Pathogen-associated self-medication behavior in the honeybee *Apis mellifera*

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Abstract Honeybees, *Apis mellifera*, have several prophylactic disease defense strategies, including the foraging of antibiotic, antifungal, and antiviral compounds of plant products. Hence, honey and pollen contain many compounds that prevent fungal and bacterial growth and inhibit viral replication. Since these compounds are also fed to the larvae by nurse bees, they play a central role for colony health inside the hive. Here, we show that honeybee nurse bees, infected with the microsporidian gut parasite *Nosema ceranae*, show different preferences for various types of honeys in a simultaneous choice test. Infected workers preferred honeys with a higher antibiotic activity that reduced the microsporidian infection after the consumption of the honey. Since nurse bees feed not only the larvae but also other colony members, this behavior might be a highly adaptive form of therapeutic medication at both the individual and the colony level.

Keywords Honeybee · Honey · Antimicrobial activity · Therapeutic self-medication · *Nosema ceranae* · Social immunity

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Introduction

Honeybees, *Apis mellifera*, are highly eusocial insects, and cooperation among colony members is essential for efficient brood care, foraging, or colony defense. The benefits resulting from cooperative behavior are the main drivers for the success of the honeybee colony (Seeley 2010). However, living in large social groups has also an important downside when it comes to pathogens and parasites. Once inside the colony, most pathogens find ideal homeostatic conditions with optimal temperatures and high humidity that greatly facilitate their spread among all colony members (Schmid-Hempel 1998; Evans and Schwarz 2011).

Indeed, honeybee colonies are threatened by a large suite of pests and pathogens, including viral, fungal, and bacterial infections (Schmid-Hempel 1998; Evans and Schwarz 2011). Surprisingly, the honeybee genome lacks many genes of the innate immune system that are typical for many insects (Evans et al. 2006). Nevertheless, the colony is by no means defenseless since honeybees use a range of the most efficient acquired immune defenses (e.g., antiseptic and hygienic behavior) to fight microbial infections (reviewed in Wilson-Rich et al. 2009). On the one hand, these defense mechanisms include behavioral adaptations of the workers ("social immunity," hygienic behavior). On the other hand, honeybees take advantage of plant-derived compounds that are foraged by the workers to boost their immune defense by self-medication (König 1985; König and Dustmann 1986; Simone et al.

2009; Simone-Finstrom and Spivak 2012). Many of these plant compounds are well studied because they have been tested in relation to human diseases (reviewed in Efem et al. 1992; Ratcliffe et al. 2011). Examples of the so-called therapeutic self-medication are known from primates ingesting plant material with antiparasitic chemicals (Clayton and Wolfe 1993; Lozano 1998). Medicinal plants have antiinflammatory, antimicrobial, immunomodulatory, and analgesic effects on primates (reviewed in Hart 2005). Also monarch butterflies use specific food plants to protect their offspring from parasite infections (Lefèvre et al. 2012). Interestingly, the effects of these compounds on honeybee health have received much less attention, yet it is here where we would expect specific evolutionary adaptations.

Honeybee foragers either collect water or plant products: nectar, pollen, and plant resins (propolis), all of which have been shown to contain suites of highly potent antimicrobial and antiviral compounds that potentially interfere with colony health (Dustmann 1979; Herbert 1992; Viuda-Martos et al. 2008; Kwakman et al. 2010). Honeybee workers have excellent skills to recognize and learn sources with rewarding forage (reviewed in Frost et al. 2012). Both visual and odor cues are known to be essential for this recognition behavior (reviewed in Menzel and Müller 1996; Galizia et al. 2011; Menzel 2012). This has been primarily shown in the context of foraging for food, but given the significance of pathogens for fitness, it would appear to be at least equally adaptive in relation to colony health (Fouks and Lattorff 2011; Schmid-Hempel 1998).

Whereas food choice for foragers in the field is primarily a matter of providing energy, protein and water, this is already different for propolis foragers who specialize on plant resins (Simone-Finstrom and Spivak 2012). For example, Simone-Finstrom and Spivak (2012) showed that chalkbrood-infected colonies increase propolis foraging leading to decreased infection intensities. Once pollen and nectar are stored inside the colony, the nurse bees which care for and feed the larval brood are in a central position for the distribution of the foraged items in the nest. On the one hand, they feed on the food stored to maintain their own development which includes developing the hypopharyngeal glands that secrete the food jelly fed to the larvae. On the other hand, they add honey and pollen to the glandular diet to feed the older worker larvae. In addition, they also feed other workers and provide the diet for the laying queen (Crailsheim 1998). Hence, they are in central position to distribute plant-derived compounds with potential relevance to colony health among the nest members.

