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Hot and sour: parasite adaptations to honeybee body temperature and pH

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Host temperature and gut chemistry can shape resistance to parasite infection. Heat and acidity can limit trypanosomatid infection in warm-blooded hosts and could shape infection resistance in insects as well. The colony-level endothermy and acidic guts of social bees provide unique opportunities to study how temperature and acidity shape insect–parasite associations. We compared temperature and pH tolerance between three trypanosomatid parasites from social bees and a related trypanosomatid from poikilothermic mosquitoes, which have alkaline guts. Relative to the mosquito parasites, all three bee parasites had higher heat tolerance that reflected body temperatures of hosts. Heat tolerance of the honeybee parasite *Crithidia mellificae* was exceptional for its genus, implicating honeybee endothermy as a plausible filter of parasite establishment. The lesser heat tolerance of the emerging *Lotmaria passim* suggests possible spillover from a less endothermic host. Whereas both honeybee parasites tolerated the acidic pH found in bee intestines, mosquito parasites tolerated the alkaline conditions found in mosquito midguts, suggesting that both gut pH and temperature could structure host–parasite specificity. Elucidating how host temperature and gut pH affect infection—and corresponding parasite adaptations to these factors—could help explain trypanosomatids' distribution among insects and invasion of mammals.

1. Introduction

Infection by parasites depends on their ability to survive and proliferate under the conditions found in their hosts [1]. Two defining characteristics of this environment are temperature and pH. Host body temperature can profoundly affect host–parasite interactions [2]. In particular, elevated host body temperature due to physiological or behavioural fever limits parasite growth and reduces infection-related morbidity in diverse animals, including insects [3–5]. pH is another driver of microbial establishment [6]. Gut pH contributes to sterilization of food and limits proliferation of opportunistic pathogens [7,8], shaping species-specific resistance to parasites in the insect gut [9]. Understanding how temperature and pH affect host specificity in insect parasites could help identify host and parasite adaptations that impact infection of economically important insects and potential insect parasite spillover into mammals.

Trypanosomatid gut parasites of insects infect a diverse range of hosts—comprising a variety of thermal niches and gut physiologies—with apparently loose host–parasite specificity that remains poorly understood [10]. The invasion of mammals by a subset of these insect-associated species—*Leishmania* and *Trypanosoma*—is thought to be limited by mammals' high body temperatures [11], which can confine infections to (cooler) peripheral body sites even in established mammalian pathogens [12]. In *Leishmania*, where the mammalian stage is intracellular, the low pH of the phagocyte lysosome poses an additional barrier to infection [12]. Nevertheless, putatively monoxenous (i.e. insect-restricted) but heat-tolerant Leishmaniinae species [13,14] occasionally infect humans [13,15]. If temperature and pH limit the establishment of insect trypanosomatids in mammals, these same factors—which vary widely across insect geographic ranges

and nutritional niches [16]—could affect the host specificity of parasites among insects as well.

The social honey and bumblebees offer unique opportunities to study parallel adaptations to high temperature and low pH in monoxenous trypanosomatids. Whereas most solitary insects have a small body size and limited ability to thermoregulate, social bees inhabit large, thermoregulated colonies with temperatures resembling those of warm-blooded mammals [17,18]. Such high temperatures increase resistance to some pathogens [19,20] and could limit infection by heat-intolerant trypanosomatids as well. Second, bee diets consist of sugar-rich nectar and polysaccharide-rich pollen, which are fermented to organic acids by gut symbionts to maintain an acidic pH in the honeybee hindgut and rectum [21,22]. This contrasts with the guts of haematophagous dipteran insects—including mosquitoes—which obtain nitrogen from low-polysaccharide animal blood and have near neutral to highly alkaline guts as adults and larvae [23–25].

To test whether host thermoregulation and diet-associated gut pH can limit trypanosomatid infection in insects, we compared the effects of temperature and pH on growth of phylogenetically related hindgut parasites from honeybees (*Crithidia mellificae* and *Lotmaria passim* from *Apis mellifera*), bumblebees (four strains of *Crithidia bombi* from *Bombus* spp., using published data [26,27]) and mosquitoes (two strains of *Crithidia fasciculata*, which infects multiple genera of Culicidae [28]). The two major honeybee trypanosomatids—*C. mellificae* [29] and the emerging parasite *Lotmaria passim*, both in the Leishmaniinae [30]—have a global distribution, can reach greater than 90% prevalence in managed colonies and have been associated with colony collapse on three continents [31–35]. Both species—as well as the bumble parasite *C. bombi* [36]—establish in the hindgut and rectum, the most acidic regions of the intestine [21,37]. Based on the thermal strategies of their host species, we predicted that parasites of highly endothermic honeybees would have greater heat tolerance than parasites from mosquitoes, with intermediate heat tolerance in parasites of bumblebees—which thermoregulate their nests at lower temperatures than do honeybees [38]. We also predicted that parasites of pollen-eating bees would tolerate acidity better than would parasites of blood-consuming mosquitoes, reflecting differences in the diets and gut pH of their hosts.

