Socially transmitted gut microbiota protect bumble bees against an intestinal parasite

Hauke Koch[1] **and Paul Schmid-Hempel**

Institute of Integrative Biology, Swiss Federal Institute of Technology (ETH) Zürich, CH-8092 Zürich, Switzerland

Edited by Nancy A. Moran, Yale University, West Haven, CT, and approved October 13, 2011 (received for review June 28, 2011)

Populations of important pollinators, such as bumble bees and honey bees, are declining at alarming rates worldwide. Parasites are likely contributing to this phenomenon. A distinct resident community of bacteria has recently been identified in bumble bees and honey bees that is not shared with related solitary bee species. We now show that the presence of these microbiota protects bee hosts against a widespread and highly virulent natural parasite (*Crithidia bombi*) in an experimental setting. We add further support to this antagonistic relationship from patterns found in field data. For the successful establishment of these microbiota and a protective effect, exposure to feces from nest mates was needed after pupal eclosion. Transmission of beneficial gut bacteria could therefore represent an important benefit of sociality. Our results stress the importance of considering the host microbiota as an "extended immune phenotype" in addition to the host immune system itself and provide a unique perspective to understanding bees in health and disease.

coevolution | immune defense | symbiont | Trypanosomatidae | *Bombus terrestris*

Pathogens are believed to play a major role in the recent worldwide decline of honey bee and bumble bee populations (1–4), raising concerns over the loss of pollination services for agricultural crops and wild flowering plants (5, 6). However, what protects bees against pathogens? In addition to the host's immune system, vertically transmitted microbial symbionts are sometimes suspected to play a role in insect defense against infection by viruses (7), bacteria (8), or eukaryotic parasites (9).

With regard to social insects, group living can facilitate the transmission of not only parasites (10) but also beneficial microbes (11). Adult honey bees and bumble bees have recently been shown to harbor a specialized and surprisingly species-poor community of bacteria in their gut (12, 13). These specific bacteria appear to be absent in solitary bee species, suggesting that a stable association with their hosts may be facilitated by sociality in these groups of corbiculate bees (12). Furthermore, experiments on honey bee larvae have demonstrated a protective effect of lactic acid bacteria against secondary infections by the bacterial pathogen *Paenibacillus larvae* (14). However, evidence is thus far lacking for a potential role of the bee gut microbiota in protecting against parasitic infections in workers, which is the caste most exposed to the environment (15). Furthermore, the role of social contact of adult bees for the establishment of these microbiota has not been tested. Here we analyze the gut microbiota found in bumble bees (*Bombus terrestris*) as a possible protection against the trypanosomatid gut parasite *Crithidia bombi*. This parasite is very common and has drastic effects on spring queens that are about to found their colonies: infection leads to a fitness loss of 40–50% compared with healthy queens (16). We separated worker pupae from the nest before eclosion and kept the emerged workers in isolation to simulate a solitary lifestyle. By exposing one treatment group to feces of nest mates, we tested the role of social contact for the establishment of the distinct microbiota of bees and its effects on subsequent parasite infections.

Results and Discussion

In a previous pilot experiment, we fed antibiotics to a sample of workers emerged from isolated pupae. This group showed high mortality during the course of the experiment (51% compared with 2% in the groups fed on sugar water without antibiotics), and the survivors did not contain detectable numbers of bacteria with our DNA-amplification protocol (13) at the end of the experiment. Infection levels after their exposure to the parasite *C. bombi* were measured and used for comparison with the outcomes in the other treatment groups (Fig. 1). The high parasite load of this group (Fig. 1) should thus give an idea of the expected maximal parasite loads in the absence of bacteria in the gut (and in an evidently weakened host, as indicated by the high mortality). As a caveat, the result of the antibiotics-fed group is not completely comparable to the results of the actual experiments because the antibiotics themselves probably strongly impaired the health of the so-treated bees. In the actual experiment, we fed workers raised from isolated pupae in a semisterile environment and from five different colonies with fresh feces from their nest mates (group a), a culture of the dominant Gammaproteobacteria that had initially been isolated from the bees (group b) (Betaproteobacteria were not cultivable so far), or sterile sugar water control (group c). Apart from one individual in group b, all workers survived until the end of the experiment (2% overall mortality).