The larval food is known to have a high antibiotic power. The food gland secretions contain potent antibiotic compounds, including the fatty acid 10-HDA (Blum et al. 1959) and the antimicrobial peptide royalisin—now known as defensin-1 (Fujiwara et al. 1990). However, the foraged compounds, honey, and pollen (Beetsma 1979) added to the larval diet have also been shown to have antibiotic activity. Yet these items must be chosen by the workers before being fed to other hive members. In a normal colony, the honey stores will comprise pollen and honey of very different floral sources with potentially very different antimicrobial characteristics.

Honey is stored in the combs in a self-organized pattern depending on the amount and availability of brood and the cell usage on the comb (Camazine et al. 1990; Camazine 1991). The major rule for a nectar-storing worker is simple: fill a cell next to a cell filled with nectar. Field observations on comb structures revealed that honey store cells are arranged in curved series on top of brood and pollen stores (Seeley and Morse 1976). Hence, nectar unloading sites, where the nectar-receiving worker bee empties the honey stomach into a honey store cell, are not at all random but are spatially well defined and dynamic over time in a self-organized way. Due to their sophisticated communication and recruitment system, honeybees specialize and are highly adapted for exploiting large nectar flows (Seeley 1997). The nectar of a given flow will be stored in the colony in specific comb regions that are ready to take up nectar at that time. It is therefore often stored in confined but coherent regions of the comb and often available as monofloral honey for the bees inside the colony. In addition, because the honey cells are sealed before the next honey flow is available, this further precludes mixing of different floral sources in a given honey cell.

As a consequence, workers can access different quality honey in the colony at any given time. It would appear to be highly adaptive, if in-hive worker bees could choose among the different stores in the colony based on their own, the larvae, and the overall colony health status. In the end, the survival and growth of the colony depends not only on the foraging capacity of the worker bees but also on the quality of the stored food both from the nutritional and also from a colony health perspective. Given the sensory skills of foraging workers, it would be highly beneficial if in-hive bees could also use this ability to enhance colony health. The question is thus if nurse workers change their choice behavior for a certain honey in an adaptive way in response to an infection in the colony.

Here, we study if nurse workers change their behavior for a certain type of honey in an adaptive way in response to an infection in the colony. We use the microsporidian parasite *Nosema ceranae* (Fries et al. 1996) as an experimental parasite model system to infect nurse honeybees. *Nosema* sp. is a gut parasite of *A. mellifera* that enters, multiplies in, and destroys the gut epithelium causing diarrhea and shortening the life span of workers (Keeling and Fast 2002; Higes et al. 2010). We compare choice behavior of infested and noninfested nurse worker honeybees among different types of honey in an olfactometer. Subsequently, also the antibiotic potential of these honeys was tested to screen if the choice behavior based

on the individual infection may have had any adaptive value for colony health.

Material and methods

Honeybee samples

Combs with sealed worker brood were taken from a single colony at our apiary, and workers were allowed to emerge in an incubator at 35 °C. Since we wanted to quantify the effects of different honeys on honeybee workers, we tried to minimize genetic variance among the workers and potential colony effects by testing workers of a single colony, which might interfere with the interpretation of the results. Because of the multiple mating of the queens (Strassmann 2001), this precaution nevertheless generated genetic variance among the workers that we did not control for. Freshly emerged bees were kept in groups of 50 for 5 days in small cages and fed with 50 % sugar (saccharose) syrup ad libitum. After day five, a microscopic examination (40× magnification, Neubauer counting chamber) of 20 randomly chosen bees confirmed the absence of *Nosema* spores in the bees' midgut.

Infection with *Nosema ceranae*

N. ceranae spores were isolated from a highly infected colony by homogenizing 30–40 worker abdomens with a pestle, diluting the homogenate in 20 ml sterile water to filter it through a 10 μm pore size filter paper and finally centrifuge the filtrate for 10 min at 2,500×*g*. The spore pellet was resolved in 2 ml sterile water, and the number of spores was determined using a Neubauer counting chamber. An RFLP assay was used to differentiate between *Nosema apis* and *N. ceranae* and to confirm the presence of *N. ceranae* in the infections (Klee et al. 2007). In all analyzed samples, exclusively *N. ceranae* was detected and used for further experiments.

The bees were starved for 3 h before they were individually fed with 2 μl of a 50 % sugar solution (saccharose) either containing 10^5 spores or 2×10^5 spores or no spores (control). After infection, the bees were kept in cages for another 3 days to ensure that the spores had reached the midgut (Higes et al., 2007).

Simultaneous choice assay

The first series of experiments were conducted to screen if honey was more attractive than plain sugar syrup and to test this as a positive control for the experimental setup. Young, noninfected worker bees (n=90) were tested using a Y-shaped olfactometer made of glass (21×15 cm). The ducts were 6 mm wide and 5 mm high in order not to restrain the bee, but to prevent reverse escape of the tested bees. Equal volumes of sugar solution (80 % saccharose) and multifloral honey were simultaneously presented at either end of the olfactometer. Left and right positions were swapped to avoid preferential side biases. Every trial was terminated when the bee arrived at one of the two ends. Bees were only used once for every trial.

