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Temperature dependence of parasitic infection and gut bacterial communities in bumble bees

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Summary

High temperatures (e.g., fever) and gut microbiota can both influence host resistance to infection. However, effects of temperature-driven changes in gut microbiota on resistance to parasites remain unexplored. We examined the temperature dependence of infection and gut bacterial communities in bumble bees infected with the trypanosomatid parasite *Crithidia bombi*. Infection intensity decreased by over 80% between 21 and 37°C. Temperatures of peak infection were lower than predicted based on parasite growth *in vitro*, consistent with mismatches in thermal performance curves of hosts, parasites and gut symbionts. Gut bacterial community size and composition exhibited slight but significant, non-linear, and taxon-specific responses to temperature. Abundance of total gut bacteria and of Orbaceae, both negatively correlated with infection in previous studies, were positively correlated with infection here. Prevalence of the bee pathogen-containing family Enterobacteriaceae declined with temperature, suggesting that high temperature may confer protection against diverse gut pathogens. Our results indicate that resistance to infection reflects not only the temperature dependence of host and parasite performance, but also temperature-dependent activity of gut bacteria. The thermal ecology of gut parasite-symbiont interactions may be broadly relevant to infectious disease, both in ectothermic organisms that inhabit changing climates, and in endotherms that exhibit fever-based immunity.

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Introduction

Temperature has strong effects on the growth and metabolism of individual species, with subsequent consequences for ecological communities (Gillooly *et al*., 2001). The effects of temperature on growth, metabolism and performance are described by the metabolic theory of ecology (Brown *et al*., 2004), which considers organismal metabolism as the combined output of numerous metabolic enzymes. Consequently, metabolic theory models predict the temperature dependence of organismal performance using equations based on enzyme kinetics (Molnár *et al*., 2017). The temperature-related changes in performance of any given species are described by its thermal performance curve, with performance generally increasing up to an optimal temperature, then declining sharply at supraoptimal temperatures (Molnár *et al*., 2017). Because species differ in their responses to temperature and thermal ranges of peak performance – a difference referred to as a 'mismatch' between two species' thermal performance curves (Cohen *et al*., 2017) – temperature can also influence the outcome of species interactions including predation, parasitism and competition (Dell *et al*., 2014; Bestion *et al*., 2018).

One context in which the effect of temperature on species interactions has been repeatedly demonstrated to reflect the predictions of metabolic theory is that of host–parasite interactions (Raffel *et al*., 2013; Kirk *et al*., 2018; Cohen *et al*., 2019). Metabolic theory predicts that the success of parasites at any given temperature reflects the relative performance of hosts and parasites at that temperature, rather than the absolute performance of either in isolation (Cohen *et al*., 2017). As a result, the temperature dependence of parasite growth may differ for parasites grown in cell cultures versus in live hosts (James, 2005; Cohen *et al*., 2017; Kirk *et al*., 2018).

Increases in body temperature – also known as fever – have been shown to ameliorate infection in plants, fish, mammals, amphibians and insects (Kluger *et al*., 1998; Thomas and Blanford, 2003; Boltaña *et al*., 2013; Heinrich, 2013; Stahlschmidt and Adamo, 2013). Both endo- and ectothermic animals may use metabolic and behavioural strategies to raise their body temperatures when infected (Starks *et al*., 2000; Campbell *et al*., 2010; Stahlschmidt and Adamo, 2013). These febrile behaviours can allow hosts to

achieve body temperatures in which the host immune system has a relative advantage over parasites (Casadevall, 2016). Consequently, fever can improve the health of individual hosts, with consequences that may be reflected at the landscape scale (Daskin *et al*., 2011; Cohen *et al*., 2019). In bees, particularly social bees that thermoregulate their nests, relatively high temperatures have been shown to ameliorate infections with microsporidia, other fungi, and viruses in both adults and larvae (Martín-Hernández *et al*., 2009; Di Prisco *et al*., 2011; Xu and James, 2012; Dalmon *et al*., 2019; Li *et al*., 2019).

Besides temperature, host microbiota – particularly gut symbionts – play an increasingly appreciated role in resistance to infection. Microbial symbionts can compete with parasites for space and resources, stimulate host immunity and produce allelopathic chemicals that inhibit parasite growth (Maslowski and Mackay, 2010; Spor *et al*., 2011). In insects, including bees, *in vitro* culturing studies, sterile rearing experiments and manipulations of the microbiota with faecal transplants have repeatedly demonstrated the ability of bacterial symbionts to suppress the growth and effects of infectious organisms within and outside of hosts (Sabaté *et al*., 2009; Koch and Schmid-Hempel, 2012; Onchuru *et al*., 2018; Praet *et al*., 2018).

Despite the recognized importance of temperature and microbial symbionts in resistance to infection, few studies have explored the effects of temperature on the microbiome, and the consequences of these effects for disease resistance have seldom been considered at all. Landscape surveys and experimental manipulations have shown that temperature can be an important driver of microbial community composition and function in soils and aquatic environments (Steinauer *et al*., 2015; Chiriac *et al*., 2017; Rubin *et al*., 2018). Exposure of rhizosphere communities to high temperatures can lead to loss of taxa and alterations in the ability of soils to support plant growth and suppress disease (van der Voort *et al*., 2016; Rubin *et al*., 2018). In insects, experimental temperature elevations reduced populations of endo- and ectosymbionts (Parkinson *et al*., 2014; Kikuchi *et al*., 2016), suggesting that fever and high temperature could be costly for host–symbiont mutualisms and their role in resistance to infection. On the other hand, in cultures of amphibian skin symbionts, high temperatures can elevate production of compounds that inhibit parasite growth (Daskin *et al*., 2014). In bees, many core gut symbionts have relatively high (34–37°C) preferred growth temperatures *in vitro* (Engel *et al*., 2013), suggesting that high temperatures could favour core gut symbionts over parasites, and thereby reinforce host–bacterial mutualisms.

The bumble bee (*Bombus* spp.)/*Crithidia* host-trypanosomatid parasite study system is well suited for investigating how temperature affects infection resistance and microbiota. First, bumble bees are facultative endotherms, capable of producing large amounts of metabolic heat to optimize nest and body temperature during foraging and incubation (Heinrich, 1972). As a result, their ranges of body temperatures and thermal strategies overlap those of both poikilo- and homeothermic animals (Heinrich, 1972), suggesting that bumble bees could be a model for study of infection–microbiota interactions in both types of host. Second, trypanosomatid infection is widespread, common, and costly in *Bombus* (Schmid-Hempel, 2001; Brown *et al*., 2003; Schmid-Hempel and Tognazzo, 2010). As a result, responses of trypanosomatid infection to environmental variables is relevant for bee conservation, and may also be relevant for the insect-vectored stage of trypanosomatids that afflict crops, livestock and humans (Maslov *et al*., 2013). Third, the well-characterized host microbiota of *Bombus* have a demonstrated role in resistance to infection (Koch and Schmid-Hempel, 2011; 2012) and higher temperatures of peak performance *in vitro* than trypanosomatid parasites (Engel *et al*., 2013; Palmer-Young *et al*., 2018b). *In vitro* experiments have suggested that the effects of core bacterial symbionts – namely, their ability to acidify the gut lumen to levels that inhibit parasite growth – are temperature-dependent (Palmer-Young *et al*., 2018b; 2019). These prior findings suggest that high temperatures could lead to symbiont-mediated increase in resistance to infection. However, no study has evaluated the effects of environmental temperature on bumble bee gut microbiota or trypanosomatid infection, nor the effects of temperature on microbiota-mediated protection against disease.

To determine the effects of environmental temperature on bumble bee gut microbiota and resistance to infection with trypanosomatid parasites, we measured gut bacterial community size and composition and infection intensity in bumble bees (*Bombus impatiens*) inoculated with the parasite *Crithidia bombi*, then incubated for 7 days at temperatures between 21 and 37°C. Because bumble and honey bee muscle performance (Gilmour and Ellington, 1993; Harrison and Fewell, 2002), bumble bee respiration rate (Kammer and Heinrich, 1974) and bacterial gut symbiont performance all have higher temperatures of peak performance than does the parasite *C. bombi* (Palmer-Young *et al*., 2018b), we predicted that infection would decrease across the temperature range previously recorded in wild bees (Heinrich, 1972). Based on metabolic theory and the concept of thermal mismatches (Cohen *et al*., 2019) – which predict that high temperatures improve performance of the host immune system and antiparasitic bacterial symbionts relative to performance of parasites – we also predicted that the temperature of peak infection in bees would be lower than the temperature of peak growth rate for parasite cell cultures. Finally, we predicted that higher temperatures would lead to lower absolute quantities of gut bacteria, due to elevation of

per capita metabolic rates and consequent reduction of the gut ecosystem's carrying capacity at higher temperatures (Bernhardt *et al*., 2018; Lemoine, 2019).