2. Material and methods

(a) Cell cultures

The honeybee parasites *C. mellificae* (ATCC 30254 [29]) and *L. passim* (strain BRL [30]) and the mosquito parasite *C. fasciculata* (strains 'CFC1' [39] and 'Wallace' (ATCC 12857)) were obtained from the American Type Culture Collection and collaborators. Honeybee parasites were grown in 'FPFB' medium including 10% heat-inactivated fetal bovine serum (pH 5.9–6.0 [40]). Mosquito parasites were grown in brain–heart infusion broth with 20 µg/ml haemin (pH 7.4). All parasites were incubated at 20°C in vented cell culture flasks and transferred to fresh media every 2 days; experiments with bee and mosquito parasites were conducted using their respective media.

(b) Temperature experiments

Parasite growth rates were measured by optical density (OD_{600}) at temperatures of 20°C and at 2°C intervals between 23°C and 41°C on a temperature-controlled microplate reader with 0.1°C resolution (Biotek 'Synergy' H1). Cultures were diluted in fresh media to a net OD of 0.040 (after accounting for the OD of the media) and aliquoted to 96-well plates containing 120 µl media per well. Measurements were taken every 5 min for 24 h, with 30 s of shaking before each measurement. Each plate contained 15 wells (treated as technical replicates) of each of the four parasite strains and six cell-free control wells—containing an equal volume of media without parasites—to control for growth-independent changes in OD during incubation. The 26 plates (one per run of the experiment) consisted of two plates at each of the 11 temperatures (to avoid confounding the effects of run and temperature), plus a third plate for each of four temperatures (25, 31, 33 and 35°C) spanning the region of primary interest.

(c) pH experiments

Parasite growth rates were measured between pH 2.1 and 11.3. Aliquots of the base medium for each parasite were first acidified (with HCl) and alkalized (with NaOH) to extreme pH levels that inhibited growth in preliminary trials. Treatments were prepared by acidified and alkalized media in varying proportions to generate 12 treatments spanning a broad pH range. To initiate the assay, a suspension of cultured cells was diluted 12-fold in each treatment for a starting OD of 0.020 in a volume of 120 µl. Each experimental block contained one well per strain plus two cell-free controls of each pH treatment. Growth rates were measured at 29°C (at which all strains grew strongly) for 24 h at 5 min intervals using a microplate reader. Final pH (after addition of fresh media to 1/12 of the final volume) was measured for each treatment using a pH electrode, calibrated immediately prior to measurement. The entire experiment was performed twice (bee parasites: range 2.14–11.2 (Block 1) and 2.45–11.3 (Block 2); mosquito parasites: 3.85–10.9 (Block 1) and 3.80–11.3 (Block 2)), yielding a single replicate well for each of 24 pH levels per strain.

(d) Comparisons with previous results

To compare thermal performance curves of honeybee parasites and their hosts, we used data for the temperature dependence of force generation during honeybee flight [41] (electronic supplementary material, figure S1). For comparison to parasites from hosts with intermediate levels of thermoregulation, we used previously published data for the thermal performance of four strains of the bumblebee parasite *C. bombi*. For these datasets, growth rates of four strains were measured across temperatures from 17 to 42°C [26], and growth rates of one strain were measured across pH values from 5.0 to 6.2 [27] (electronic supplementary material, figure S2). We used the mean value from a meta-analysis on 88 traits to depict the peak performance temperature of mosquitoes (28.4°C [42]).

(e) Statistical analysis

Analyses were conducted using R for Windows v4.0.3 [43]. Models were fit using package 'rTPC' [44]. Figures were made with packages 'ggplot2' and 'cowplot' [45,46].

(i) Growth rates

Net OD was calculated by subtracting the mean OD from cell-free controls of the corresponding media, treatment and time point. Growth rates for each well were calculated as the maximum slope of the curve of ln(OD) versus time, obtained by fitting a rolling linear regression to each 4 h (48-measurement) window of the growth curve [47]. The first 2 h of each run were excluded to allow OD readings to stabilize. We used only slopes with r^2 values of greater than 0.95 and greater than 0.90 for the temperature and pH experiments, respectively, and assigned a growth rate of zero

to samples where the average slope of the growth curve was negative (to avoid spurious rate estimations based on low cell densities). For temperature experiments, we used the median growth rate among the 15 replicates within each plate, to avoid pseudoreplication within each plate-level implementation of the temperature treatment [48].

(ii) Temperature models

We modelled the temperature dependence of growth for each trypanosomatid strain using a Sharpe–Schoolfield equation modified for high temperatures [47,49,50].

$$\text{rate}(T) = \frac{r_{T_{\text{ref}}} \cdot e^{\frac{-E}{k}\left(\frac{1}{T}-\frac{1}{T_{\text{ref}}}\right)}}{1 + e^{\frac{E_h}{k}\left(\frac{1}{T_h}-\frac{1}{T}\right)}}. \quad (2.1)$$

In equation (2.1), *rate* refers to the maximum specific growth rate (in [h^{-1}]); $r_{T\text{ref}}$ is the growth rate (in [h^{-1}]) at the calibration temperature T_{ref} (293 K, i.e. 20°C); E is the activation energy (in eV), which primarily affects the upward slope of the thermal performance curve (i.e. sensitivity of growth to temperature) at suboptimal temperatures; k is Boltzmann's constant (8.62×10^{-5} eV·K^{-1}); E_h is the deactivation energy (in eV), which determines how rapidly the thermal performance curve decreases at temperatures above the temperature of peak growth T_{pk} (in K); T_h is the high temperature (in K) at which growth rate is reduced by 50% (relative to the value predicted by the Arrhenius equation—which assumes a monotonic, temperature-dependent increase) [50] and T is the experimental incubation temperature (in K). An identical model was fit to the honeybee force data.