Testing preference for monofloral honeys

In a second set of experiments, four different types of undiluted monofloral honeys (verified by melissopalynological analysis) were used: linden (*Tilia* sp.), sunflower (*Helianthus annuus*), black locust (*Robinia pseudoacacia*), and honeydew (sugar-rich liquid, secreted by aphids or scale insects) honey. Characteristic sugar profiles of each honey are shown in Table S1, determined using a method described in Dezmirean et al. (2012). Analyzing all honey samples for the dominant pollen present on every microscopic slide confirmed their declared origin. Worker bees from the three different experimental groups (0, 10^5, 2×10^5 spores feeding regime, for each group 100 bees) were used for the choice assay with different honey types. All types of honeys were tested against each other in a pair-wise comparison based on the relative preference (in %) for a certain honey with another honey.

Chi-square tests were used to test for significant differences in honey preference for all tested groups (control, low, and high infected) on three levels: (1) control vs. infected with 10^5 spores, (2) control vs. infected with 2×10^5 spores, and (3) infected with 10^5 spores vs. infected 2×10^5 spores. At each step, we compared the numbers of individuals preferring one or the other type of honey and if this number changed with level of infection. Bonferroni correction was used to correct for multiple testing (corrected significance level $P<0.025$).

Effect of diet on *Nosema* infection

All types of honey were tested for potential antibiotic contaminants (tetracycline and oxytetracycline) using standard HPLC techniques (AOAC Method, Carson 1993). To assess the effect of the honey on the infection, freshly emerged workers were individually infected with *N. ceranae* (10^5 spores per bee) and kept in groups of 100 bees for 6 days following the protocol of Huang et al. (2012). The groups were fed ad libitum with the identical honeydew and sunflower honey showing the strongest impact at the previous choice assay. After 6 days, the bees were sacrificed and the infection level was microscopically determined with gut spore counts in five bees per group as described above. Mann–Whitney U test was used to test for significant differences between both groups.

Antimicrobial activity on *Escherichia coli*

Antimicrobial activity of honey is usually tested using bacteria growth inhibition assays. All honeys from the choice assay were used to quantify the antimicrobial activity of each honey using a classic bacterial growth inhibition test and if this might also explain the tested spore reducing activity of honeydew and sunflower honey.

E. coli (GM2163, Fermentas, St. Leon-Rot, Germany) was cultivated in standard LB medium, and the bacterial solution at an OD_{600} of 0.01 was used as a starting culture for the subsequent growth assay. The bacteria were mixed with every type of honey and pipetted into 96-well microtiter plates to reach a honey concentration of 5, 10, 25, and 50 % in the final volume of 200 μl. The bacteria solution (with honey), positive controls (only medium), and sugar controls (5, 10, 25, and 50 % sugar solution—100 %: glucose (0.32 g/ml) and fructose (0.42 g/ml)) were analyzed in four replicates, over 12 h. The optical density was measured every 15 min using a fully automated plate reader (Synergy 2 Multi-Mode Microplate Reader, BioTek, Winooski, Vermont, USA) at 37 °C and medium shaking speed.

We determined the slope of the growth curve during the log-phase with a linear regression, and quantified the growth inhibition of the honeys in relation to the corresponding sugar controls. Here, we used the relative difference between the honey and control (sugar) slopes for each concentration to calculate the relative bacterial growth inhibition as follows:

$$\text{inhibition}\ (\%) = \left(1 - \left(\frac{b_h}{b_c}\right)\right) \times 100 \qquad (1)$$

with

b_h Slope of the linear regression for the honey
b_c Slope of the linear regression for the sugar control

At each concentration, an ANOVA and post hoc Scheffé test was used to determine significant differences of the antimicrobial activity between the four tested honeys. STATISTICA 8.0 (StatSoft, Tulsa, OK, USA) was used for all statistical analysis.

Results

Decision making in infected worker bees

The olfactometer experimental set proved functional when testing sugar solution vs. multifloral honey. The multifloral honey was generally preferred over the sugar control (χ^2 test, χ^2=4.06, *df*=1, *P*=0.04), and the assay did not show any significant side preferences (χ^2=1.14, *df*=1, *P*=0.286) confirming the absence of experimental errors generated by the choice assay setup.

The choice assay among the various tested honeys showed a significant and clear-cut ranking in preference among the four honey types (sunflower>linden=black locust>honeydew honey). Pair-wise comparisons of control and low infected bees (10^5 spores) revealed significant preferences for sunflower honey when tested against honeydew honey (*P*<0.025, Table 1). This phenomenon became even more obvious in pair-wise comparisons of control and highly infected bees (2×10^5 spores). Thus, sunflower honey was increasingly preferred with increasing degree of infection with *N. ceranae* (Fig. 1, Table 1). The lowest numbers of choices were observed for honeydew honey that decreased with increasing *N. ceranae* infection (Fig. 1). Linden and black locust honey failed to show any trend with changing treatments (control or infection) and were less chosen when tested against sunflower honey by infected bees (*P*<0.025, Table 1).