Results

Overview of experiments

We tested the effects of experimental inoculation with parasites and rearing temperature on *C. bombi* infection intensity, gut bacteria, sugar water consumption and mortality in the common Eastern bumble bee, *B. impatiens*. Bumble bee workers were inoculated with 10^4 *C*. *bombi* parasite cells or a sham inoculum without parasite cells. Each inoculated bee was then reared individually for 7 days at one of five temperatures (21, 25, 29, 33 or 37°C), chosen to capture the range of body temperatures previously recorded in bumble bees (Heinrich, 1972), and to include temperatures above and below the temperature of peak parasite growth rate (Palmer-Young *et al*., 2018b). Seven days after inoculation, bees were frozen for dissection and analysis of microbiota and infection intensity by amplicon sequencing and qPCR. The experiment was conducted in four temporal blocks of inoculations, with three colonies used per block, and incubator temperatures reassigned after the first two blocks to avoid confounding the effects of incubator with those of temperature. Data were analysed by generalized linear mixed models to examine the effects of temperature and infection treatment on *C*. *bombi* infection intensity, gut bacterial composition and abundance, sugar water consumption and mortality. We also tested for correlations between *C*. *bombi* infection and abundances of specific families of gut bacteria.

Crithidia bombi *infection*

Experimental inoculation resulted in detectable *C*. *bombi* infection after 7 days in 67% of the 239 experimental bees; prevalence ranged from 42% at 37°C to 84% at 21°C. Infection intensity declined quadratically with temperature, as shown by the significance of models terms for both temperature and temperature^2, that is, the square of the mean-centred temperature (temperature: $\beta = -0.10 \pm 0.017$ SE, $\chi^2_1 = 36.02$, $p < 0.001$; temperature^2: $\beta = -0.0075 \pm 0.0034$ SE, $\chi^2_1 = 4.89$, $p = 0.027$). After exponentiation from the log scale, intensity declined by 81% over the range of incubation temperatures, from 7.34×10^5 cells bee^{-1} at 21°C to 1.40×10^5 cells bee^{-1} at 37°C (Fig. 1). Infection intensity varied significantly among bees from different colonies ($\chi^2_4 = 47.84$, $p < 0.001$) and was positively correlated with ln(bacterial abundance) ($\beta = 0.22 \pm 0.094$ SE, $\chi^2_1 = 5.31$, $p = 0.021$, Fig. 3).

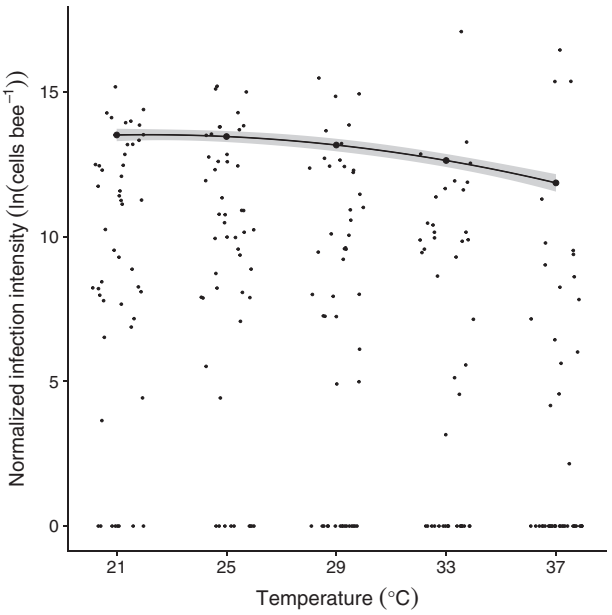

Fig. 1. Effects on temperature on *C. bombi* infection intensity. Infection decreased with increasing temperature. Line and shaded band show fitted means and standard errors from generalized linear mixed model of normalized infection intensity after inverse log transformation from the scale of the linear predictor. Predictions are averaged over infection treatments and colonies and calculated for a bee of average gut bacterial abundance. Shaded bands indicate uncertainty from the fixed effects portion of the model only. Large points show the five tested incubation temperatures; small points show raw data, ln(x + 1)-transformed and randomly jittered by a standard deviation of ±1°C to reduce overplotting.

Bacterial community composition

Processing of 16S rRNA gene amplicon sequences resulted in a data set of 7,044,293 total sequences comprising 128 Exact Sequence Variants (ESVs). Samples were rarefied to a depth of 10,233 reads per sample (see rarefaction curves in Supporting Information Fig. S1). Temperature had slight but significant, bacterial family-specific effects on gut communities. Permutational MANOVA of proportional composition (based on weighted UniFrac distances between samples) indicated that temperature explained more than twice as much variation than any other experimental factor ($F_{4, 411} = 12.46$, $R^2 = 0.10$, $p < 0.001$). There were also significant but smaller effects of colony ($F_{4, 411} = 4.32$, $R^2 = 0.036$, $p < 0.001$) and infection treatment ($F_{1, 411} = 3.59$, $R^2 = 0.007$, $p = 0.027$), although the latter explained less than 1% of variation across samples. There was no significant interaction between infection and temperature ($F_{4, 411} = 1.08$, $p = 0.38$).

Total and family wise bacterial abundance

Total bacterial abundance (normalized 16S copy number) varied quadratically with temperature (temperature^2: $\beta = 0.0098 \pm 0.0022$ SE, $\chi^2_1 = 20.49$, $p < 0.001$, Fig. 2). The lowest total abundance occurred at intermediate temperatures (Fig. 2). Averaged across colonies for a bee of average

size, model-predicted abundance declined by 46% from 6.16×10^7 copies per bee at 21°C to 3.30×10^7 copies per bee at 29°C, then rose to 6.22×10^7 copies per bee at 37°C (Fig. 2). Bacterial abundance (normalized to quantities of host DNA) also differed significantly among bees from different colonies ($\chi^2_4 = 15.37$, $p = 0.0040$), and was negatively correlated with bee size (marginal cell length, $\beta = -0.99 \pm 0.18$ SE, $\chi^2_1 = 28.75$, $p < 0.001$). The simplest explanation for the negative relationship between bee size and normalized bacterial abundance is that larger guts have a lower ratio of luminal volume (i.e., space available to bacteria) to surface area (i.e., host cells). Bacterial abundance was not significantly affected by infection treatment ($\chi^2_1 = 1.69$, $p = 0.19$, Supporting Information Table S2).

Because we used the same primer set for qPCR and amplicon sequencing, we combined the total absolute abundance values from the 16S rRNA gene qPCR with the proportional abundance values from the amplicon sequencing to estimate absolute abundances of each bacterial family. The gut bacterial community was dominated by bacteria from four families – Orbaceae, (32.7%), Neisseriaceae (31.6%), Lactobacillaceae (17.4%) and Bifidobacteriaceae (16.3%). Only one additional family, Enterobacteriaceae, accounted for >1% of reads (1.67%). Collectively, these five families accounted for 99.7% of detected bacteria.

Like total bacterial abundance, abundances of the main symbiont families were generally weakly but significantly affected by temperature, with changes of less than twofold across the range of experimental temperatures. However, the effect of temperature was statistically significant for all except Orbaceae (Fig. 2). Abundances of Orbaceae and Neisseriaceae, which were found in almost exactly equal copy numbers, both tended to decline with temperature (Orbaceae: $\beta = -0.014 \pm 0.0077$ SE, $\chi^2_1 = 3.43$, $p = 0.064$; Neisseriaceae: $\beta = -0.018 \pm 0.0078$ SE, $\chi^2_1 = 5.41$, $p = 0.02$). This effect was only significant for Neisseriaceae and of small magnitude, with fitted models predicting just a 26% drop in copy numbers from 21 to 37°C (Fig. 2).

Abundances of Lactobacillaceae and Bifidobacteriaceae varied quadratically with temperature (Fig. 2). The strongest effects were seen for Lactobacillaceae, where model-fitted abundances roughly doubled (99% increase after exponentiation from the log scale) across the temperature range (temperature: $\beta = 0.042 \pm 0.0084$ SE, $\chi^2_1 = 25.15$, $p < 0.001$; temperature^2: $\beta = 0.0039 \pm 0.0018$ SE, $\chi^2_1 = 4.84$, $p = 0.028$, Fig. 2). Abundances of Bifidobacteriaceae most closely mirrored the patterns of total bacterial abundance, with an initial 34% decrease in abundance as temperatures approached 33°C, followed by a 12% rise at the highest temperature (temperature: $\beta = -0.019 \pm 0.0074$ SE, $\chi^2_1 = 6.25$, $p = 0.012$; temperature^2: $\beta = 0.0039 \pm 0.0016$ SE, $\chi^2_1 = 6.19$, $p = 0.013$, Fig. 2). Together, the slight decreases in abundances of Orbaceae, Neisseriaceae and Bifidobacteriaceae account for the initial fall in total bacterial abundance from 21°C through 29°C, whereas the relatively strong increase in abundance of Lactobacillaceae accounts for the rebound in total abundance above 29°C.