(iii) pH models

To describe the effects of pH on growth rates, we used a biphasic logistic model that describes sigmoidal decreases in growth rate at low and high pH.

$$\text{rate}(\text{pH}) = \frac{r_{\max}}{1 + e^{-E_L((1/pH_L)-(1/pH))} + e^{E_h((1/pH_h)-(1/pH))}}. \quad (2.2)$$

In equation (2.2), $r_{\max}$ is the specific growth rate at the optimum pH; E_L and E_h correspond to the rates of deactivation at low and high pH, respectively; and pH_L and pH_h represent the pH values at which growth rate is reduced by 50% relative to $r_{\max}$. For *C. bombi*, the absence of high-pH data precluded use of a biphasic model; we therefore used a standard (monophasic) logistic model instead, which omitted the second term of the denominator in equation (2.2).

Models were optimized using nonlinear least squares, implemented with R packages rTPC and nls.multstart [44]. Confidence intervals on parameter values and predicted growth rates were obtained by bootstrap resampling of the residuals (10 000 model iterations, R package 'car' [51]). We also used the bootstrap model predictions to estimate the following traits: temperatures of peak growth rate (T_{pk}) and 50% inhibition relative to the peak value (IT_{50}); pH of peak growth (pH_{pk}) and pH niche breadth (i.e. the number of pH units between pH_L and pH_h). The 0.025 and 0.975 quantiles for parameter estimates, predicted growth rates at each temperature and traits derived from bootstrap predictions were used to define 95% confidence intervals. Strains were considered significantly different from each other when their 95% confidence intervals did not overlap.

3. Results

(a) Temperature experiments

The two honeybee parasites showed higher heat tolerance than the mosquito parasites (figures 1 and 2). One honeybee parasite (*Crithidia mellificae* (T_{pk}: 35.4°C, 95% CI: 34.9–35.9°C; IT_{50}: 38.7°C, CI: 38.5–38.9°C)) grew well throughout the temperature range found in honeybee hives during brood-rearing (33.8–37°C [18]) and exhibited the peak growth temperature closest to that of *A. mellifera* (38.4°C [41]; electronic supplementary material, figure S1). The heat tolerance of the emerging honeybee parasite *L. passim* (T_{pk}: 33.4°C, CI: 32.6–34.4°C; IT_{50}: 37.0°C, CI: 36.5–37.4°C) was significantly (2°C) less than that of *C. mellificae*, with predicted growth rates reduced by greater than 50% at the upper end of the thermal range found in colonies (figure 1). Thermal performance curves and parameter estimates were similar for the two strains of the mosquito parasite *C. fasciculata*, where temperatures of peak growth (strain CFC1: 31.1°C, CI: 30.2–31.9°C; strain Wallace: 31.6°C, CI: 31.0–32.3°C) and 50% inhibition (CFC1: 35.3°C, CI: 34.8–35.9°C; Wallace: 35.5°C, CI:35.3–35.9°C) were significantly lower than for either honeybee parasite (by approximately 2°C relative to *L. passim* and approximately 4°C relative to *C. mellificae*) (figure 2). Nevertheless, both strains had peak growth temperatures (T_{pk}) that exceeded the mean T_{pk} for a variety of traits in diverse mosquito species (28.4°C [42], figure 2).

Thermal performance curves of *C. bombi* from bumblebees (mean T_{pk}: 33.7°C; mean IT_{50}: 37.90°C, figure 2; electronic supplementary material, figure S2) most resembled that of *L. passim* from honeybees. Although the coarser 5°C temperature interval for the published *C. bombi* data resulted in higher uncertainty, all four strains of this species had significantly (approx. 2°C) higher inhibitory temperatures (IT_{50}) than did the mosquito parasite *C. fasciculata* (figure 2).

(b) pH experiments

We observed the greatest tolerance to acidity in the two parasites of honeybees, each of which grew at nearly two units' lower pH than either *C. fasciculata* from mosquitoes or the previously tested *C. bombi* from bumblebees. Both honeybee parasites maintained strong growth at the pH of the honeybee rectum (pH 5.2 [21] (figure 3). *Crithidia mellificae* had the broadest pH niche, with the greatest tolerance of both acidity (50% low-pH inhibition (pH_L): 3.07, 95% CI: 2.97–3.25) and alkalinity (50% high-pH inhibition (pH_h): 9.93, CI: 9.55–10.21, figure 4). *Lotmaria passim* was nearly as tolerant of acidity as *C. mellificae* (pH_L: 3.44, CI: 3.35–3.53) but grew weakly above pH 7 (pH_h: 7.33, CI: 7.24–7.43), with peak growth pH (5.57, CI: 5.20–5.76) closely matched to that of the host rectum (figures 3 and 4; note that neither strain's curve showed a well-defined peak).