Microbial community analyses by terminal restriction fragment length polymorphism (TRFLP) showed that the gut microbiota of workers taken from inside the source colonies and experimentally raised workers fed with feces from their source colonies (group a) were indistinguishable (Fig. 2). The microbial community of group a was also very close to the "wild type" that we observed in field-caught *Bombus terrestris* workers (Fig. 2). In a previous study (13), we showed that, based on a 16S rRNA gene clone library, the bacterial communities of *Bombus* spp. are species-poor and host species-specific and matched both the TRFLP findings as well as a recent study by Martinson et al. (12). Altogether, our experimental animals appear to be fairly characteristic with respect to their bacterial communities and, hence, our results should have a general bearing. Note that these findings are unlikely to be just an artifact of the TRFLP method. In fact, the method has repeatedly been shown to be highly reproducible and to monitor changes in community composition with great precision (see, e.g., refs. 17–19). If anything, the method might be problematic for highly diverse communities, such as in soil (18), but the community in the bumble bee gut is species-poor (12, 13) and can therefore be reliably monitored by

Author contributions: H.K. and P.S.-H. designed research; H.K. performed research; H.K. and P.S.-H. analyzed data; and H.K. and P.S.-H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. JF813790).

[1]To whom correspondence should be addressed. E-mail: hauke.koch@env.ethz.ch.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1110474108/-/DCSupplemental.

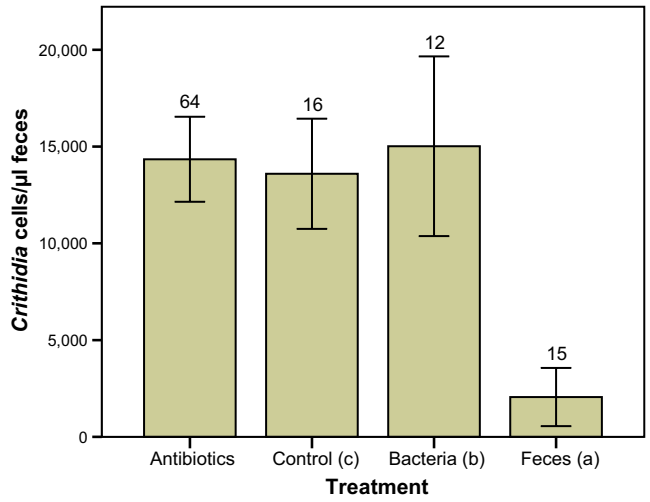

Fig. 1. Comparison of the number of *C. bombi* cells per µL of feces at 7 d postinfection for the three treatment groups and the antibiotics-fed individuals. The sample size of each group is listed above the bars. (Error bars: ±1 SE.)

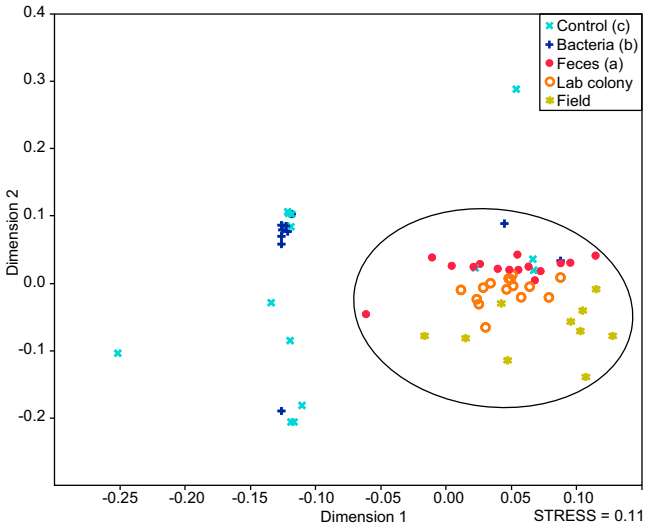

Fig. 2. Two-dimensional nonmetric multidimensional scaling (NMDS) analysis of the gut bacterial communities of the three experimental treatment groups (a, feces; b, bacteria; and c, control) and three randomly selected individuals from each source laboratory colony (Lab colony) and uninfected individuals from the field source population (Field). The distances are based on a Bray–Curtis dissimilarity matrix from the TRFLP profiles. The ellipse marks the community dominated by Gamma- and Betaproteobacteria (clades I and III in ref. 13) and is considered "normal" in this study.

TRFLP analyses. Furthermore, our results are completely in line with previous findings (12) that used a sequencing approach with potentially higher resolution.