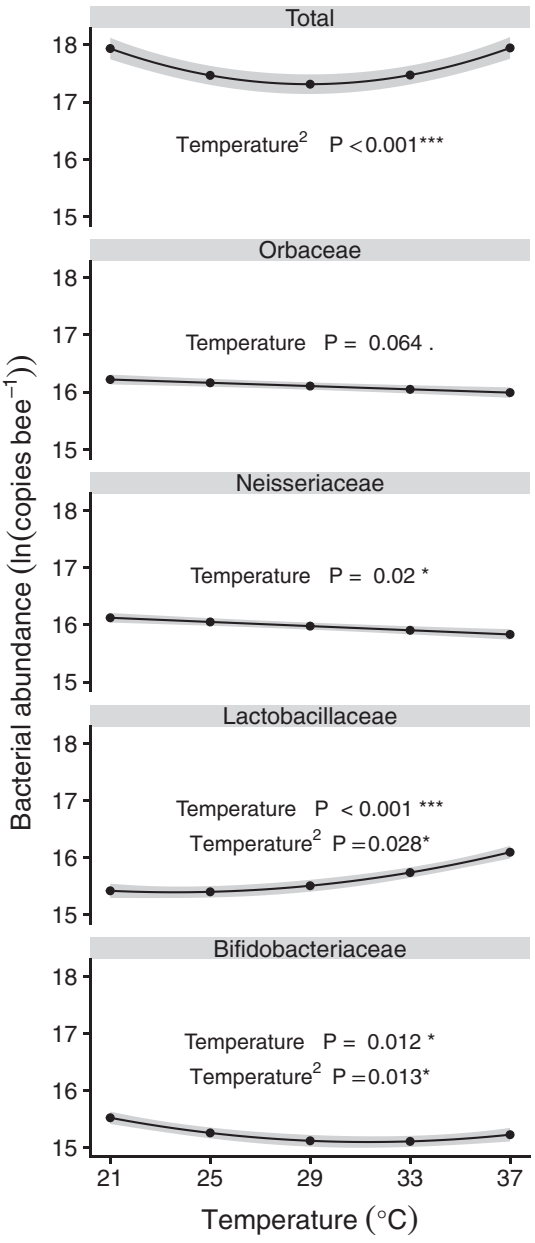

Fig. 2. Effects of temperature on abundances of the four most abundant families of bumble bee gut bacteria. Top panel depicts total bacteria abundance, as measured by qPCR of the 16S rRNA gene. Lower panels depict abundances of individual families, estimated for each sample by multiplying total abundance by the proportion of amplicon sequence reads associated with each family. Lines and shaded bands show fitted means and standard errors from negative binomial linear mixed model, averaged across colonies and plotted at the mean values for temperature and wing size. Annotations indicate significance of temperature and, where significant, temperature^2 terms in models of abundance, as assessed by Wald χ^2 tests ('.': $p < 0.10$, '*': $p < 0.05$, '**': $p < 0.01$, '***': $p < 0.001$). Effects of temperature and/or temperature^2 were statistically significant for all families except Orbaceae ($\chi^2_1 = 3.42$, $p = 0.064$). See Supporting Information Table S2 for full model summaries.

In contrast to the significant effects of temperature, abundances of the four most abundant bacterial families were not significantly affected by infection treatment (Supporting Information Table S2). Abundances of each family were negatively correlated with wing size, which was a significant predictor in every model, and varied across colonies for all families except Neisseriaceae (Supporting Information Table S2).

Correlations between bacterial abundance and C. bombi *infection intensity*

After accounting for effects of temperature and colony, infection intensity was positively correlated with total bacterial abundance ($\beta = 0.22 \pm 0.094$ SE, $\chi^2_1 = 5.31$, $p = 0.021$, Fig. 3; also noted in Results: *Crithidia bombi* infection) and with abundance of Orbaceae ($\beta = 0.28 \pm 0.11$ SE, $\chi^2_1 = 7.17$, $p = 0.0074$, Fig. 3), but not with abundance of Neisseriaceae, Lactobacillaceae or Bifidobacteriaceae (Fig. 3; see Supporting Information Table S3 for full model summaries).

Occurrence of the next most abundant family, Enterobacteriaceae (1.7% of overall abundance), was also influenced by temperature. However, unlike abundance of the major symbiont families, Enterobacteriaceae was also affected by infection treatment. Prevalence declined by over 50% with increasing temperature ($\beta = -0.052 \pm 0.024$ SE, $\chi^2_1 = 4.74$, $p = 0.029$), from an average of 27% at 21°C to 13.1% at 37°C (Fig. 4). Presence of this family was overall less than half as likely for bees in the infection treatment (13.1 vs. 28.4% prevalence, Fig. 4), an effect that was highly significant ($\beta = 0.97 \pm 0.27$ SE, $\chi^2_1 = 13.24$, $p < 0.001$).

Temperature had a quadratic effect on bacterial alpha diversity, measured as the number of observed ESV's per sample (Fig. 4). Both temperature ($\beta = 0.030 \pm 0.0027$ SE, $\chi^2_1 = 124.0$, $p < 0.001$) and temperature^2 ($\beta = 0.0036 \pm 0.00058$ SE, $\chi^2_1 = 38.28$, $p < 0.001$) terms were highly significant predictors of richness. Model-fitted ESV richness declined slightly between 21 and 25°C, then increased by 70% at the highest temperature (Fig. 4). ESV richness was slightly (9.3%) lower in parasite-inoculated bees (infection treatment: $\beta = 0.098 \pm 0.032$ SE, $\chi^2_1 = 9.61$, $p = 0.002$, Fig. 4). Richness also varied significantly among bees from different colonies ($\chi^2_4 = 25.41$, $p < 0.001$).

Sugar water consumption

Rates of sugar water consumption declined strongly and significantly with temperature ($\beta = -0.021 \pm 0.0019$ SE, $\chi^2_1 = 171.52$, $p < 0.001$, Fig. 5). Covariate-adjusted model predictions, averaged across colonies and infection treatments, showed an 82% decrease in rate of sugar water consumption over the range of incubation temperatures, from 0.256 g bee^{-1} days^{-1} at 21°C to 0.046 bee^{-1} days^{-1} at 37°C (Fig. 5). Although infected bees tended to have higher consumption rates at low temperatures but lower consumption rates at high temperatures, the effects of infection

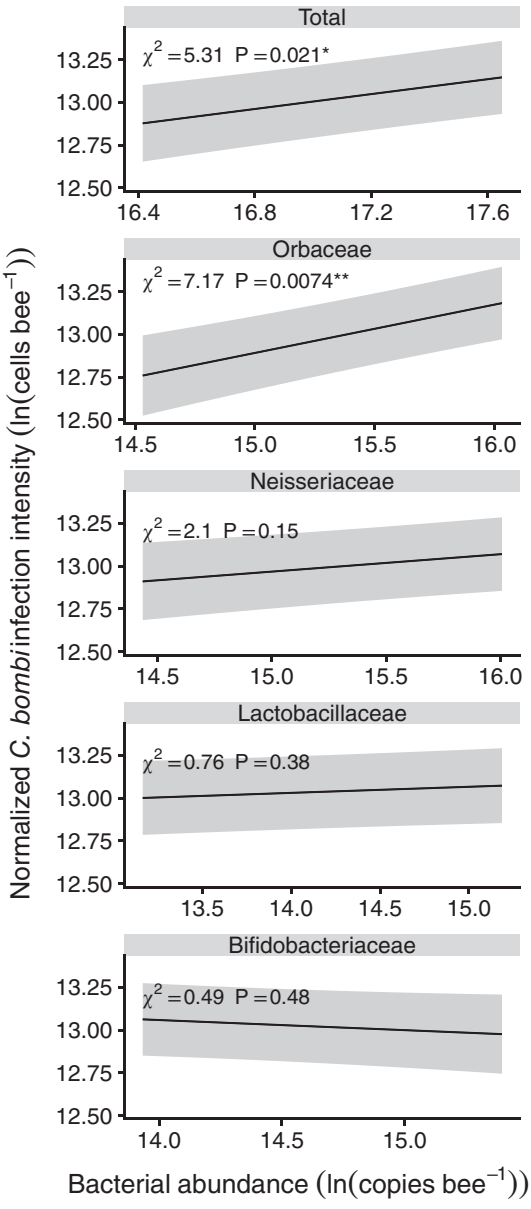

Fig. 3. Relationship of *C. bombi* infection intensity to total and family wise gut bacterial abundance. Abundance of Orbaceae, but not other main families of gut bacteria, was positively correlated with *C. bombi* infection intensity. Panels depict relationship between infection intensity and total gut bacteria (top panel) or family wise abundance (lower panels). Lines and shaded bands show fitted means and standard errors from negative binomial linear mixed model, averaged across colonies and plotted at the mean value for temperature and, where significant, bee size. *X*-axes span the interquartile ranges of abundances of total bacteria and of each family. Shaded bands indicate uncertainty from the fixed effects portion of the model only. Annotations indicate significance of ln(abundance) term in negative binomial mixed model ('*': $p < 0.05$, '**': $p < 0.01$); degrees of freedom for χ^2 statistic equals 1 for all panels. See Supporting Information Table S3 for full model summaries.