By contrast, both strains of the mosquito parasite *C. fasciculata* grew fastest at neutral to weakly basic pH (pH_{pk} for CFC1: estimate 7.58, CI: 6.90–8.10; Wallace: estimate 7.42, CI: 7.05–7.73, figures 3 and 4). Although tolerance of acidity was significantly less than in the honeybee parasites (pH_L for CFC1: 5.01, CI: 4.71–5.24; Wallace: 5.08, CI: 4.86–5.39), the two strains were tolerant of alkaline conditions (pH_h for CFC1: 9.62, CI: 9.39–9.84; Wallace: 9.24, CI: 9.01–9.47; both significantly higher than for *L. passim*) that approached those in the midgut of their host *Culex pipiens* [25] (figures 3 and 4). The acidity tolerance of *C. bombi* (pH_L 5.18, CI: 5.17–5.19) was indistinguishable from that of *C. fasciculata* (figure 4; see electronic supplementary material, figure S4 for full *C. bombi* curves). *Crithidia bombi* was also notable for its steep decline in growth rate between pH 6 and pH 5 [27],

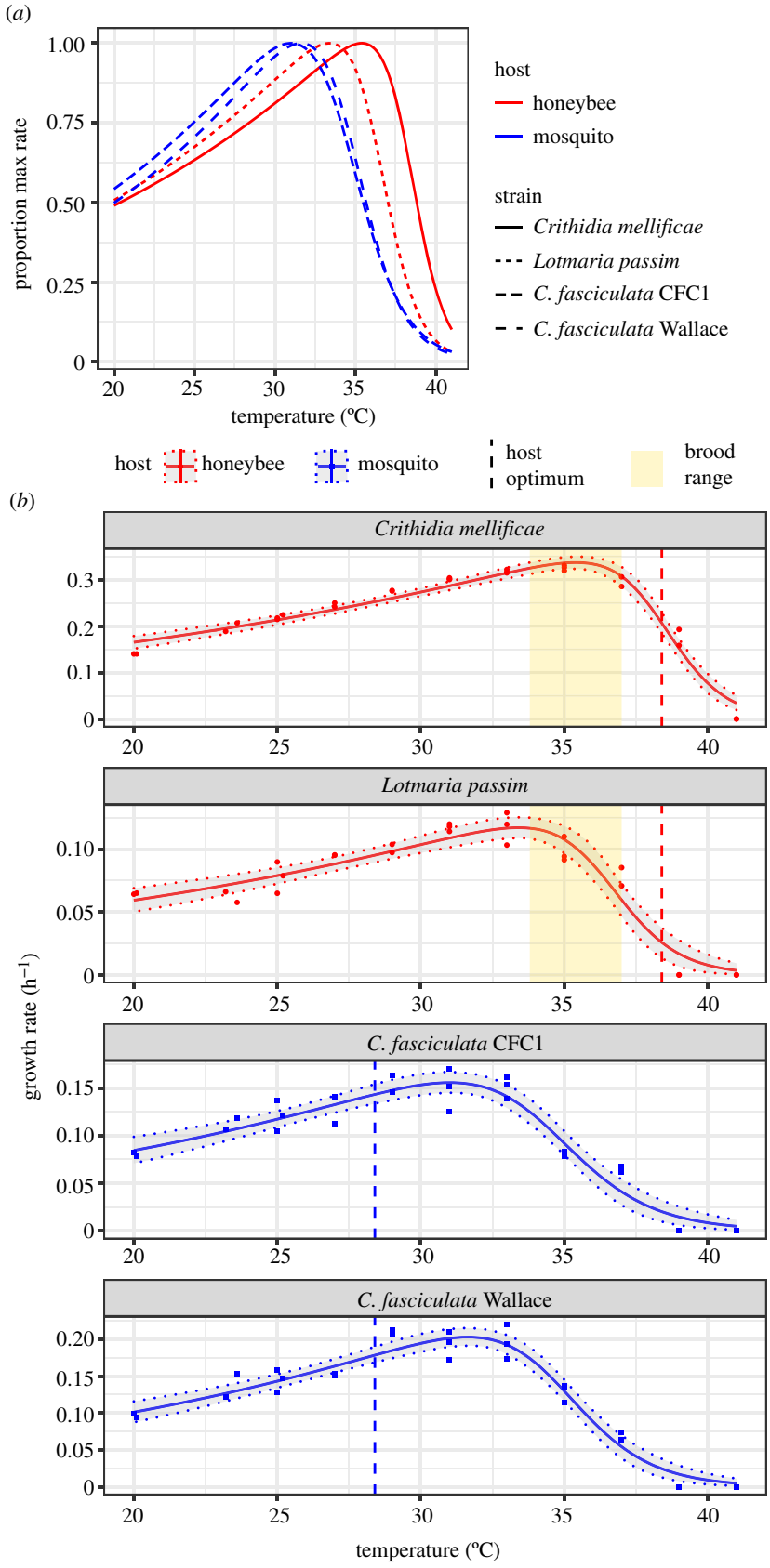

Figure 1. Thermal performance curves for trypanosomatid parasites from honeybees (*Crithidia mellificae, Lotmaria passim*) and mosquitoes (*Crithidia fasciculata*). (*a*) Scaled curves for all strains. (*b*) Details for each strain. Each point represents the median specific growth rate (h^{-1}) from one 15-replicate experiment, with colour and shape corresponding to the parasite's host. Lines and shaded bands show predictions and 95% bootstrap confidence intervals from Sharpe–Schoolfield models [44,50]. Vertical lines show optimum temperatures for honeybees (estimated from force production during flight [41]) and mosquitoes (mean of 88 traits [42]). Vertical band (in yellow) shows temperature range for honeybee brood incubation [18]. See electronic supplementary material, figure S1 for full thermal performance curve of honeybee force production. (Online version in colour.)

which was reflected in an estimate for deactivation energy (parameter E_l) more than sixfold higher than that of the strains tested here (electronic supplementary material, figure S5).