As demonstrated previously (13), the TRFLP profiles of the communities analyzed here are also strongly dominated by peaks corresponding to two groups of Gamma- and Betaproteobacteria. These were the most abundant bacterial taxa (clades I and III in ref. 13) in the previous study of wild bumble bees, too. For the individuals fed on a sterile control (group c), the 16S rRNA gene either did not amplify, indicating the number of bacteria in the gut was below the detection threshold of our method, or mostly showed highly aberrant TRFLP profiles compared with those found in the source colonies or with those of wild-caught individuals (Fig. 2). Notably, these control profiles lacked the peaks corresponding to the Gamma- and Betaproteobacteria.

Workers of group b were fed with cultured Gammaproteobacteria (Betaproteobacteria could not be cultured). However, we did not detect the TRFLP peak characteristic for the strain of bacteria fed to this treatment group (with the exception of a single individual), suggesting that these bacteria had most likely been cleared from the gut by the end of the experiment. The reasons for the apparent failure of the in vitro-cultured Gammaproteobacteria to successfully establish in treatment group b remain unclear. Perhaps in vitro culturing had rendered them noninfectious because bees fed with fresh feces from bees infected with the Gammaproteobacteria became readily infected. A rapid loss of infectivity during in vitro culturing has been described before for bacteria closely associated with their hosts (20, 21), including one report for honey bees exposed to an undescribed bacterium referred to as "Bacterium eurydice" (22). Altogether, therefore, the combined results indicate that feeding feces from nest mates leads to the establishment of the microbiota comparable to that present in workers within the source colony and also similar to the microbiota observed in healthy bumble bees in the field. Bumble bees without contact with the feces of their nest mates, or that were fed the dominant Gammaproteobacteria from a culture, had either an untypical or a largely absent microbiota.

Our experimental treatment significantly affected the parasite infection load (Kruskal–Wallis $H = 12.77$, df $= 2$, $P = 0.002$, $n = 43$). Infection intensity of *C. bombi* was high and did not differ between the two treatment groups with untypical gut bacteria (control group c and bacteria-fed group b; Fig. 2) (Dunnett's post hoc test, two-sided, $t = -0.22$, $P = 0.77$). These groups also had similarly high infection levels as did the antibiotics-treated individuals from the previous experiment (Fig. 1). In contrast, those individuals fed with feces, and thus obtaining a wild-type bacterial microbiota (Fig. 2), were significantly less infected than the control group c (Dunnett's post hoc test, two-sided, $t = -3.67$, $P < 0.001$), with an average parasite load almost one order of magnitude lower than in the other treatments (Fig. 1). For all individuals from the experiment combined, parasite load was also significantly negatively correlated with the sum of the relative TRFLP peak heights of Gamma- and Betaproteobacteria (Spearman rank correlation $\rho = -0.45$, $P = 0.002$) or Gammaproteobacteria ($\rho = -0.56$, $P < 0.001$) and Betaproteobacteria separately ($\rho = -0.40$, $P = 0.005$) (Fig. S1).

At the end of the experiment, we also typed the diversity of strains that had established after exposure to the experimental mixture of *C. bombi* strains in the inoculum. The experimental treatment affected the diversity of *C. bombi* strains (Kruskal–Wallis $H = 6.43$, df $= 2$, $P = 0.04$, $n = 43$). We found that the two treatment groups lacking the normal gut microbiota (control group c and bacteria-fed group b) did not differ from each other in strain diversity [control: 2.50 ± 0.35 strains (±SE); bacteria-fed: 2.67 ± 0.40 strains; Dunnett's post hoc test, two-sided, $t = 0.23$, $P = 0.57$], but the control group c had significantly higher numbers compared with the feces-fed group with intact microbiota (1.47 ± 0.37 strains; Dunnett's post hoc test, two-sided, $t = -2.41$, $P = 0.03$). Hence, the normal microbiota of *B. terrestris* workers seems to protect them against diverse and intense infections, both of which have been shown to facilitate the transfer of the parasite to daughter queens (23) and to reduce their fitness (16).