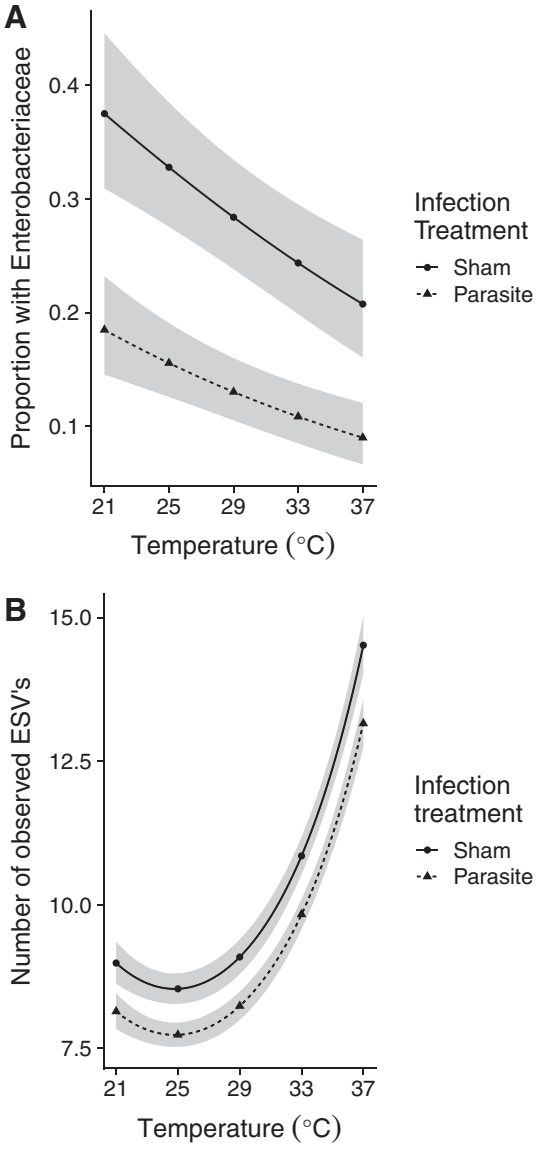

Fig. 4. Effects of temperature and infection treatment on (A) prevalence of Enterobacteriaceae and (B) bacterial alpha diversity (number of Exact Sequence Variants (ESV's) per sample). Enterobacteriaceae prevalence and bacterial alpha diversity exhibited opposite responses to temperature, but both metrics were lower in parasite-inoculated bees. Lines and shaded bands show fitted means and standard errors from binomial (A) or Poisson (B) linear mixed models, back-transformed to probabilities (from the logit scale) or counts (from the log scale). Circles and solid line: parasite infection treatment; triangles and dotted line: sham infection treatment. Predictions in (B) are averaged over colonies. Shaded bands indicate uncertainty from the fixed effects portion of the model only. Points show the five tested incubation temperatures.

($\chi^2_1 = 0.05$, $p = 0.82$) and the temperature × infection interaction were non-significant ($\chi^2_1 = 3.60$, $p = 0.058$), as was the overall effect of infection treatment ($\chi^2_1 = 0.050$, $p = 0.82$). Consumption was positively correlated with bee size (marginal cell length, $\beta = 0.11 \pm 0.025$ SE, $\chi^2_1 = 20.07$,

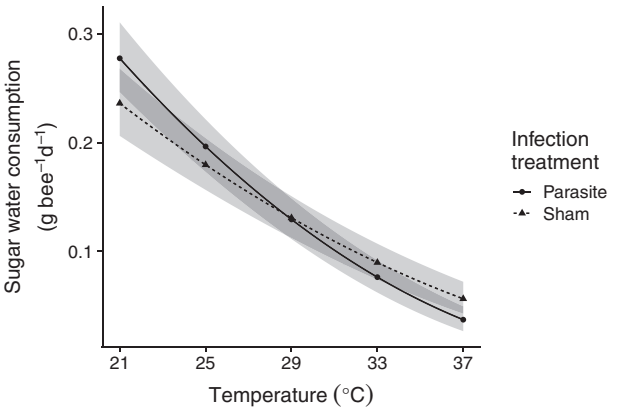

Fig. 5. Effects of temperature and infection treatment on sugar water consumption. Consumption declined with temperature ($\chi^2_1 = 171.52$, $p < 0.001$), with no significant effect of infection treatment ($\chi^2_1 = 0.050$, $p = 0.82$) or the infection × temperature interaction ($\chi^2_1 = 3.60$, $p = 0.058$). Lines and shaded bands show fitted means and standard errors from general linear mixed model on square root-transformed consumption, back-transformed to the original scale of the measurement. Circles and solid line: parasite infection treatment; triangles and dotted line: sham infection treatment. Predictions are averaged over colonies and calculated for a bee of average size. Shaded bands indicate uncertainty from the fixed effects portion of the model only. Points show the five tested incubation temperatures.

$p < 0.001$) and varied significantly among bees of different colonies ($\chi^2_4 = 16.34$, $p = 0.0026$).

Mortality

The probability of premature death ranged from 7.5% (three deaths) in sham-infected bees at 29°C to 26% in sham-infected bees at 33°C (10 deaths). However, survival analysis showed no effect of temperature treatment ($\chi^2_4 = 5.93$, $p = 0.20$), infection treatment ($\chi^2_1 = 0.90$, $p = 0.34$), or their interaction ($\chi^2_4 = 1.61$, $p = 0.81$) on rates of mortality, although rates differed significantly among colonies ($\chi^2_4 = 21.96$, $p < 0.001$).

Discussion

High temperatures reduced infection intensity

In agreement with predictions from *in vitro* experiments and temperature-dependent changes in performance of parasites, bees, and bacterial symbionts, we found reductions in trypanosomatid infection intensity as temperature increased over the typical range of bee body temperatures (Heinrich, 1972). Parasites in cell culture had optimal growth temperatures of 27–32°C (Palmer-Young *et al*., 2018b). Hence, reductions in infection above 32°C could be due to direct inhibition of growth, but reductions between 21 and 32°C likely reflect relative, rather than absolute, disadvantage of parasites in

comparison to the bee immune system and gut bacterial symbionts. Bee muscle and whole-body metabolism have temperatures of peak performances >37°C (Kammer and Heinrich, 1974; Gilmour and Ellington, 1993; Harrison and Fewell, 2002). If the immune system has a similar temperature of peak performance, then metabolic theory would predict improvement in host immune function across the 21–37°C temperature range examined here. The core bee gut bacterial symbionts also have relatively high temperatures of peak performance (35–37°C (Engel *et al*., 2013)). In the case of *L. bombicola*, growth and production of *C. bombi*-inhibiting acids increased exponentially across the 21–37°C range (Palmer-Young *et al*., 2018bb). Thus, reductions in infection with increasing temperature could reflect increases in metabolic rates of acid-producing, parasite-inhibiting bacteria. With the exception of Neisseriaceae, all major bee gut symbionts ferment carbohydrates to short-chain fatty acids (Kešnerová *et al*., 2017), and can acidify the gut to levels that inhibit growth of trypanosomatids (Zheng *et al*., 2017; Palmer-Young *et al*., 2019). However, the temperature dependence of gut pH remains unexplored.

Experiments that compare the temperature dependence of infection between bees raised under normal versus germ-free conditions could help to clarify the importance of microbiota in temperature-mediated resistance to parasites. If microbiota drive the temperature dependence of infection, then the relationship between temperature and infection should more closely resemble the thermal performance curve of isolated parasites in germ-free than in symbiont-colonized bees. Alternatively, if infection primarily reflects performance of the host immune system relative to growth of parasites, similar temperature dependence of infection would be predicted in symbiont-colonized and germ-free bees.