4. Discussion

Our results show an association between social thermoregulation and parasite heat tolerance, suggesting a possible role

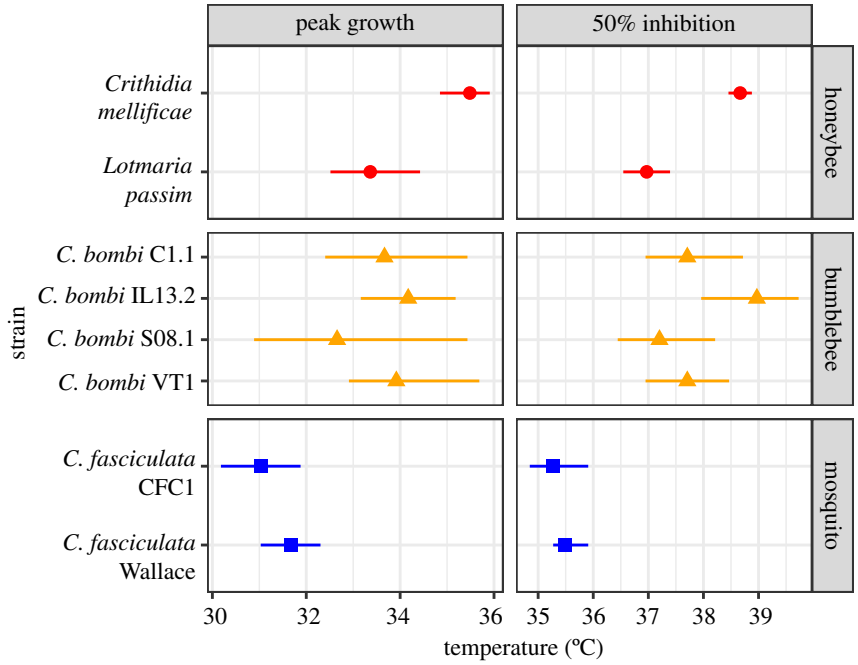

Figure 2. Temperatures of peak growth and 50% inhibition of growth rate for parasites of honeybees (*Crithidia mellificae, Lotmaria passim*), bumblebees (*C. bombi*, tested in [26]) and mosquitoes (*C. fasciculata*). Points and error bars show estimates and 95% bootstrap confidence intervals for predictions from Sharpe–Schoolfield models. See electronic supplementary material, figure S2 for full thermal performance curves for *C. bombi*. Estimates for additional model parameters are shown in electronic supplementary material, figure S3. (Online version in colour.)

for colony-scale endothermy in social bees as a filter for gut parasites. Although only four species were examined, all the parasites from endothermic social bees showed greater heat tolerance than did parasites from mosquitoes. Heat tolerance of *C. mellificae* exceeds that of all previously studied, poikilothermic tropical insect-associated trypanosomatids that were noted for heat tolerance, but nevertheless grew more slowly at 37°C than at 28°C [52–54]. Growth of *Leptomonas seymouri*—which occasionally infects humans [15]—was likewise poor at 37°C [55]. By contrast, the growth of *C. mellificae* was approximately 30% faster at 37°C than at 28°C. Such heat tolerance was suggested by Cosgrove & Mcghee [56], whose review stated that an unnamed trypanosomatid from *Vespula squamosa* (presumably ATCC strain 30862 of *C. mellificae*) grew in avian embryos at 37°C with no prior acclimation. However, the relevant reference [57] did not mention *C. mellificae*. The species that maintained growth in embryos at 37°C was *Crithidia acanthocephali* [58]. Although originally isolated from a hemipteran [58], sequences matching this species were recently amplified from honeybees in Spain [59]; the parasite's heat tolerance could facilitate its survival in bees.

The warm-blooded mammal-like temperatures of a breeding honeybee colony [18] likely preclude infection by trypanosomatids with low heat tolerance and could exert positive selection for heat tolerance within parasite lineages. For parasites that do establish in colonies, our results suggest that high colony temperatures might reduce infection intensities. Even growth of the most heat-tolerant parasite (*C. mellificae*) peaked at a lower temperature than did flight performance of honeybee hosts (38.4°C, figure 1). Peak performance temperatures of flight muscle [60] and respiration [61] in bumblebees are also high (greater than 40°C). This suggests that increases in temperature could favour increases in host metabolic performance—perhaps including immune function—while inhibiting parasite growth. Honey and bumblebee gut symbionts—which enhance resistance to *C. bombi* [62]—are likewise heat-tolerant. Honeybee symbionts have standard culturing temperatures of 35–37°C [63], can grow at temperatures up to 44°C [64] and tolerate hour-long heat shock at 52°C [64]. A *Lactobacillus* species from bumblebees was similarly thermophilic, with a peak growth temperature of approximately 40°C [26]. High temperatures could therefore enhance the antiparasitic activities of these symbionts as well as performance of the bee immune system [27], harnessing the bees' socially enabled thermoregulation and core gut microbiota for defense against infection.