Effect of diet on *Nosema* infection

In this experiment, the bees were fed ad libitum with different types of honey, and the level of *Nosema* (*N. ceranae*) infection was quantified 6 days post-infection. HPLC diagnostics revealed that all tested honeys were free of any tested antibiotics (tetracycline and oxytetracycline). Based on the results from the choice assay, we only tested the honeys with the most extreme differences in the choice behavior experiments. The sunflower honey-fed bees showed a significant lower *N. ceranae* spore load ($7.66\times10^5\pm0.077\times10^5$) compared to bees fed with honeydew honey ($8.24\times10^5\pm0.045\times10^5$) (Mann–Whitney *U* test *Z*=2.6112, *P*=0.009).

Antimicrobial activity on *Escherichia coli*

The growth inhibitory effect of the sugar controls was subtracted from the results of the honey samples to reveal better the residual effects of the four honeys tested. Not any bacterial growth was detected at 50 % honey concentration; that is not surprising as the sugar control also completely prevented bacterial growth (Fig. 2). At the 25 % concentration, all honeys showed an inhibition of bacterial growth of more than 50 % (Table S2). Sunflower honey had the highest antimicrobial activity at both 10 % (ANOVA *F*=37.71, *df*=3, *P*<0.001; post hoc Scheffé test, sunflower vs. black locust and sunflower vs. honeydew, each *P*<0.001) and 5 % (ANOVA *F*=149.66, *df*=3, *P*<0.001; post hoc Scheffé test, sunflower vs. black locust and sunflower vs. honeydew, each *P*<0.001) honey concentration compared to honeydew and black locust honey (Table S2; Fig. 2, only showing sunflower and honeydew for clarity of presentation as the most extremes from the choice assay and those honeys used for the *Nosema* infection assay).

Table 1 Statistics for the honey choice assay results using uninfected (control) and *Nosema ceranae* (*N. c.*)-infected (two different doses) honeybees

Honey choices	Control vs. *N. c.* (10^5)		Control vs. *N. c.* (2×10^5)		*N. c.* 10^5 vs. 2×10^5	
	χ^2	*P*	χ^2	*P*	χ^2	*P*
Linden vs. black locust	0.19	0.66	0.2	0.65	0.79	0.37
Linden vs. honeydew	0.23	0.63	0.11	0.74	0.66	0.42
Linden vs. sunflower	3.41	0.07	10.58	*0.01*	2.04	0.15
Black locust vs. honeydew	0.38	0.54	2.82	0.09	5.23	*0.02*
Black locust vs. sunflower	1.68	0.20	5.49	*0.02*	1.12	0.29
Honeydew vs. sunflower	9.9	*0.002*	25.4	*<0.0001*	4.19	0.04

P levels were Bonferroni adjusted to account for multiple testing and significant *P* values are marked in italics (*P*=0.025)

Discussion

Self-medications as type of "group defense" behavior represent a special case of "social immunity" that includes mechanisms to reduce host exposure to parasites and reduce infection risk through behavioral and/or physiological defense (de Roode and Lefèvre 2012). Such antiparasitic behavior can result in reducing infection probability ("prophylactic self-medication") and parasite burdens once the host is infected ("therapeutic self-medication") (Hart 1990). Most examples for therapeutic self-medication are mainly based on field observations on mammals ingesting plant material with antiparasitic chemicals (Clayton and Wolfe 1993) and several behavioral traits in invertebrates (Parker et al. 2011).

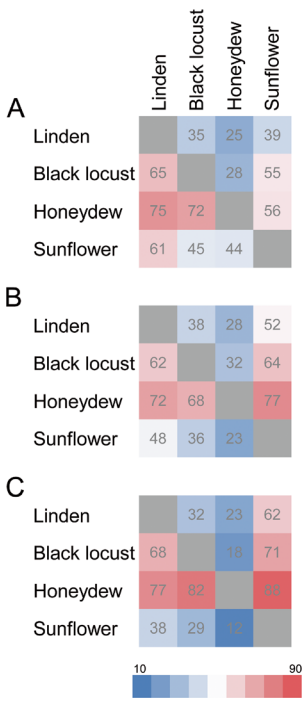

Fig. 1 Honey preference depending on *N. ceranae* degree of infection; **a** control (*no spores*), **b** 10^5 spores, and **c** 2×10^5 spores. Each plot shows the percentage of bees being attracted by type of honey listed on *top* of the table (*columns*), in comparison to the honey listed on the *left* (*rows*). With increasing level of infection, sunflower honey is more often chosen by the workers

Here, we showed the potential for therapeutic self-medication in an invertebrate with complex social structure to reduce or probably even prevent pathogen infections. Nurse bees infected with *N. ceranae* preferentially chose the food source that decreased the infection intensity after feeding the selected honey type. This is not only important for their own health status but very likely also for the other nestmates they would feed under natural conditions. Because the preferred honey had also a higher antibiotic potential, this may also be relevant for bacterial diseases including the brood fed by the nurse bees.