We also compared our experimental findings with the bacterial communities of wild-caught workers. In the field data, we observed a negative association between the presence of *C. bombi* and the fraction of the bacterial community that was made up by the Betaproteobacteria (clade III in ref. 13) and a weak trend for the Gammaproteobacteria (clade I in ref. 13) in the same direction (Fig. 3). A logistic regression with presence or absence of *C. bombi* infections as response variable showed that the

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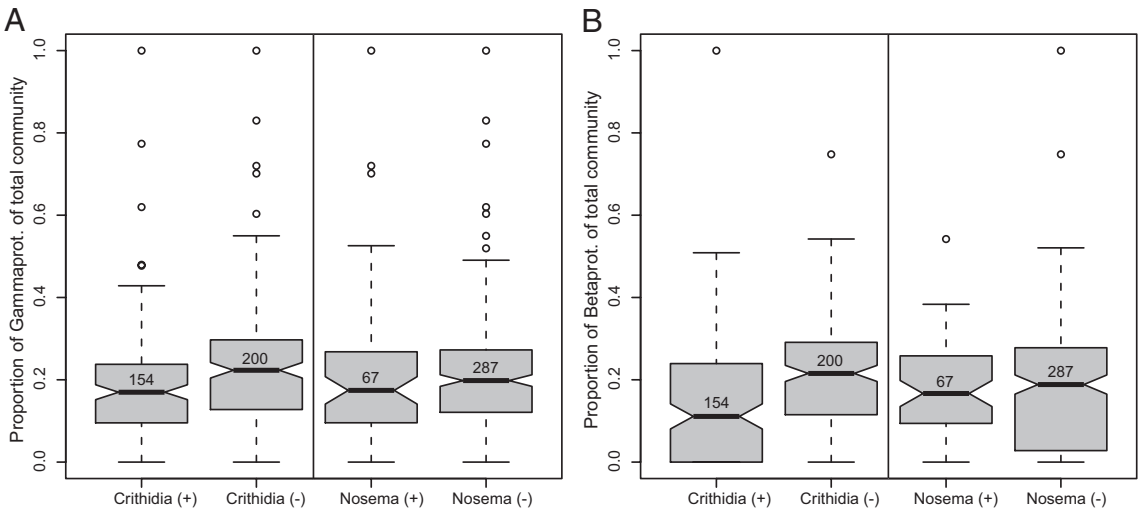

Fig. 3. Box plot showing comparisons between field-caught individuals either infected (+) or uninfected (–) with *Crithidia* or *Nosema* for the relative contribution of the dominant Gammaproteobacteria (*A*) and Betaproteobacteria (*B*) to the whole gut bacterial community. Numbers on each box plot indicate the sample sizes for each group. The notches on the box plots indicate an ~95% confidence interval of the median calculated as median $\pm$ 1.58 $\times$ IQR/$\sqrt{(n)}$, with IQR being the difference between the third and first quartiles (48). The relative contribution of the two bacteria was calculated as the area of the TRFLP peak corresponding to either of the bacteria divided by the sum of all peaks in an individual profile.

proportion of Betaproteobacteria significantly predicted an infection (P = 0.004); however, a significant effect was not found for the proportion of Gammaproteobacteria (P = 0.37). No significant relationship emerged for the presence or absence of the intracellular microsporidian parasite *Nosema bombi* and the fractions of Gammaproteobacteria (P = 0.75) or Betaproteobacteria (P = 0.52), respectively, in a logistic regression (Fig. 3).

C. bombi is genotypically diverse and abundant in the field (24, 25) and can have serious fitness effects on spring queens that are founding a colony (16). In this article, we show that experimental addition of the gut microbiota reduces the load by this parasite. The average parasite load of ≈ 2,000 cells per μL of feces in group a is in line with the parasite load of previous experiments using similar conditions [e.g., refs. 26 (≈ 2,000 cells per μL after 7 d), 27 (≈ 3,000 cells per μL after 7 d), and 28 (≈ 300 cells per μL averaged over 22 d after infection)], whereas the absence of the specific microbiota led to parasite loads almost an order of magnitude higher (Fig. 1). Furthermore, field data suggest that a high percentage of Betaproteobacteria in the gut correlates with a low likelihood of infections by the gut parasite *C. bombi* but not by the concurrent intracellular parasite *N. bombi*.