Temperature had smaller but significant effects on bacterial symbiont communities

In comparison to *C. bombi*, the size and general composition of the gut bacterial community was relatively robust to changes in temperature, as well as to infection treatment, but some changes were nevertheless statistically significant. In contrast to *C. bombi* infection levels, which declined with temperature, total bacterial abundance was lowest at intermediate temperature (29°C). This represents the centre of the range of body temperatures recorded in workers (Heinrich, 1972), is close to the set point at which bumble bees incubate developing brood (30°C (Vogt, 1986)), and matches the 28–30°C temperature recommended for bumble bee rearing (Velthuis and van Doorn, 2006). We hypothesize that the bumble bee immune system has optimal control over the bacterial community in this temperature range, and is able to curtail overgrowth that might otherwise deplete host resources or facilitate establishment of opportunistic infections. Research with honey bees and heat shock proteins (McKinstry *et al*., 2017) has suggested a tradeoff between temperature tolerance and immune function. Direct measurements of bee immune function could clarify the effects of temperature on hosts, while experimental augmentation or suppression of the immune response could clarify the importance of the host immune system relative to interbacterial community dynamics in the observed patterns of gut bacterial abundance. Another approach to clarify the relative importance of host- and microbiota-mediated immunity could be to compare the effects of temperature elevation on injected or systemic versus enteric pathogens; the latter would be expected to be more directly affected by gut microbes, although gut symbiont-mediated alteration of systemic immunity shows that microbiota may mediate resistance to systemic as well as gut infection (Dillon and Dillon, 2004; Kwong *et al*., 2017).

Although temperature explained more variation in bacterial composition than did any other experimental factor, average abundance of total bacteria and of specific core symbiont families did not vary by more than twofold across the experimental temperature range. Abundances of Neisseriaceae and Orbaceae were exceptionally stable, with abundances not varying by more than 30% (Fig. 2). Populations of Lactobacillaceae were the most responsive to temperature, with mean abundances increasing twofold between the lowest and highest temperatures. Experiments *in vitro* with the widespread symbiont *L. bombicola* – the most abundant member of this family among our samples – indicate that growth rate of this species increases throughout the experimental temperature range (Palmer-Young *et al*., 2018bb). Whether the Lactobacillaceae are more thermophilic than other major symbionts, or their maximum population size is simply less constrained by host surface area than those of the biofilm-forming Neisseriaceae and Orbaceae (Martinson *et al*., 2012), remains to be determined. Regardless of the exact mechanism, the Lactobacillaceae appear to have greater abundance in the gut as temperature increases.

Despite the relative stability of the core bacterial symbiont community, the twofold changes in abundance of total and specific gut bacteria across temperatures may still be biologically meaningful. These changes are equivalent to the perturbations caused by treatment of honey bees with the herbicide glyphosate and the antibiotic tetracycline, which resulted in spontaneous proliferation of non-core *Serratia* (Enterobacteriaceae) species and doubling of mortality among workers in the colony and those challenged with *Serratia* pathogen inoculation (Raymann *et al*., 2017; Motta *et al*., 2018). Moreover, in the case of glyphosate, treatment had more profound effects on microbiota when administered

to newly emerged bees than to bees that had prior exposure to the colony (Motta *et al*., 2018), as was the case in our study. It is therefore possible that temperature variation early in adult life has even stronger effects on infection and microbiota than those observed here.

Bacterial abundance was positively correlated with infection

Although the reduction in *C. bombi* infection at high temperature corresponded with higher populations of Lactobacillaceae (Fig. 2), which produced parasite-inhibiting organic acids *in vitro* (Palmer-Young *et al*., 2018b), our findings indicate that an abundance of acid-producing bacteria does not necessarily confer resistance to parasites. In models controlling for temperature, there was no significant correlation between Lactobacillaceae abundance and *C. bombi* infection (Fig. 3), whereas the effect of temperature itself remained significant (Supporting Information Table S3). Moreover, abundances of total bacteria, and specifically of Orbaceae – the most abundant family among our samples (32.7% of total 16S rRNA gene copy numbers) – were positively correlated with infection intensity in our study (Fig. 3).

We hypothesize that proliferation of both bacteria and parasites may be driven by a common factor, possibly related to suboptimal host immune function. For example, in honey bees, the same *Snodgrassella alvi* pre-treatment that resulted in high gut bacterial abundance and proliferation of *Gilliamella apicola* (Orbaceae) also led to higher levels of infection with the trypanosomatid *L. passim* (Schwarz *et al*., 2016). Although previous studies with bumble bees have implicated *Gilliamella*-rich microbiota in resistance to *C. bombi* infection (Koch and Schmid-Hempel, 2012; Mockler *et al*., 2018), the Orbaceae clade is phenotypically diverse, varying in traits such as carbohydrate metabolism and resistance to antimicrobial peptides despite conserved 16S rRNA gene sequences (Engel *et al*., 2014; Kwong *et al*., 2017), and may harbour strains associated with both health and disease. For example, a cross-colony survey correlated high levels of *Gilliamella* ('Gamma-1') with honey bee colony collapse (Cox-Foster *et al*., 2007), intensity of infection with the honey bee-infective microsporidian *Nosema ceranae* (Rubanov *et al*., 2019), and general 'dysbiosis' associated with low adult bee mass, high mortality, the scab-forming bacterium *Frischella perrara*, and *Nosema* infection (Maes *et al*., 2016).

High temperature and trypanosomatid exposure conferred protection against potentially pathogenic bacteria

Although the focus of this study was to determine how temperature affects resistance to *C. bombi*, we also found that high temperatures conferred resistance to colonization with presumably pathogenic members of the family Enterobacteriaceae. This family includes opportunistic pathogens of bees, such as *Serratia marcescens* (Raymann *et al*., 2018), as well as clinically and agriculturally important pathogens such *Salmonella*, *Klebsiella*, *Yersinia*, *E. coli* and *Erwinia* spp. Previous studies with indoor-reared bumble bees showed that when colonies were moved to outdoor environments, core symbionts were displaced by members of Enterobacteriaceae of presumed environmental origin (Parmentier *et al*., 2016). Another study suggested the presence of two 'enterotypes' among wild bumble bees, one characterized by dominance of core symbionts and the other by *Serratia* and other Enterobacteriaceae (Li *et al*., 2015), again suggesting that presence of these taxa is suboptimal for bee health. Proliferation of enteric Enterobacteriaceae has also been linked to inflammatory bowel diseases in humans (Nagalingam and Lynch, 2012). Endothermic maintenance of high body temperatures has been proposed as a factor that limits the establishment of most environmental bacteria in mammals, both by direct inhibition of parasite growth and by augmentation of the host immune response (Casadevall, 2016). We propose that high nest and body temperatures, like those found among social bees, also provide a comparative advantage to thermophilic core gut symbionts, reinforcing mutualistic relationships with bacteria while limiting establishment of potential pathogens.

Whereas abundances of core symbiont families were unaffected by infection treatment, we found that inoculation with *C. bombi* resulted in lower prevalence of Enterobacteriaceae and lower gut bacterial alpha diversity. Both results suggest that the infection treatment provoked an immune response that enhanced resistance to colonization by non-core symbionts. Previous research in bumble bees has documented effects of the microbiota on trypanosomatids (Koch and Schmid-Hempel, 2011; Mockler *et al*., 2018), but not of exposure to *C. bombi* on either beneficial or pathogenic bacteria. However, *C. bombi* inoculation can cause an immune response that resembles the reaction to injection with heat-killed bacteria, characterized by production of antimicrobial peptides, other antibacterial effector proteins and reactive oxygen species (Barribeau and Schmid-Hempel, 2013; Brunner *et al*., 2013). In mosquitoes, exposure to *Plasmodium* (malarial) parasites likewise upregulated antibacterial immune pathways (Dong *et al*., 2006). Importantly for our study, where one-third of bees cleared the experimental infection, induction of antibacterial defences did not require successful replication of parasites (Dong *et al*., 2006). Hence, mere transient exposure to trypanosomatids could act like a vaccination that elevates immunity to Enterobacteriaceae and other non-essential gut bacteria. However, detection of these effects at the landscape scale may be difficult, as different parasite strains can vary substantially in the extent to which they elicit immune

responses, and responses to the same strain can vary across colonies (Barribeau and Schmid-Hempel, 2013; Barribeau *et al*., 2014).