Our results suggest that maintenance of high, 'social fever'-like colony temperatures would be particularly effective against the relatively heat-susceptible *L. passim* and *C. bombi*. Growth rates of *L. passim* dropped by approximately 50% over the 3.2°C range found in brood-rearing honeybee colonies (figure 1). Similarly, the infection of *C. bombi* was 81% lower at 37°C than at 21° [65]. Inoculations of honeybees with *C. mellificae* were likewise less successful at 35°C than at 29°C (albeit in separate experiments [29]). Our results also suggest that bees may become increasingly susceptible to infection as they transition from activities at the well-heated colony core to the cooler and more variable periphery, or to foraging outside (at age 10–25 days [66]). Observations of experimentally infected, colony-reared bees—which showed a 10-fold increase in parasite mRNA between ages 7 and 27 days [67]—are consistent with these predictions. However, similar age-related infection dynamics were observed in caged bees at constant temperatures [67], suggesting that other age-related factors could also contribute to this pattern.

Honeybee trypanosomatid infection intensities are inversely related to temperature in field colonies [68]. In managed US colonies, *L. passim* infection intensity (originally described as *C. mellificae* [30,69]) peaked in mid-winter,

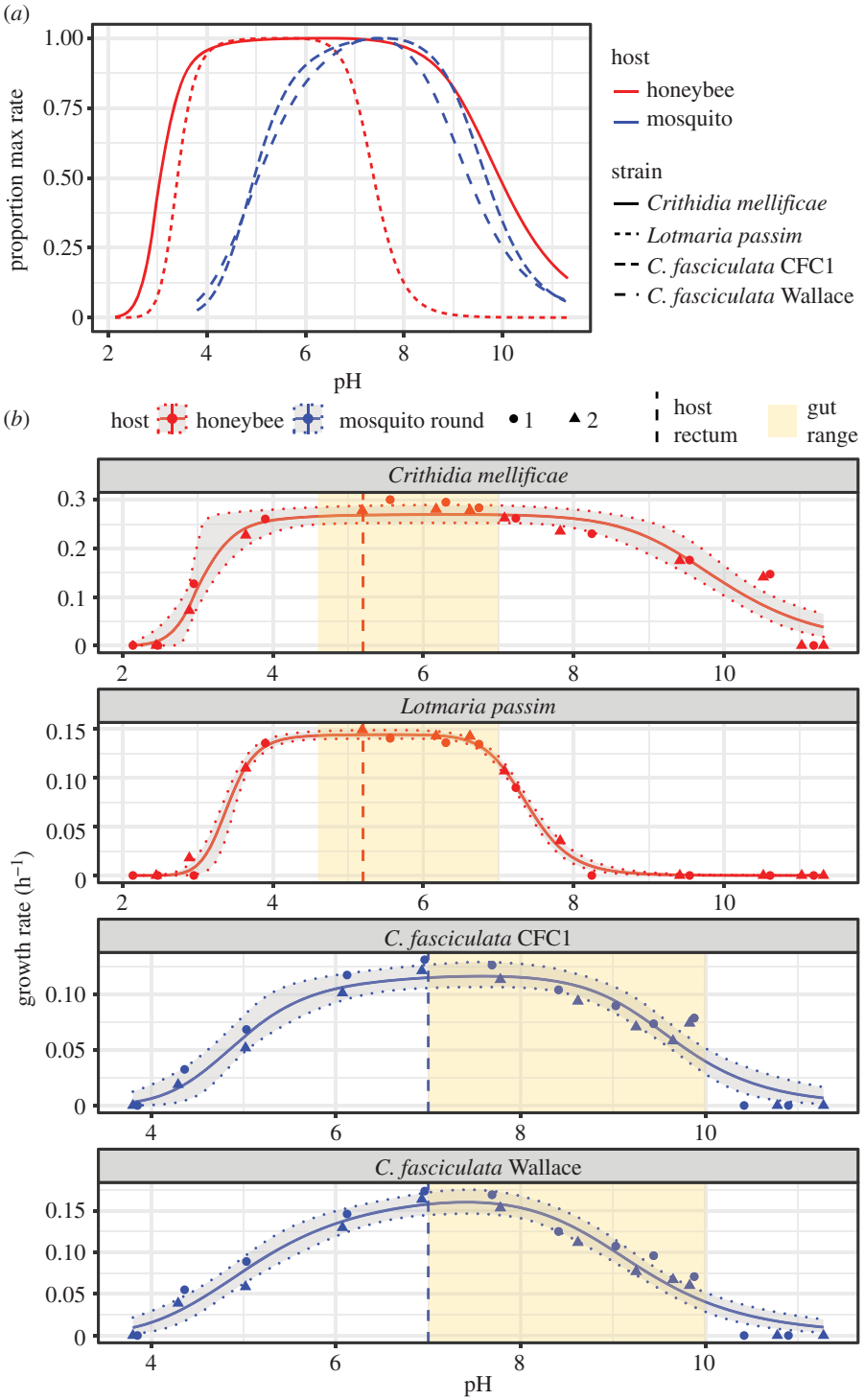

Figure 3. Effects of pH on growth of trypanosomatid parasites from honeybees (*Crithidia mellificae*, *Lotmaria passim*) and mosquitoes (*Crithidia fasciculata*). (*a*) Scaled curves for all strains. (*b*) Details for each strain. Each point represents the specific growth rate (h^{-1})) from one sample. The experiment was conducted over two experimental blocks (Round 1: circles; Round 2: triangles). Lines and shaded bands show predictions and 95% bootstrap confidence intervals from biphasic logistic models. Vertical lines and shaded regions show pH of the rectum (primary site of parasite infection) and range of the gut overall, as measured previously in honeybees [21,37] and *Culex pipiens* mosquitoes [25]. (Online version in colour.)