The mechanism by which the gut microbiota reduce the parasite load remains unclear. Several observations, however, suggest a direct interaction between the bacteria and the *Crithidia* parasites in the gut rather than a general fitness effect of a lack of the microbiota on the bees. First, the absence of the microbiota was not linked directly to a higher host mortality because all workers from the control group c survived over the course of the experiment. The high mortality in the antibiotics-fed individuals was therefore likely caused by a direct toxic effect of the antibiotics rather than by an increase in host mortality because of the absence of gut bacteria. Second, a weakening of the host immune system because of a poorer nutritional status caused by a reduction in digestion efficiency in bees lacking the resident microbiota is an equally unlikely explanation. Because bees were fed ad libitum with sugar water and pollen in our experiment, a reduced efficiency in digestion could have been compensated for by an increased food intake. Furthermore, poorer host nutritional status of *B. terrestris* has been shown to lead to a reduction in *C. bombi* parasite load (29), making our results the opposite of what would be expected for the control group c in the case of a limited supply of nutrients caused by the absence of the resident microbiota. Third, although ingesting bacteria has been shown to stimulate the immune system in honey bees (30), the bees fed with a high concentration of Gammaproteobacteria in our experiment did not show a reduction in parasite load; therefore, this is also unlikely to be the cause of the observed effect. Successful establishment of the resident microbiota therefore appears to be necessary. Possible mechanisms for the reduction in parasite load may be increased competition for resources in the gut by the presence of gut microbiota (8) or the production of antimicrobial substances by the bacteria (14, 31).

During metamorphosis, holometabolous insects undergo a major gut reorganization in which the larval gut is replaced completely and shed as meconium after emergence (32). During development, the pupal midgut furthermore becomes sterilized as a potent mixture of antimicrobial substances is produced (33). The newly formed gut therefore has to become recolonized by bacteria from the environment. As seen in the TRFLP profiles, our workers indeed tended to lack the specific gut bacteria if they were raised under semisterile conditions and not infected with feces from their nest mates after emergence from the pupa (Fig. 2). Clearly, the social environment of bumble bees (or honey bees) must facilitate the uptake of bacteria from nest mates, which may lead to a predominantly vertical transmission and subsequent coevolution of specific gut bacteria with these bees. Accordingly, solitary bees lack these specific microbiota (12). Predominantly vertically transmitted symbionts can spread in a host population by conferring a benefit to their host, for example, against pathogens (34). This host protection appears to have evolved numerous times in insects; so far it has mostly been studied with intracellular symbionts (7, 9), although recently extracellular symbiotic Actinobacteria have been found to have a protective function for digger wasps (35), leaf cutter ants (36), and pine beetles (37). Theoretical models predict that if (horizontally transmitted) parasites reduce fecundity rather than increase host mortality, selection for protection by symbionts is more likely (38). This theoretical prediction would fit well with the horizontal transmission mode (39), weak effect on host mortality (40), but high reduction in host fecundity (16, 41) of the parasite *C. bombi*.

The reduction of parasite load by gut microbiota in bumble bees observed in this study may have wider implications for other

host–parasite systems. Similar gut microbiota have been observed in honey bees (12). These microbiota have been speculated to play a role in honey bee health (15, 42), but studies are lacking so far. In addition, gut bacteria may influence infection success by trypanosomatid parasites in vectors of human diseases. For example, the Tsetse fly bacterial symbiont *Sodalis glossinidius* increases susceptibility of its host toward *Trypanosoma brucei*, the cause of sleeping sickness (43). In contrast, *Trypanosoma cruzi*, the cause of Chagas disease, is inhibited by a strain of *Serratia marcescens* in its insect vector *Rhodnius prolixus* (31). Recently, a specific gut bacterial community has also been found in the phlebotomine sand-fly vectors of leishmaniasis, which might interact with *Leishmania* parasites (44). Similar phenomena occur when transplanting feces from humans with a healthy gut flora to patients with an antibiotics-induced disturbed gut flora that is associated with severe *Clostridium difficile* infections. In this case, the reestablishment of the gut microbiota can cure *C. difficile* infections (45). As in bees, humans appear to have a distinct resident community of gut bacteria that may be predominantly vertically transmitted through social contact (46, 47). Because the gut bacterial communities in bumble bees are much simpler than the one found in humans (13), and they can also easily be manipulated experimentally, bumble bees represent a good model system for understanding interactions among the host, its microbiota, and potential pathogens.

We provide experimental evidence that the microbiota of social bees play a major role in protecting adult bees and could pertain to the field situation too. This finding will have major consequences for our understanding of bees in health and disease. For example, bumble bees and their *Crithidia* parasites have extensively been used in the past and are now a generally acknowledged model system to study the ecology of host–parasite interactions. Our results now suggest that the microbiota could be a key part of this interaction. If so, bumble bees, their microbiota, and *Crithidia* will also be a good model system to study these interactions more generally on an ecological scale because the microbiota are specific and simple, and experimental techniques are well developed to conduct experiments with bumble bees in both a laboratory setting and under natural conditions in the field.