Resource availability could alter thermoregulation in wild bees

Maintenance of high temperatures may be optimal for resistance to parasites, but thermoregulation in wild bees might be constrained by the availability of floral resources. Like the immune response itself, endothermic elevation of nest or body temperature incurs a substantial metabolic cost, particularly at low ambient temperatures and in colonies of small size. Even under the solitary conditions of our experiments, where bees did not need to fly or incubate brood, rates of sugar water consumption were greatly (~5-fold) elevated at low temperature (Fig. 5), as previously demonstrated in bee colonies (Esch, 1960; Heinrich, 1972). Each spring, solitary queen bumble bees face thermoregulatory challenges as they emerge from hibernation to establish new colonies. This is also the stage of the life cycle at which *C. bombi* can be most virulent (Brown *et al*., 2003; Fauser *et al*., 2017). The availability of adequate early season floral resources may be a key determinant of the ability of queens to maintain body temperatures necessary for foraging, brood incubation and parasite inhibition. However, in some regions, changing climates have resulted in higher frequency of spring frosts that damage early blooming flowers, compromising key sources of nectar and pollen (Inouye, 2008). Honey bees and bumble bees, like other animals, rapidly become torpid when sugar reserves are depleted (Esch, 1960; Heinrich, 1972; Angilletta *et al*., 2010). In bats, torpor exacerbates susceptibility to fungal infections, but infection is generally cleared when animals are fed sufficiently to allow maintenance of high body temperatures (Meteyer *et al*., 2011). The consequences of periods of low body temperature for the gut microbiota of facultative endotherms remain unknown, but could affect resistance to infection.

Conclusions

The effects of high body temperatures (fever) on resistance to infection have previously been considered to reflect changes in parasite performance relative to host immune function (Casadevall, 2016; Cohen *et al*., 2017). However, non-pathogenic gut microbiota can augment resistance to infection in many ecto- and endothermic organisms (Dillon and Dillon, 2004; Spor *et al*., 2011). As a result, the effects of temperature on infection might not be fully understood without considering how temperature affects the structure and function of the gut symbiont community. Our findings show that in bumble bees, high temperature can ameliorate infection with parasites without apparent harm to the major symbiont families. On the other hand, temperature elevation did harm key symbionts of other insects (Parkinson *et al*., 2014; Kikuchi *et al*., 2016), suggesting that temperature elevation may be costly for microbiome-mediated benefits in some systems. In comparison to social bumble and honey bees, where the colony provides both gut microbial inoculum and thermoregulation, solitary insects experience a wide range of temperatures and gut microbiota (Corby-Harris *et al*., 2007; McFrederick *et al*., 2012; Moran *et al*., 2012). As a result, the effects of environmental temperature on microbiota may be considerably stronger in these purely ectothermic hosts, such as *Plasmodium*-vectoring mosquitoes, where microbiota also mediate resistance to infection (Cirimotich *et al*., 2011). As a topic with relevance to both endothermic animals that use fever as an immune strategy and ectothermic taxa that face novel infections in changing climates, interactions between temperature, infection and gut symbionts warrant further investigation, and could be a key factor in diseases of clinical and conservation concern.

Experimental procedures

Bumble bees

Five colonies of *B. impatiens* were obtained from a commercial rearing facility (Koppert Biological Supply, Howell, MI). Colonies were reared at 27°C in constant darkness, with red light illumination during periods of handling. Colonies were fed weekly with 50% (w/w) sugar water and every 2–3 days with pollen (Brushy Mountain Biological Supply, Moravian Falls, NC).

To facilitate collection of age-controlled bees, existing workers in each colony were marked on the thorax with white correction fluid (Wite-Out, BIC, Clichy France) 3 days prior to the beginning of the experiment. Thereafter, newly emerged workers (identified by their absence of thoracic marking) were collected twice weekly for experimental inoculations. Adult worker bees were isolated from the colony on the day prior to inoculation. Hence, they ranged in age from 1 to 5 days post-pupal eclosion at the time of inoculation, and 8–12 days old at the 7 days post-inoculation sampling. This protocol allowed us to pick bees that were old enough to have had time to acquire normal microbiota from the colony, which occurs within <1 days (Anderson *et al*., 2016; Billiet *et al*., 2017), but were young enough to still have microbiota in the formative period (4–6 days post-eclosion (Meeus *et al*., 2013; Powell *et al*., 2014)). Binning bees of mixed ages into a limited number of inoculation blocks allowed us to minimize variation in infection intensity due to differences in inoculum vigour across blocks, and also to obtain a more general picture of how temperature affects resistance to infection and microbiota across bees that began treatment at different ages.

Three strains of *C. bombi* were obtained from infected wild *B. impatiens* and *B. terrestris* by single cell sorting:

Strains '12.6' (from *B. impatiens* in Lufkin, TX in 2014 by Hauke Koch, 'IL13.2' (from *B. impatiens* in Normal, IL in 2013 by Ben Sadd), and 'C1.1' (from *B. terrestris* in Corsica in 2012 by Ben Sadd) (Palmer-Young *et al*., 2016). Parasites were grown at 27°C in vented culture flasks with modified Mattei growth medium as previously described (Salathé *et al*., 2012).

Inoculation, rearing and consumption measures

Experimental bees were removed from their colonies on the day prior to inoculation and housed overnight with access to 1:1 sugar water in 500 ml plastic cups containing 20–30 newly emerged workers each. Prior to inoculation, bees were transferred to individual 30 ml plastic vials and deprived of food for ~5 h.

Infection success of individual parasite strains can vary widely across colonies (Sadd and Barribeau, 2013). To improve the chances of successful infection in bees from a variety of colonies, the inoculum consisted of a 'cocktail' that included equal cell number of each of the three parasite strains (total 10^4 cells in 10 μl (Näpflin and Schmid-Hempel, 2018)). The inoculum consisted of a 1:1 mixture of 16 mM sucralose (as 1 g Splenda (Heartland Food Products, UK) in 8 ml water +16 μl red #40 food dye) and parasite cells in growth medium. Sucralose (rather than sugar water) was used to reduce osmotic shock to parasites during inoculation, and thereby improve probability of successful infection. High concentrations of sugar are lethal to *C. bombi* (Cisarovsky and Schmid-Hempel, 2014), and we observed that cells rapidly became deformed and immotile in sugar water. To control for effects of the inoculation procedure, bees in the sham infection treatment were inoculated with the same 1:1 mixture of 16 mM sucralose with dye and growth medium, but without *C. bombi* cells.

In all, 525 bees were used (N = 90–129 per colony, N = 63–67 per temperature in the *C. bombi* infection treatment and 39–43 per temperature in the sham infection treatment, Supporting Information Table S1). Final sample sizes are unequal for the parasite and sham-infection treatments because of uncertainty in rates of compliance during the inoculation. We conservatively expected only ~50% compliance during the inoculation among the bees fed the parasite inoculum. Consequently, we attempted to inoculate two bees with parasites for every one bee in the sham-infection treatment. Because rates of compliance generally exceeded 50%, final sample sizes are higher in the parasite-infection treatment.

After inoculation, bees were transferred to individual, inverted 60 ml translucent polystyrene deli cups, lined with a disk of filter paper to absorb excess moisture. Each bee was provided with a ~50 mg ball of autoclaved pollen paste, and provisioned *ad libitum* with 50% sugar water from a 1.7 ml microcentrifuge tube. Sugar water tubes were checked daily and replaced with fresh sugar water as needed. Mortality was recorded daily at time of feeding (ca. 1200 local time). Bees that escaped during the experiment were scored as not having died; they were given an end time corresponding to the first date at which they were observed missing (N = 6).

Sugar water consumption was recorded during at least two 24 h intervals, normally 3–4 days and 4–5 days post-inoculation, for one bee per unique combination of infection treatment, temperature treatment, colony and inoculation block. Consumption was calculated as the change in mass of the feeder tube from the beginning to the end of the trial. Net consumption, corrected for mass loss due to evaporation and handling, was determined by subtracting the mass loss of tubes in identical rearing setups, but without bees, at the corresponding temperature. At 7 days post-inoculation, bees were frozen in 2 ml microcentrifuge tubes on dry ice, then stored at −80°C until dissection.

Dissection and DNA extraction

Bees were dissected to remove the mid- and hindgut using standard methods described in the BeeBook (Engel *et al*., 2013). The body was thawed on ice and surface-sterilized by rinsing for 3 min in 1% household bleach (0.05% sodium hypochlorite (NaOCl)) and 3 × 1 min in doubly deionized water. The gut was removed by pulling on the distal segment of the abdomen with sterile forceps. The mid- and hindgut of the alimentary tract were drawn out onto a UV-sterilized piece of aluminium foil, then transferred to a 96-well plate for DNA extraction. Length of the marginal cell on the right forewing (in mm) was measured as an index of bee size (Wilfert *et al*., 2007).