when colony core temperatures average 14°C lower than in summer [18]. Such temperature-dependent infection dynamics could explain the associations between trypanosomatid infection and overwinter colony collapse [32]. Seasonal susceptibility of colonies to infection could be exacerbated by landscape, chemical and nutritional factors that impair thermoregulation [70,71]. For example, colonies from agricultural areas had average winter temperatures 8°C lower than did colonies from grasslands [72], highlighting how land use changes could affect temperature-mediated resistance to an emerging infectious disease.

Lotmaria passim's low heat tolerance relative to *C. mellificae*, susceptibility to the high temperatures found in honeybee colonies, and apparently recent global emergence in *A. mellifera* [30] invite speculation of a recent host shift from a less endothermic bee species. The Asian honeybees *Apis cerana* [73] and *A. dorsata* [74] have approximately 2°C lower brood temperature optima relative to *A. mellifera*

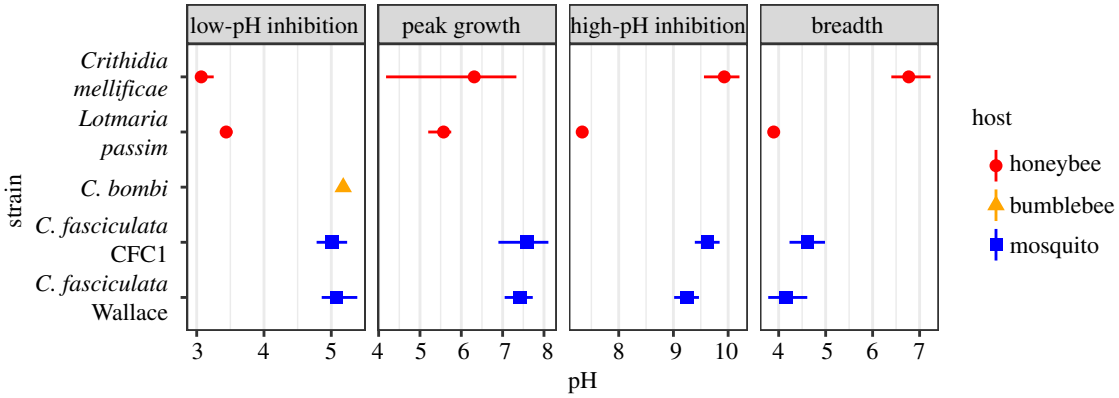

Figure 4. Estimates for pH of peak growth, 50% inhibition of growth rate due to low and high pH, and pH niche breadth (i.e. difference between estimates of 50% inhibition due to low and high pH) for parasites of honeybees (*Crithidia mellificae*, *Lotmaria passim*), bumblebees (*C. bombi* strain IL13.2, tested in [27]) and mosquitoes (*C. fasciculata*). Points and error bars show estimates and 95% bootstrap confidence intervals for predictions from biphasic logistic models. Colours and shapes correspond to host of origin. See electronic supplementary material, figure S4 for full model predictions for *C. bombi*. Estimates for additional model parameters are shown in the electronic supplementary material, figure S5. (Online version in colour.)

[18]—matching the approximately 2°C difference in optimal and inhibitory temperatures between *C. mellificae* and *L.* passim. *Apis cerana* harboured an *L. passim* haplotype basal to the strains found on other continents [75], providing circumstantial phylogenetic evidence for an Asian parasite origin. Such a host shift could parallel the worldwide dispersal of the now ubiquitous microsporidian *Nosema ceranae* from *A. cerana* [76].

The acid tolerance in parasites of honeybees and alkaline tolerance in parasites of mosquitoes suggests that gut pH—itself a reflection of diet, digestive physiology and microbiota—could also be an important driver of host specificity in trypanosomatid parasites of insects. The tolerance of acidic conditions shown by honeybee parasites—and the low optimum pH of the emerging parasite *L. passim*—reflect the typically acidic pH found in the honeybee rectum where these parasites establish [21,29,30]. This tolerance of acidity was noted by Langridge & McGhee [29] in their isolations of *C. mellificae*. The honeybee's low gut pH results from fermentation of pollen polysaccharides by the characteristic bee gut microbiota [21,22]. In humans, acidic intestinal and faecal pH levels likewise reflect the intake and subsequent fermentation of dietary polysaccharides [77], with consequences for microbiome composition and growth of opportunistic pathogens [7,78]. The pH of the bee rectum—which at pH 5.2 is over a full pH unit more acidic than the already pathogen-inhibiting faeces of humans consuming fibre-rich vegan diets (pH 6.3 [78])—may likewise provide protection against opportunistic invaders, including non-specialist trypanosomatids.

