

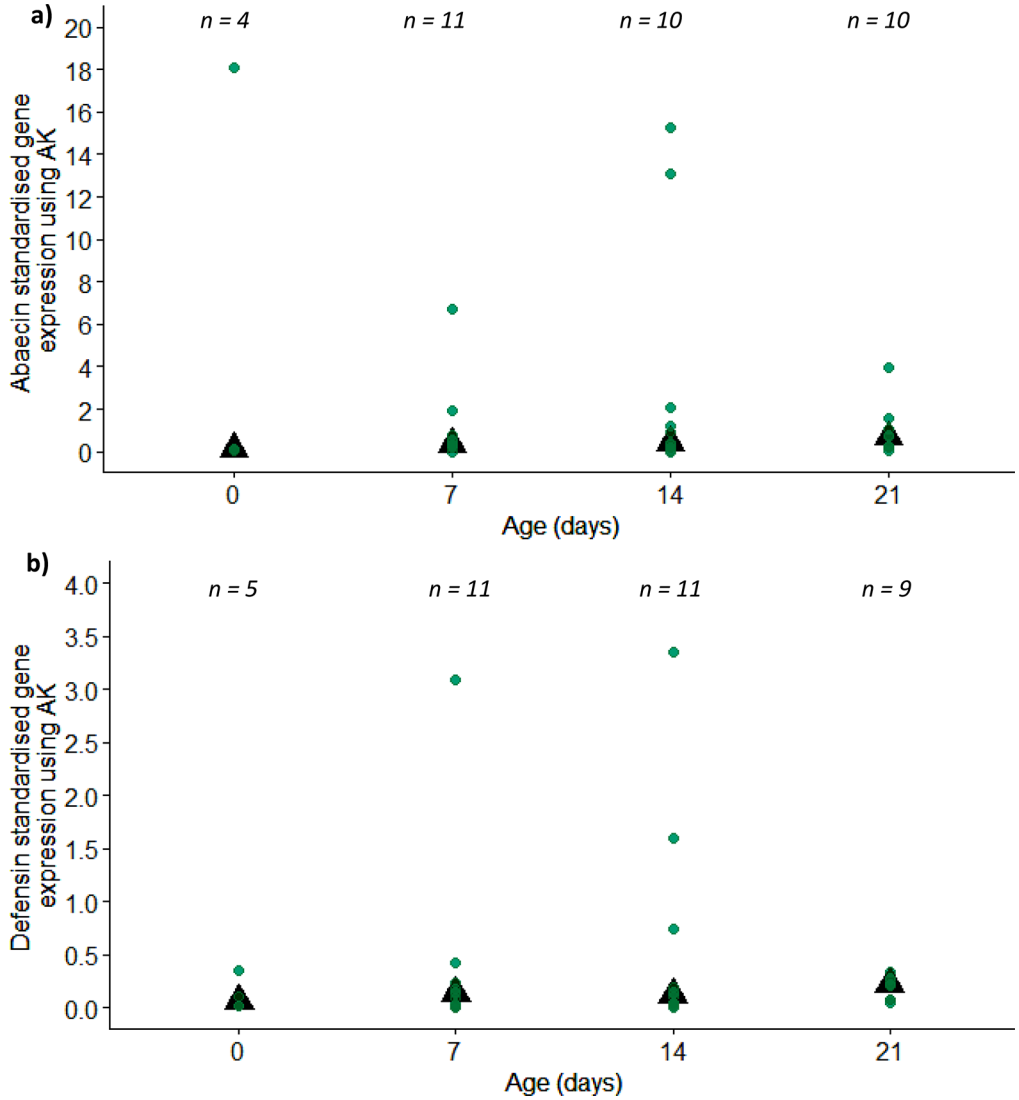

Fig. 2 (**a**) Abaecin standardised gene expression in *B. terrestris* of four different ages 18 h after inoculation with *Crithidia sp.* Abaecin gene expression is standardised against the reference gene AK. The triangle shows the median and smaller green circular datapoints show the raw datapoints. In two samples abaecin was not amplified (one from the 0 day and from the 14 days treatment group) and these were excluded because standardised gene expression could not be calculated. Sample sizes are given above datapoints. There was no significant difference in abaecin expression between ages. (**b**) same as (**a**) for defensin gene expression. One extreme outlier has been excluded from the 0 day treatment group for easier visualisation. A plot of all data is shown in Fig. S5