DNA was extracted using the Qiagen DNEasy blood and tissue kit (Qiagen, Hilden, Germany). Samples were treated with 180 μl lysis buffer (Qiagen buffer 'ATL') and 20 μl proteinase K solution, then homogenized for 6 min at 30 Hz in a TissueLyser (Qiagen) with a 3.2 mm diameter steel ball and 50 μl of 0.1 mm glass beads. Homogenized samples were incubated overnight at 56°C in a convection oven. Subsequent DNA extraction was performed according to the manufacturer's instructions, including two reagent blanks per plate. Extracted DNA was stored at −80°C until use in PCR-based assays.

Quantification of infection intensity and bacterial abundance by qPCR

Bees in both infection treatments (parasite and sham control) were first screened for presence or absence of *C. bombi* by PCR using the primers CB-SSUrRNA-F2 (CTTTTGACGA ACAACTGCCCTATC) and CB-SSUrRNA-B4 (AACCGAAC GCACTAAACCCC) (Schmid-Hempel and Tognazzo, 2010). The product was visualized on a 1.5% agarose gel. This initial

screen confirmed the absence of infection among bees in the sham infection treatment, and strongly suggests that our experimental inoculations with *C. bombi* cell cultures were the sole source of trypanosomatid infections among experimental bees.

Infection intensity. For bees in the parasite infection treatment, infection intensity was quantified by qPCR of *C. bombi* DNA; *C. bombi* quantities were then normalized to quantities of bumble bee actin in the corresponding sample to correct for extraction efficiency (Palmer-Young *et al*., 2018a). Quantification of *C. bombi* was made for each sample in triplicate with primers for the *C. bombi* 18s rRNA gene ('CriRTF2' (GGCCACCCACGGGAATAT) and 'CriRTR2' (CAAAGCTTTCGCGTGAAGAAA) (Ulrich *et al*., 2011). The assay used 20 μl reaction volume consisting of 2 μl DNA extract and 300 nM of each primer in 1x Power SYBR Green Mastermix (Applied Biosystems, Foster City, CA). Thermocycle conditions included 10 min initial denaturation at 95°C followed by 40 cycles of denaturation (15 s at 95°C) and annealing-extension (60 s at 60°C). Absolute quantifications (number of parasite cell equivalents) were made relative to a standard curve consisting of eight dilutions of *C. bombi* DNA (equivalent of 3.9×10^3 to 2.5×10^5 cells) extracted from cell cultures of known concentration. The standards were run in triplicate on each assay plate, along with three no-template controls. Analysis of standard curves showed typical amplification efficiency of 90%–100% with $R^2 > 0.98$.

Bacterial abundance. Quantification of gut bacteria was made using universal primers for the bacterial 16S rRNA gene (forward: 799F-mod3 (CMGGATTAGATACCCKGG) (Hanshew *et al*., 2013), reverse: 1115R (AGGGTTGCG CTCGTTG) (Kembel *et al*., 2014)), chosen to minimize amplification of DNA from plastids in pollen (McFrederick and Rehan, 2016; Rothman *et al*., 2018). The assay used 15 μl reaction volume with 1.5 μl of 10x diluted DNA extract. (An initial round of qPCR with undiluted extracts resulted in failure of amplification in >60% of samples.) Thermocycle conditions consisted of 3 min denaturation at 95°C, followed by 40 amplification cycles of 10 s denaturation at 95°C and 30 s simultaneous annealing and extension at 59°C.

Absolute quantifications of bacteria (16S copies bee^{-1}) were made relative to an 8-concentration standard curve (10^2 to 10^8 copies μl^{-1}), run in triplicate on each plate, along with three no-template controls. Analysis of standard curves showed typical amplification efficiency of 90%–100% with $R^2 > 0.99$. The standards were generated by cloning an amplicon of the 799–1115 V5-V6 region of the 16S rRNA gene from a stock culture *of Lactobacillus micheneri* (courtesy Hoang Vuong, (McFrederick *et al*., 2018)). The amplicon was cloned into *E. coli* using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA); the reaction was confirmed by sequencing the insert of plasmids that were purified from transformed cultures (Purelink Plasmid Purification Kit, Invitrogen). The plasmid was linearized with the Pst1 restriction enzyme (New England Biolabs, Ipswich, MA). Concentration of 16S gene copies in the stock solution of linearized plasmid were estimated by fluorescence-based quantification of DNA concentration using a Qubit (Invitrogen).

Normalization. For normalization, the amount of host DNA was determined by a separate qPCR assay, also run in triplicate, for the *B. impatiens* actin 5C gene (primers Forward: CAAACGCTCGCTCAAACG, Reverse: GTGTACGTGAATGGTCTTGCAC (Palmer-Young *et al*., 2018aa)). The assay used 20 μl reaction volume consisting of 2 μl DNA extract and 300 nM of each primer in 1x Power SYBR Green Mastermix. Thermocycle conditions consisted of 10 min denaturation at 95°C, followed by 40 amplification cycles of 15 s denaturation at 95°C and 31 s simultaneous annealing and extension at 60°C. Specificity was confirmed by melt-curve analysis. Quantifications were made in units of proportion of host DNA relative to a pooled DNA extract from 10 randomly selected experimental bees (Palmer-Young *et al*., 2018a). A standard curve, consisting of eight dilutions of the pooled DNA extract, was run in triplicate on each plate, along with three no-template controls.

Characterization of gut bacterial communities

Library preparation. Gut bacterial community composition was determined for all bees by amplicon sequencing of the V5-V6 region of the 16S rRNA gene (McFrederick and Rehan, 2016; Rothman *et al*., 2018) on an Illumina (San Diego, CA) MiSeq using standard methods (Engel *et al*., 2013). Libraries were prepared using the same bacterial 16S rRNA primers (799F-mod3 and 1115R) used for bacterial qPCR. Use of the same primers for qPCR and amplicon sequencing enabled us to estimate absolute, as well as relative, abundances of individual taxa from sequence data. Libraries were prepared using two rounds of PCR as previously described (McFrederick and Rehan, 2016; Rothman *et al*., 2018). The first round of PCR amplified the target region and barcoded each sample's amplicons with unique 8-nucleotide sequences appended to the forward and reverse primers; the second round added the forward or reverse Illumina sequencing primer.

The first round of PCR (20 μl reaction volume) used 2 μl of 10x diluted DNA extract, 1 μl each of 10 μM barcoded forward (799F-mod3) and reverse (1115R) primers (final concentration: 500 nM), 10 μl of 2x Pfusion High-Fidelity DNA polymerase master mix (New England Biolabs, Ipswich, MA), and 6 μl ultrapure water. PCR was performed in a C1000 Touch thermal cycler (BioRad, Hercules, CA) (3 min

denaturation at 94°C; 24 cycles of 45 s at 94°C, 60 s at 52°C, 90 s at 72°C; 10 min at 72°C). To remove residual primers and dNTPs, each sample's product was treated with 10 μl of a solution containing 0.05 units μl^{-1} exonuclease I (New England Biolabs) and 0.025 units μl^{-1} alkaline phosphatase (Sigma-Aldrich, St. Louis, MO). Samples were incubated for 30 min at 37°C, followed by 5 min at 95°C to inactivate the enzymes. The second round of PCR used 1 μl of the treated PCR product as template, 1 μl each of 10 uM forward and reverse primers (PCR2F: CAAGCAGAAGACG GCATACGAGATCGGTCTCGGCATTCCTGC) and PCR2R: AATGATACGGCGACCACCGAGATCTACACTCTTTCC-CTACACGACG) to generate the forward and reverse Illumina adapter sequences; 10 μl of 2x Phusion MasterMix, and 13 μl ultrapure water. Thermocycle conditions consisted of 3 min at 95°C; 14 cycles of 45 s at 95°C, 60 s at 58°C, 90 s at 72°C; and 10′ at 72°C.

To equalize concentrations of DNA from each sample in the final pooled amplicon library, 18 μl of the product from the second round of qPCR was bound to, then eluted from a SequalPrep (Thermo Fisher, Waltham, MA) normalization plate according to the manufacturer's instructions. The normalized products were pooled (5 μl $sample^{-1}$), then purified with the Purelink PCR product purification kit (Invitrogen). Amplicon size and abundance were checked on a 2100 Bioanalyzer (Agilent, Santa Clara, CA). Libraries were sequenced at the UC Riverside Genomics Core Facility on an Illumina MiSeq Sequencer (Illumina, San Diego, CA) using a MiSeq V3 Reagent Kit. The sequencing run consisted of 2 × 300 PCR cycles. Raw sequence data are available on the NCBI Sequence Read Archive (SRA) under Accession number PRJNA532469.