Although standard trypanosomatid culture media is neutral to weakly basic (e.g. brain–heart infusion broth, pH 7.4), enhancement of growth under acidic conditions has been reported before. For example, the growth of *H. samuelpessoai* occurred between pH 4 and pH 9 [54]. In addition to *C. mellificae*—described as 'acidophilic', with optimum growth at pH 5 [29]—McGhee described enhanced growth under acidic conditions (pH 5 versus pH 8) in three additional trypanosomatids and found growth exclusively at low pH in two others [79]. All these acidophilic species were isolated from hemipteran hosts; two were from the giant milkweed bug *Oncopeltus fasciatus*, whose gut pH (4.6–5.4 [80]) resembles that of honeybees—suggesting potential for bee–hemipteran parasite exchange.

By contrast—and concordant with our results—the parasite species that thrived under basic conditions (including *C. fasciculata*) were from dipterans [79], where gut pH is typically extremely alkaline. For example, the original host of our *C. fasciculata* (*Culex pipiens*) has a midgut pH greater than 10 in larvae [25]—yet this life stage can still be infected by *C. fasciculata* [28]. Similarly high pH values occur in the larval guts of other Diptera (e.g. midgut pH of 11 in bibionid larvae [24]). In mosquito adults, the midgut is near pH 6 in sugar-fed adults [81], but is alkalized to pH 8.5–9.5 following ingestion of blood [23]. Adaptations to these conditions are reflected in our results, with both *C. fasciculata* strains growing fastest near neutral pH (6–8) and remaining viable up to pH 10 (figure 3), consistent with previous characterizations [82]. Intriguingly, the difference in pH optima between the honeybee parasite *L. passim* and the mosquito parasite *C. fasciculata* matched almost exactly the differences between the optima for the mammalian tissue (amastigote, pH 5.5) and insect (promastigote, pH 7–7.5) stages of *Leishmania* [12]. This raises the question of whether differences in pH tolerance among species of monoxenous taxa and between life stages of dixenous taxa can be explained by similar mechanisms, and whether tolerance of acidity is correlated with the tolerance of high temperature (as in *Leishmania* [12]).

Contrary to predictions, the bumblebee parasite *C. bombi* did not exhibit the high tolerance of acidity found in the honeybee parasites. The single report of bumblebee gut hindgut pH that we could locate (pH 6.25 from *Bombus fervidus* [16]) is substantially higher than the pH < 5.2 measured in honeybees [21,37], but a close match to the pH 6.0–6.2 that yields optimal growth of *C. bombi* (electronic supplementary material, figure S4, [27]). Although honey and bumblebees have similar pollen- and nectar-based diets and gut microbial communities [83]—which might be expected to result in similar gut pH—they exhibit marked differences in physiology and behaviour. Bumblebees have a more rapid intestinal transit time than do honeybees [84], leaving less time for acid-generating fermentation driven by host and symbiont processes. By contrast, honeybees not only have slower baseline transit times, but also

fastidiously refrain from defaecation in the colony—a behaviour not exhibited by bumblebees [85]. As honeybees spend the first 10–25 days in the colony before they forage outdoors [66], the pollen-rich rectal contents have considerable time to acidify. During the winter, honeybees commonly retain rectal contents for several months while confined in the colony [86]. Meanwhile, they continue to ingest pollen, with their distended guts exhibiting increases in populations of fermentative hindgut bacteria [87]. We hypothesize that these behaviours result in lower gut pH—and greater selection on parasites for tolerance of acidity—in honeybees than in bumblebees.

The same heat tolerance that allows insect trypanosomatids to infect endothermic bees could also pre-adapt parasites for infection of warm-blooded mammals. Several supposedly monoxenous species have been found in humans—often together with the expected *Leishmania* [13,15,56]—and proven infectious in the glands of opossums and the skin and organs of mice [13,88], demonstrating the ability to proliferate at 37°C. Intriguingly, trypanosomatids with DNA sequences identical to *C. mellificae* were recently isolated from the blood of numerous wild mammals in Brazil [89,90]. The viability of these parasites at 37°C [90]—consistent with our findings—would permit survival in the mammalian bloodstream, perhaps additionally aided by parasite acclimation to high temperatures in honeybee colonies. Given that *L. seymouri*—one of the closest known relatives of *C. mellificae* [30]—occasionally infects humans [15] despite minimal growth at 37°C [55], corresponding infection of mammals by *C. mellificae* seems plausible. Although pathways of transmission remain unclear, we have shown that *C. mellificae* from honeybees can proliferate in bees of other families—including halictids, which are attracted to mammalian perspiration [91]. The impressive range of pH tolerance shown here could also support its survival in other, possibly haematophagous hosts with diverse gut physiologies.

5. Conclusion

Our interspecific comparisons—including the first tests of temperature and pH tolerance in the emerging parasite *L. passim*—suggest colony-level endothermy and diet- and microbiome-related changes in gut acidity as drivers of host specificity in insect trypanosomatids. Our results also provide a mechanistic explanation for the relative resistance of honeybees to trypanosomatids from other insects [92] and the recent findings of *C. mellificae*—a presumed monoxenous parasite—in a variety of warm-blooded mammals [89,90]. Escape from parasites could be one factor that favours the evolution of energetically costly social endothermy and maintenance of gut symbiont communities in insects, providing infection-related benefits that parallel those found in homeothermic vertebrates while exerting parallel selective pressures on parasites.

Data accessibility. All data are supplied in the electronic supplementary material, data S1 [93]. An earlier version of this article is available from *bioRxiv*, a preprint server for biology (https://doi.org/10.1101/2021.07.03.447385) [94].

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Competing interest. We declare we have no competing interests.

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