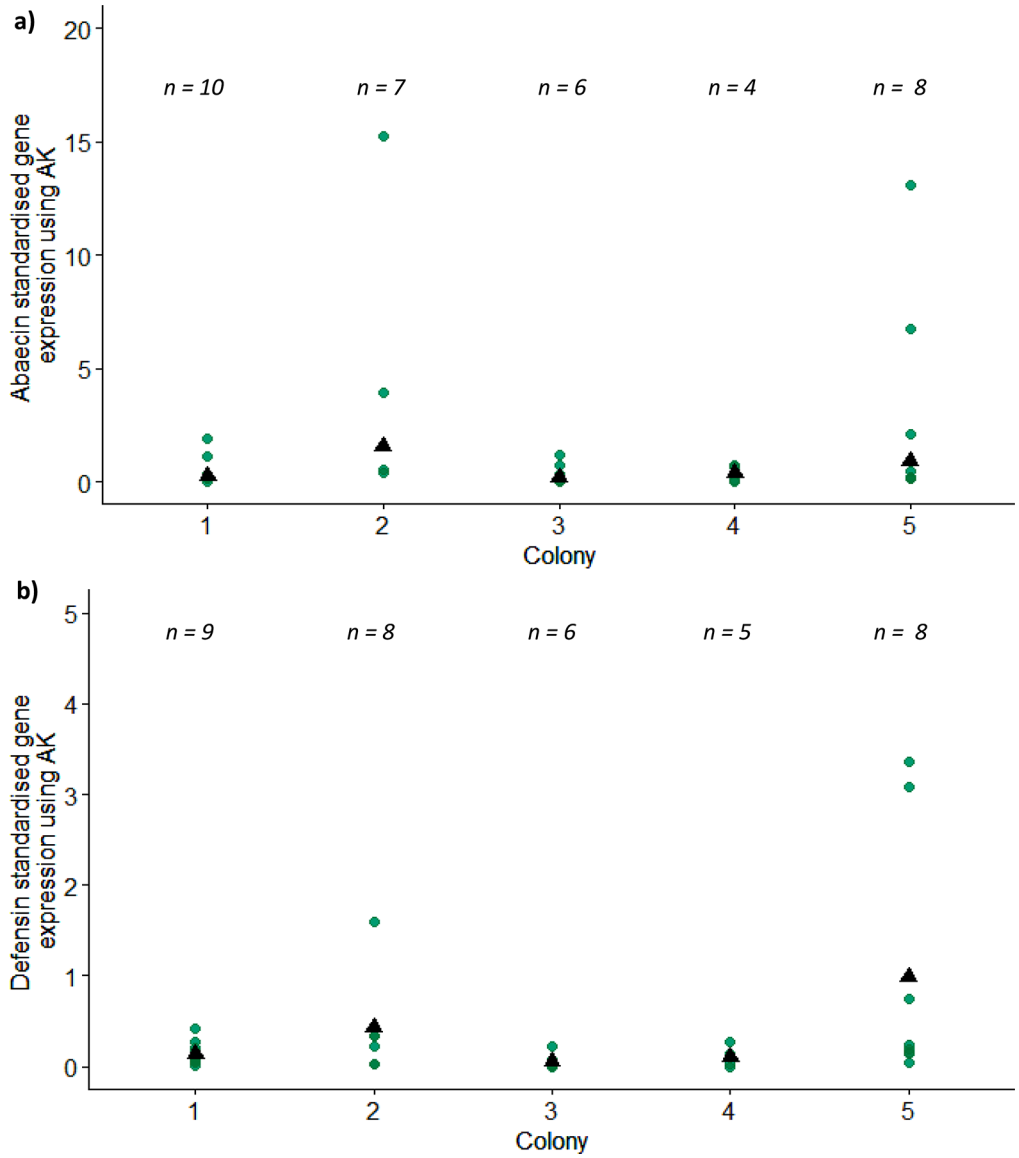

Fig. 3 (**a**) Abaecin standardised gene expression against AK in *B. terrestris* from five different colonies 18 hours after inoculation with Crithidia sp. Triangles show the median and smaller, circular, green datapoints the raw datapoints. Sample sizes are shown above the boxplots. Two samples (one from colony 2 and one from colony 4) were excluded as abaecin was not amplified. Colony significantly affected defensin gene expression but Tukey host poc tests showed no significant pairwise differences between colonies. (**b**) Same as (**a**), for defensin gene expression. One extreme outlier from colony 1 was excluded for easier visualisation

tests found no pairwise significant differences in defensin expression between colonies.