Other social insect species have also been reported to collect antimicrobial plant material to reduce pathogen loads in their colonies. Wood ants (*Formica paralugubris*) incorporate pieces of solidified conifer resin into their nests. Resin inhibits growth of bacteria and fungi in nest material and protects the ants against harmful microorganisms (Christe et al. 2003; Chapuisat et al. 2007). However, by measuring immune response, the prophenoloxidase activity (part of the humoral innate immune response of insects) was very low and not affected by the presence of resin. Additionally, only a light decrease in antibacterial and lytic activities was observed (Castella et al. 2008).

Not only ants use resin, also honeybees collect resin to prevent parasite infections and decrease parasite growth (Walker and Crane 1987; Simone et al. 2009; reviewed in Simone-Finstrom and Spivak 2010). Exposure of honeybees to extracts from honeybee propolis (a mixture of resins and wax) led to a significantly lowered expression of two immune-related genes (*hymenoptaecin* and *AmEater*) and lowered bacterial loads (Simone et al. 2009). The case of reduced immune-related gene expression upon pathogen infection and collecting antimicrobial plant products may present an evolutionary adaptation in social insects that can explain the reduced number of immune genes, especially in honeybees.

It has been suggested that honeybees self-medicate with plant resins and collect more resin in response to honeybee-specific fungal infections (i.e., chalkbrood, *Ascosphaera apis*) (Simone-Finstrom and Spivak 2012). Resin-treated colonies showed decreased infection intensities of this fungal parasite (Simone-Finstrom and Spivak 2012) as it is the case for

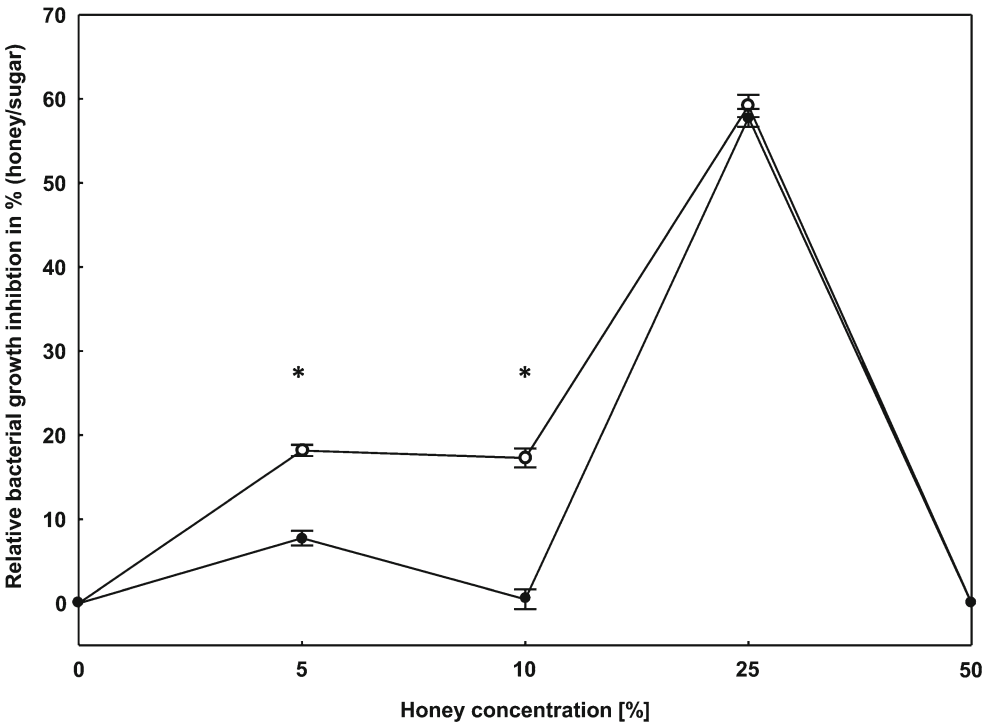

Fig. 2 The difference in inhibition (% mean±SE) of *E. coli* growth between sugar control and honey when treated with several concentrations of honeydew (*filled circles*) and sunflower honey (*open circles*). Only the results of honeydew and sunflower honey are plotted as both showed the strongest differences in the preceding behavioral choice assay. All other details are summarized in Table S2. For details on the calculation, see formula 1 in "Antimicrobial activity on *Escherichia coli*" section. The inhibition effect of honey decreases at 50 % because of the strong antibiotic effect by the sugar concentration alone. *Asterisks* indicate significant differences between honey types (ANOVA and post hoc Scheffé test, $P<0.001$)

N. ceranae infection in this study. The whole bee colony can be protected by resin ("group level defense"), as resin is an effective surface-antimicrobial agent. Many other bee products and hive substances also serve as antimicrobial or protective agents and might be candidates as active self-medication agents (Dustmann 1979; Viuda-Martos et al. 2008).