Materials and Methods

Experiments were carried out on five *B. terrestris* colonies kept in the laboratory and originating from first laboratory-generation parasite-free queens derived from laboratory colonies of queens collected in central Switzerland (Neunforn, 47°35′40″N, 8°47′30″E) in 2010. To keep workers free from potential infections, cocoons were removed from the colonies and kept at 30 °C with 70% relative humidity. Upon emergence, the workers were transferred into sterile individual plastic boxes and fed ad libitum throughout the experiment with filter-sterilized (0.2-μm pore size) sugar water and pollen (heated for 30 min at 85 °C). At 2 d after emergence, bees were fed with 15 μL of (*a*) a mixture of fresh feces from four individuals of the same colony (source) and sugar water (1:2 mixing ratio), (*b*) a mixture of a culture (10^9 cells·mL^{-1}) of the dominant Gammaproteobacteria in bumble bees (*SI Materials and Methods*) and sugar water (1:2 mixing ratio), or (*c*) a sugar water control. The Gammaproteobacteria were selected for treatment group b because they were previously found to be one of the two dominant bacterial members of the bumble bee gut community (12, 13), and we did not succeed in culturing the other dominant member from the Betaproteobacteria (*SI Materials and Methods*).

After 5 d more, all bees were fed with 15 μL of a mixture of sugar water and five *C. bombi* strains (with 3,000 cells of each strain; see *SI Materials and Methods* for inoculum preparation). After 7 d more, feces were collected from each individual bee, *C. bombi* cell concentrations were quantified with Neubauer-type counting chambers, and individuals were frozen for further analyses.

To examine the bacterial communities of field-caught *B. terrestris* workers, infected or uninfected with *C. bombi* and *N. bombi*, workers were collected from 40 colonies kept in the field near the collection site of our experimental queens (*SI Materials and Methods*). The dissection, DNA extraction, and analysis of bacterial TRFLP profiles for the bees from both the laboratory experiment and the field collection followed the method described in ref. 13. For the laboratory experiment, *C. bombi* strains surviving in the gut through the experiment were genotyped via microsatellites (24) (*SI Materials and Methods*). We previously characterized the gut microbiota of *B. terrestris* by using a combination of 16S rRNA gene clone libraries and TRFLP profiles (13). The findings of this study indicated a dominant role of two bacterial taxa from the Gamma- and Betaproteobacteria for the gut microbiota based on both clone library and TRFLP data. These bacteria could unambiguously be associated with two characteristic TRF peaks in our method (13). The 16S rRNA gene sequence data also suggested that these bacteria were highly specific to bumble bees (with related bacteria in honey bees). We therefore focused our analysis of the TRFLP profiles particularly on the TRFs of these two bacterial taxa. As a measure of the relative contribution of the dominant Gamma- and Betaproteobacteria to the entire gut bacterial community, the TRF peak area of the peak corresponding to each of the two taxa was divided by the sum of all peak areas of an individual sample. Although TRF peaks do not provide reliable information on the absolute abundance of the corresponding taxon, they can be used to monitor differences in relative abundance of taxa within a community with high repeatability and precision if a standardized TRFLP protocol is used (17–19).

In the year before this experiment, a group of bumble bees was fed, for 3 d after emergence, on an antibiotics mixture of 250 μg/mL each of rifampicin, streptomycin, ampicillin, and tetracycline dissolved in sugar water. Bees originated from 10 laboratory colonies founded by field-collected queens from the same population as the other bees used in this study (*SI Materials and Methods*). After a further 7 d on sugar water free of antibiotics, the bees were infected with *C. bombi*, and infections were assessed after 7 d. The presence or absence of gut bacteria was checked by using the dissection, DNA extraction, and 16S rRNA gene-amplification protocol described in ref. 13. The *C. bombi* strains used for infection and the methods for infecting and counting parasite cells in feces samples were identical to the ones described above. The bees from this experiment were included for comparative purposes in Fig. 1, reflecting the parasite loads in individuals cured of their microbiota by antibiotics.

For details on all statistical analyses, see *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank G. Cisarovsky, R. Schmid-Hempel, Y. Ulrich, S. Büchel, M. Jales Hone, and M. Dal Molin for help and comments and the Genetic Diversity Centre at the Swiss Federal Institute of Technology Zürich for providing a work platform. This work was supported financially by Swiss National Science Foundation Grant 31003A-116057 (to P.S.-H.).

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