Discussion

Here we show that younger bumblebees are more susceptible to infection by a trypanosome parasite than their older siblings. On average, individuals inoculated at 0 days of age had an infection intensity seven days later of 40,325 cells/µl, which was more than double the infection intensity of individuals inoculated when they were 7 days old, and more than quadruple the infection intensity of individuals inoculated at 21 days of age. In contrast, the gene expression of two AMPs known to protect against *Crithidia bombi*, abaecin and defensin, did not significantly vary with age. However, we emphasise that these gene expression results are inconclusive since power analysis showed that our sample sizes were only able to detect an extremely large age effect.

We found that 0 day old individuals, or callows, were most susceptible to *Crithidia sp.* infection, confirming our hypothesis that susceptibility would be highest in younger individuals. While there appeared to be more variation in infection intensity in younger age groups, testing for

differences in the coefficient of variation showed this was not significant. In contrast to our hypothesis, susceptibility was not higher in the oldest individuals. Despite infection intensity not being significantly different between those inoculated on day 0 and 14, the mean infection intensity of those inoculated at 7, 14 and 21 days old are very similar. The high susceptibility of callows to infection may reflect a broader increased susceptibility to gut parasites in young bees, as two day old *B. terrestris* are also more susceptible to another gut parasite, *Vairimorpha* (*Nosema) bombi* compared to 10 day old individuals (Rutrecht et al. 2007). A reduced immune response might explain such higher susceptibility, however, we found no age-related difference in the expression of either abaecin or defensin after inoculation (which also argues against immunosenescence in the expression of these immune genes). It is unclear whether this finding is a result of an actual lack of difference in gene expression or experimental limitations. Our power analyses suggests that we only have a sufficient sample size to detect a very large difference in AMP expression, specifically our sample size could detect a significant difference if age explained over 85% of the variation in infection intensity. Furthermore, we measured AMP expression at a single time point post-infection and therefore, we could not detect whether temporal patterns of AMP expression varied with age. Furthermore, we cannot rule out the role of AMPs in the susceptibility of callows because lower AMP expression has previously been observed in younger honeybees and bumblebees (Hammer et al. 2023; Lourenço et al. 2019). Hammer et al. (2023) found abaecin and defensin gene expression was significantly lower in the hindgut of individuals aged 0–1 days compared to 3–19 days old. However, this study measured baseline expression, whereas we measured expression after an immune-challenge (in this case, inoculation with *Crithidia sp.*). Furthermore, gene expression was only measured in the hindgut. These differences in methodology may limit the comparability of our results, but this previous study provides some evidence that reduced abaecin and defensin expression could explain the increased susceptibility of callows to *Crithidia sp.* in our experiment.

It is important to acknowledge that we only measured the expression of two immune-related genes when, in reality, the immune system is very complex and many genes are likely upregulated following infection. It is possible that following *Crithidia sp.* inoculation, the expression of abaecin and defensin do not vary with age but the expression of other immune-related genes, not measured here, do vary. For example, peroxidase, which produces reactive oxidative species (ROS), is upregulated early in *C. bombi* infection (Riddell et al. 2011). Indeed, Hammer et al. (2023) found that the expression of dual oxidase, which generates ROS, increases as bumblebees mature, suggesting that other aspects of the immune response, not measured here, may vary with age.