Nectar alkaloids of bee-pollinated plants have been shown to reduce pathogen loads in bumble bees infected with protozoan parasites (Manson et al. 2010). Honey as the main carbohydrate nutrient of the honeybee (*A. mellifera*) might therefore be an excellent candidate to serve as a self-medication agent against various bee pathogens. Honey mainly consists of foraged water, nectar, and traces of pollen. Several components contribute to the antimicrobial nature of honey to kill pathogens in the colony and microbes found in honey (e.g., bacteria, yeasts, and molds). The main antibacterial activity of honey results from sugar (Gilliam et al. 1988), H_2O_2 (White et al. 1963), methylglyoxal (Adams et al. 2008), and bee defensin-1 (Kwakman et al. 2010). Although sugar profiles are known to vary with the nectar source and also the viscosity of the honey may interfere with the preference of honeybees (Nicolson et al. 2013), the decisions of the bees in the olfactometer was based on the odoral perception of the honey and not by contact perception. Therefore, neither the sugar profile nor the viscosity was a likely driver of the results of the choice assay. Other factors including volatile odors, secondary plant compounds, nectar symbiotic bacteria, or plant pathogen metabolites contributing to the overall honey bouquet may be more relevant (McArt et al. 2014). The volatile bouquet of the nectar is a crucial signal for the foragers visiting flowers in the field. Although many volatiles may evaporate over time, many odor signals will remain after the nectar has been processed to honey. There is no reason why workers in the hive should not use these signals to choose and distribute nectar in the hive. Though the workers may be able to feed the nectar to nestmates directly, without processing to honey, this would not systematically contribute to colony health, because the nectar may come in at a time when not needed. The power of the system is based on the ability to store the nectar and use it when needed to fight a certain disease or infection.

Manuka and multifloral honey was tested on microsporidian parasites (Malone et al. 2001). Although *N. apis* spores started losing viability after several days, no general effect on parasite prevalence was observed (Malone et al. 2001). In contrast, our study demonstrates a reduction in the degree of infection with *N. ceranae* within 6 days. Although we only tested the two honeys with the most extreme differences in the choice assay, the honey specificity could be confirmed with its effect on the infection intensity. Sunflower honey was more frequently chosen by the infected than by the noninfected nurse bees, with a 57 % increase in attractivity in choice tests with honeydew. Its higher antimicrobial activity might be explained by its much higher H_2O_2 concentration compared to black locust and honeydew honey (Oelschlaegel et al. 2012), which was confirmed by the bacterial growth inhibition assay (Fig. 2). However, generalizing the results of the bacterial growth assay has to be seen with caution. Any antimicrobial activity against bacteria will not directly reflect

the antimicrobial activity against *Nosema*. Since there is currently no stable cell system to study intracellular *Nosema* cell proliferation in vitro, a standardized screening of bacteria offers the only option to quantify antimicrobial activity. Although this allows for indirect conclusions only, honey type-specific antimicrobial activity fitted well with both the behavioral choice of the workers and the in vivo *Nosema* infection intensity.

We need to stress that we here tested individual workers in response to a single pathogen. At the colony level, an exclusive monofloral sunflower honey diet may not be that beneficial, and for example, monofloral sunflower nectar resources have recently been suspected to have contributed to colony losses (Pirk et al. 2014). Indeed, it may well be the diversity of honey stores that facilitates colony-level immunity against the full spectrum of pathogens the colony is exposed to. Although we cannot exclude that some of the responses found in this study may be very specific to the workers of the one tested colony, the pathogens, and the bacterial strains we used, we nevertheless show that the variability among different honeys in a colony may be an important principle for colony health. We cannot exclude that other pathogens or other colonies may respond in a different way, but the variance among the stored honeys may just be important from a nutrition perspective but also may have profound effect on colony immunity.

By storing and accessing specific food, honeybees may be in a position to enhance the colony defense not only against nutritional but also against parasite stress. Protein levels for instance can decrease in bees from colonies with low pollen stores which in turn can lead to reduced individual immunocompetence (Alaux et al. 2010). Moreover, the protein–carbohydrate ratio is important for the nutritional intake and physiological demands of stressed or sick bees (Altaye et al. 2010). We here show that the in-hive worker bees might be in an exceptionally important position to distribute selectively honey in the colony that affect their own health but potentially also that of other nestmates. Whereas this is an important pathway for colony health in the wild, also beekeepers might use this knowledge to profit from the natural antimicrobial substances. In the specific case of *N. ceranae*, moving colonies to foraging sites with rich sunflower honey flows might be an important step in the fight against Nosemosis. At the same time, apiculturists should be aware that the increasing demand for monofloral honeys might put an additional health stress on managed colonies, facilitating the spread of diseases and undesired colony losses.