Bioinformatics. Sequences of 16S rRNA amplicons were processed in macQIIME and QIIME2 (Caporaso *et al*., 2010; Bolyen *et al*., 2018). Reads were trimmed to removed low-quality regions, then binned to exact sequence variants ('ESV's', i.e., bacteria with identical 16S amplicon sequences) with DADA2 (Callahan *et al*., 2016). Taxonomic classification of each ESV was inferred using the SILVA database (Quast *et al*., 2013). Proportional composition and ESV richness were estimated for each sample after removal of ESV's found in only one sample or in blanks (i.e., reagent controls) and rarefaction to a read depth of 10 233 reads. A phylogeny of the observed ESV's was built using maximum likelihood in FastTree2 (Price *et al*., 2010) and used to estimate unifrac distances between samples (Lozupone and Knight, 2005).

Statistical analyses

Statistical analyses were conducted in R v3.5 for Windows (R Core Team, 2014). Models were fitted with packages lme4 (for general linear models) (Bates *et al*., 2015) and glmmTMB (for negative binomial models) (Magnusson *et al*., 2017). Significance of individual predictor terms was tested with Wald χ^2 tests, implemented with the Anova function in package car (Fox and Weisberg, 2011). Predictions from models of each response variable were estimated with package emmeans (Lenth, 2019). Plots were created with packages ggplot2 and cowplot (Wickham, 2009; Wilke, 2016). Temperature was centred at the mean value (29°C) to permit estimation of a quadratic term for the effect of temperature (i.e., $temperature^2$).

Infection intensity. For bees in the parasite-infection treatment, effects of temperature on infection intensity were tested with a negative binomial family linear mixed-effects model in R package glmmTMB (Magnusson *et al*., 2017). The negative binomial model is suited to overdispersed, non-negative count data (Bliss and Fisher, 1953), while the zero inflation term allows us to simultaneously account for two separate processes that might generate zeroes – in this case, whether or not the infection established (a binomial process), and the resulting infection intensity in bees when the infection did establish (Martin *et al*., 2005).

Infection intensity was normalized to amount of host actin to control for gut size and DNA extraction efficiency. Normalized infection intensity was computed for each sample as the qPCR estimate of number of *C. bombi* parasite cells per gut divided by the same sample's quantity of bumble bee actin. Actin quantities were expressed as the proportion of actin found in a reference extraction. The reference extraction consisted of a pooled DNA extract from 10 randomly selected experimental bees (see Methods: Quantification of infection intensity and (Palmer-Young *et al*., 2018a). Samples with less than 10% of the actin found in the reference extraction were excluded *a priori*. The model used normalized infection intensity as the response variable; temperature (centred at the mean temperature, 29°C), $temperature^2$, and bee colony as fixed predictor variables. We chose to use bee colony as a fixed effect because we had fewer than 7 levels of this factor (Bolker *et al*., 2009). Models also used ln(normalized bacterial abundance) as a covariate that was previously correlated with *C. bombi* infection (Mockler *et al*., 2018) and inoculation block as a random effect. Size of the forewing marginal cell (an index of bee size (Wilfert *et al*., 2007)) was initially included a covariate, but excluded from the final model because it did not explain significant variation in infection ($\chi^2_1 = 2.57$, $p = 0.11$).

Microbiome composition. Predictors of microbiome community structure were assessed with permutational MANOVA (Oksanen *et al*., 2017). The weighted UniFrac distance matrix of between-sample dissimilarity was used as the multivariate response variable. Infection treatment, temperature treatment (coded as a factor in this analysis only), their interaction, and bee colony were tested as predictor variables.

Significance of individual terms was assessed with *F* tests. Proportion of variation explained by each term (R^2) was determined as the ratio of the sum of squared variation associated with the predictor relative to that of that of the full model.

Total bacterial abundance. Effects of temperature and infection treatments on gut bacterial abundance were tested with a negative binomial family linear mixed-effects model in R package glmmTMB (Magnusson *et al*., 2017). Normalized 16S copy number (raw copy number divided by proportion of bumble bee actin in a reference extraction, as in the analysis of infection intensity) was the response variable. As in the model for infection, samples with less than 10% of the actin found in the reference extraction were excluded *a priori*; samples with no measurable bacteria in the qPCR assay were also excluded. Infection treatment, temperature (centred at the mean temperature treatment, 29°C) and temperature^2, and bee colony were used as fixed predictor variables; size of the forewing marginal cell was used as a covariate, and inoculation block was included as a random effect. The infection × temperature interaction was included in the initial model, but excluded from the final model because it did not explain significant variation in bacterial abundance ($\chi^2_1 = 0.36$, $p = 0.55$).

Family wise bacterial abundance. Responses of family wise abundances to temperature and infection treatments was assessed for each of the four bacterial families (Orbaceae, Neisseriaceae, Lactobacillaceae and Bifidobacteriaceae, all found in >98% of samples) that accounted for >10% of the total number of reads in the 16S amplicon sequencing. To calculate absolute abundances of each family in each sample, we multiplied total normalized abundance by the proportion of reads corresponding to each family. Changes in abundances were assessed with negative binomial family generalized linear mixed models of the same structure used to evaluate temperature-dependent changes in total bacterial abundance. Infection treatment; temperature and, where significant, temperature^2; and bee colony were used as fixed predictor variables. Size of the forewing marginal cell was used as a covariate, and inoculation block was included as a random effect. The infection × temperature interaction term was not a significant predictor in any of the models ($p > 0.20$ for all) and was excluded from the final analyses.

The fifth most abundant family, Enterobacteriaceae, was present in only 20% of samples. Its abundance was analysed with a standard binomial model, rather than the negative binomial used for the four more abundant families. Terms for the infection × temperature interaction ($\chi^2_1 = 0.57$, $p = 0.45$), temperature^2 ($\chi^2_1 = 2.11$, $p = 0.15$), bee colony ($\chi^2_4 = 5.93$, $p = 0.20$) and wing size ($\chi^2_1 = 0.45$, $p = 0.50$) were excluded from the final model because they did not explain significant variation in prevalence.

Correlations between family wise abundance and C. bombi *infection intensity.* Correlations between abundances of each of the four most abundant bacterial families and *C. bombi* infection were assessed with negative binomial family generalized linear mixed models of the same structure used to evaluate temperature-dependent changes in infection intensity, except that ln(abundance +1) of the individual family was substituted for ln(total bacterial abundance) covariate. Temperature, temperature^2 and bee colony were used as fixed predictor variables. Inoculation block was included as a random effect. Size of the forewing marginal cell was included as a covariate in the model with Neisseriaceae abundance, but was removed from the other models because it did not explain significant variation in infection ($p > 0.05$ for Wald chi-squared tests).

Alpha diversity (ESV richness). Number of unique ESV's per sample was analysed with a Poisson family linear mixed model that used infection treatment, temperature and temperature^2, and bee colony as fixed predictor variables. Inoculation block was included as a random effect. The infection × temperature interaction ($\chi^2_1 = 0.81$, $p = 0.37$) and wing marginal cell size ($\chi^2_1 = 0.05$, $p = 0.82$) were excluded from the final model because they did not explain significant variation in ESV richness.

Sugar water consumption. Effects of temperature and infection treatments on sugar water consumption (g bee^{-1} day^{-1}) were tested with a linear mixed-effects model in R package lme4 (Pinheiro and Bates). Net consumption values that were less than 0 after correction for evaporation were assigned a trivial positive mass of 1 mg. Square root-transformed net mass of sugar water consumed was the response variable; infection treatment, temperature treatment, their interaction and bee colony were used as predictor variables. Size of the forewing marginal cell was used as a covariate. Inoculation block was included as a random effect, as was individual bee identity to account for non-independence of repeated measures on the same individual. A temperature^2 term was initially included in the model, but excluded from the final model because it did not explain significant variation in consumption ($\chi^2_1 = 0.79$, $p = 0.37$).

Mortality. Effects of temperature and infection treatment on mortality were tested with a Cox proportional hazards mixed-effects model (Therneau, 2015). Death hazard rate was the response variable; infection treatment, temperature treatment, their interaction and bee colony were used as predictor variables; and inoculation block was

included as a random effect. Exploratory plots and models showed no linear trends of mortality by temperature ($\chi^2_1 = 0.20$, $p = 0.65$), and models that included a temperature^2 term failed to converge; therefore, this analysis treated temperature as a factor rather than as a continuous variable. Marginal cell size was initially included in the model, but excluded from the final model because it did not explain significant variation in the response ($\chi^2_1 = 1.26$, $p = 0.26$).

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Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

Raw sequence data are available on the NCBI Sequence Read Archive under Accession number PRJNA532469. All other data are supplied in the Supporting Information, Data S1.

Author Contributions

ECPY and QSM conceived the study. ECPY, TRR and QSM designed experiments. ECPY, LN and RBN conducted experiments. EPY and JAR analysed data. EPY drafted the manuscript. All authors revised the manuscript and gave approval for publication.

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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: Supporting Information 1.
Appendix S2: Data S1.