The gut microbiome provides another explanation for the increased susceptibility of callows to infection. Callows emerge without a gut microbiome (Hakim et al. 2009; Kapheim et al. 2021) and 24 h post-emergence gut bacteria in the mid- and hindgut of bumblebees are scarce and exhibit reduced diversity (Hammer et al. 2023). Post-emergence, bumblebee gut bacteria exhibit logistic growth that stabilises after four days (Hammer et al. 2023). The gut microbiome is a vital predictor of *C. bombi* infection intensity (Koch and Schmid-Hempel 2011, 2012) and individuals without a gut microbiome have exhibited increased susceptibility to *C. bombi* (Koch and Schmid-Hempel 2011). Furthermore, the abundance of *Gilliamella*, which confers resistance to *C. bombi* (Cariveau et al. 2014; Mockler et al. 2018), significantly increases with age (Hammer et al. 2023). Combined, this indicates that a reduced gut microbiome in callows could contribute to the higher susceptibility of callows to *Crithidia sp.*

It is possible that the housing conditions throughout the experiment could have affected our results. Following removal from colonies callows were housed in multi-colony groups consisting of only workers. Workers start developing ovaries when housed in worker-only groups (Bloch and Hefetz 1999) which could divert resources away from the immune system. Indeed, a trade-off between the immune response and energy expended on foraging has been observed (Doums and Schmid-Hempel 2000). Furthermore, workers were housed in mixed colony groups for varying lengths of time depending on their age treatment. However, if there is an effect of housing conditions on the immune response, we would expect to see an increase in infection intensity with age, which was not observed.

The increased susceptibility of callows to *Crithidia sp.* infection has implications for the susceptibility of the whole colony to infection. If callows develop higher infection intensities, *Crithidia sp.* will be transmitted faster through the colony, because of an increased concentration of propagules in the faeces. Consequently, *Crithidia sp.* might be more likely to be transmitted through the colony during the early compared to late stages of the colony lifecycle, when the average age of workers is lower. The susceptibility of the colony may also peak and trough in accordance with the emergence of cohorts of callows. However, when considering the implications of our findings on within colony transmission, callow behaviour in the colony needs to be considered. In larger colonies, callows spend the majority of their time hidden underneath pupae and often do not feed in the first hours after emergence (HWG pers. obs). Consequently, in spite of their high susceptibility to infection at 0 days of age, their initial low exposure in the colony may

reduce the likelihood, or magnitude, of peaks in infection during the emergence of cohorts of callows, particularly in larger, older colonies. Previously, callows have been used in studies of *C. bombi* epidemiology, due to their lack of infection (Shykoff and Schmid-Hempel 1991b; Cisarovsky et al., 2012). Hence, the increased susceptibility we show here is particularly interesting in this host-parasite system. Importantly, our results show, that if inoculating individuals less than one week old, results may not be applicable to adult bees due to differences in infection susceptibility.

The lack of a colony effect on infection intensity in our experiment is surprising, given previous studies which show that this host parasite system exhibits specific host-parasite genotype-genotype interactions (Baer and Schmid-Hempel 2003; Cisarovsky et al., 2012; Barribeau and Schmid-Hempel 2013). In contrast, abaecin and defensin expression did vary between colonies. Previous work has shown that different colonies produce contrasting patterns in AMP expression in response to both different *C. bombi* strains and immune challenges, for example inoculation with *C. bombi* compared to bacteria (Barribeau and Schmid-Hempel 2013). Furthermore, Riddell et al. (2009) found that different *C. bombi* strains produced contrasting defensin, but not abaecin, expression across different colonies. The lack of relationship in our study between colony variation in immune gene expression and parasite susceptibility again suggests that the gut microbiome of the colony may play a role in determining the infection outcome (Koch and Schmid-Hempel 2012).

Overall, these results suggest that age does affect susceptibility to *Crithidia sp.* infection in *B. terrestris*, with callows exhibiting higher infection intensities than other age treatment groups. We found no difference in AMP expression, however, our low sample size reduced our power to detect an effect of age on AMP expression. Therefore, we cannot disregard the role of AMPs in the susceptibility of callows to *Crithidia sp.* infection. Higher susceptibility of callows may have implications for the susceptibility of colonies at different stages of their lifecycle, due to the contrasting age demography of workers in the colony. However, the size of this impact is unclear because the risk of infection for callows in the colony may be relatively low due to their behaviour and therefore, exposure to *Crithidia sp.* within the colony.

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Declarations

Competing interests The authors declare no competing interests.

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