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References

Adams CJ, Boult CH, Deadman BJ, Farr JM, Grainger MN, Manley-Harris M, Snow MJ (2008) Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (*Leptospermum scoparium*) honey. Carbohydr Res 343:651–659. doi:10.1016/j.carres.2007.12.011

Alaux C, Ducloz F, Crauser D, Le Conte Y (2010) Diet effects on honeybee immunocompetence. Biol Lett 6:562–565. doi:10.1098/rsbl.2009.0986

Altaye SZ, Pirk CW, Crewe RM, Nicolson SW (2010) Convergence of carbohydrate-biased intake targets in caged worker honeybees fed different protein sources. J Exp Biol 213:3311–3318. doi:10.1242/jeb.046953

Beetsma J (1979) The process of queen-worker differentiation in the honeybee. Bee World 60:24–39

Blum MS, Novak AF, Taber S 3rd (1959) 10-Hydroxy-delta 2-decenoic acid, an antibiotic found in royal jelly. Science 130:452–453. doi:10.1126/science.130.3373.452

Camazine S (1991) Self-organizing pattern formation on the combs of honey bee colonies. Behav Ecol Sociobiol 28:61–76. doi:10.1007/BF00172140

Camazine S, Sneyd J, Jenkins MJ, Murray JD (1990) A mathematical model of self-organized pattern formation on the combs of honeybee colonies. J Theor Biol 147:553–571. doi:10.1016/S0022-5193(05)80264-4

Carson MC (1993) Simultaneous determination of multiple tetracycline residues in milk using metal chelate affinity chromatography. J AOAC Int 76:329–34

Castella G, Chapuisat M, Moret Y, Christe P (2008) The presence of conifer resin decreases the use of the immune system in wood ants. Ecol Entomol 33:408–412. doi:10.1111/j.1365-2311.2007.00983.x

Chapuisat M, Oppliger A, Magliano P, Christe P (2007) Wood ants use resin to protect themselves against pathogens. Proc R Soc B 274:2013–2017. doi:10.1098/rspb.2007.0531

Christe P, Oppliger A, Bancalà F, Castella G, Chapuisat M (2003) Evidence for collective medication in ants. Ecol Lett 6:19–22. doi:10.1046/j.1461-0248.2003.00395.x

Clayton DH, Wolfe ND (1993) The adaptive significance of self-medication. Trends Ecol Evol 8:60–63. doi:10.1016/0169-5347(93)90160-Q

Crailsheim K (1998) Trophallactic interactions in the adult honeybee (*Apis mellifera* L.). Apidologie 29:97–112. doi:10.1051/apido:19980106

de Roode JC, Lefèvre T (2012) Behavioral immunity in insects. Insects 3:789–820. doi:10.3390/insects3030789

Dezmirean GI, Mărghitaș LA, Bobiș O, Dezmirean DS, Bonta V, Erler S (2012) Botanical origin causes changes in nutritional profile and antioxidant activity of fermented products obtained from honey. J Agric Food Chem 60:8028–8035. doi:10.1021/jf3022282

Dustmann JH (1979) Antibacterial effect of honey. Apiacta 1:1–4

Efem SE, Udoh KT, Iwara CI (1992) The antimicrobial spectrum of honey and its clinical significance. Infection 20:227–229. doi:10.1007/BF02033065

Evans JD, Schwarz RS (2011) Bees brought to their knees: microbes affecting honey bee health. Trends Microbiol 19:614–620. doi:10.1016/j.tim.2011.09.003

Evans JD, Aronstein K, Chen YP, Hetru C, Imler JL, Jiang H, Kanost M, Thompson GJ, Zou Z, Hultmark D (2006) Immune pathways and defence mechanisms in honey bees *Apis mellifera*. Insect Mol Biol 15:645–656. doi:10.1111/j.1365-2583.2006.00682.x

Fries I, Feng F, da Silva A, Slemenda SB, Pieniazek NJ (1996) Nosema ceranae n. sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian

honey bee Apis cerana (Hymenoptera, Apidae). Eur J Protistol 32: 356–365. doi:10.1016/S0932-4739(96)80059-9
Fouks B, Lattorff HMG (2011) Recognition and avoidance of contaminated flowers by foraging bumblebees (*Bombus terrestris*). PLoS One 6:e26328. doi:10.1371/journal.pone.0026328
Frost EH, Shutler D, Hillier NK (2012) The proboscis extension reflex to evaluate learning and memory in honeybees (*Apis mellifera*): some caveats. Naturwissenschaften 99:677–686. doi:10.1007/s00114-012-0955-8
Fujiwara S, Imai J, Fujiwara M, Yaeshima T, Kawashima T, Kobayashi K (1990) A potent antibacterial protein in royal jelly. purification and determination of the primary structure of royalisin. J Biol Chem 265: 11333–11337
Galizia G, Eisenhardt D, Giurfa M (2011) Honeybee neurobiology and behavior: a tribute to Randolf Menzel. Springer, Berlin
Gilliam M, Taber S, Lorenz BJ, Prest DB (1988) Factors affecting development of chalkbrood disease in colonies of honey bees, *Apis mellifera*, fed pollen contaminated with *Ascosphaera apis*. J Invertebr Pathol 52:314–325. doi:10.1016/0022-2011(88)90141-3
Hart BL (1990) Behavioral adaptations to pathogens and parasites: five strategies. Neurosci Biobehav R 14:273–294. doi:10.1016/S0149-7634(05)80038-7
Hart BL (2005) The evolution of herbal medicine: behavioural perspectives. Anim Behav 70:975-989. doi:10.1016/j.anbehav.2005.03.005
Herbert EW Jr (1992) Honey bee nutrition. In: The hive and the honey bee (edt. Graham, JM). Published by Dadant & Sons, pp.197–233.
Higes M, García-Palencia P, Martín-Hernández R, Meana A (2007) Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (microsporidia). J Invertebr Pathol 94:211–217. doi:10.1016/j.jip.2006.11.001
Higes M, Martín-Hernández R, Meana A (2010) *Nosema ceranae* in Europe: an emergent type C nosemosis. Apidologie 41:375–392. doi:10.1051/apido/2010019
Huang Q, Kryger P, Le Conte Y, Moritz RFA (2012) Survival and immune response of drones of a Nosemosis tolerant honey bee strain towards *N. ceranae* infections. J Invertebr Pathol 109:297–302. doi:10.1016/j.jip.2012.01.004
Keeling PJ, Fast NM (2002) Microsporidia: biology and evolution of highly reduced intracellular parasites. Annu Rev Microbiol 56:93–116. doi:10.1146/annurev.micro.56.012302.160854
Klee J, Besana AM, Genersch E, Gisder S, Nanetti A, Tam DQ, Chinh TX, Puerta F, Ruz JM, Kryger P, Message D, Hatjina F, Korpela S, Fries I, Paxton RJ (2007) Widespread dispersal of the microsporidian Nosema ceranae, an emergent pathogen of the western honey bee, Apis mellifera. J Invertebr Pathol 96:1–10. doi:10.1016/j.jip.2007.02.014
König B (1985) Plant sources of propolis. Bee World 66:136–139
König B, Dustmann JH (1986) Propolis und Viren: der gegenwärtige forschungsstand. Apidologie 17:334–336
Kwakman PH, te Velde AA, de Boer L, Speijer D, Vandenbroucke-Grauls CM, Zaat SA (2010) How honey kills bacteria. FASEB J 24:2576–2582. doi:10.1096/fj.09-150789
Lefèvre T, Chiang A, Kelavkar M, Li H, Li J, de Castillejo CL, Oliver L, Potini Y, Hunter MD, de Roode JC (2012) Behavioural resistance against a protozoan parasite in the monarch butterfly. J Anim Ecol 81:70–79. doi:10.1111/j.1365-2656.2011.01901.x
Lozano GA (1998) Parasitic stress and self-medication in wild animals. Adv Stud Behav 27:291–317. doi:10.1016/S0065-3454(08)60367-8
Malone LA, Gatehouse HS, Tregidga EL (2001) Effects of time, temperature, and honey on *Nosema apis* (Microsporidia: Nosematidae), a parasite of the honeybee, *Apis mellifera* (Hymenoptera: Apidae). J Invertebr Pathol 77:258–268. doi:10.1006/jipa.2001.5028
Manson JS, Otterstatter MC, Thomson JD (2010) Consumption of a nectar alkaloid reduces pathogen load in bumble bees. Oecologia 162:81–89. doi:10.1007/s00442-009-1431-9
McArt SH, Koch H, Irwin RE, Adler LS (2014) Arranging the bouquet of disease: floral traits and the transmission of plant and animal pathogens. Ecol Lett 17:624–636. doi:10.1111/ele.12257
Menzel R (2012) The honeybee as a model for understanding the basis of cognition. Nat Rev Neurosci 13:758–768. doi:10.1038/nrn3357
Menzel R, Müller U (1996) Learning and memory in honeybees: from behavior to neural substrates. Annu Rev Neurosci 19:379–404. doi:10.1146/annurev.ne.19.030196.002115
Nicolson SW, de Veer L, Köhler A, Pirk CWW (2013) Honeybees prefer warmer nectar and less viscous nectar, regardless of sugar concentration. Proc R Soc B 280:20131597. doi:10.1098/rspb.2013.1597
Oelschlaegel S, Pieper L, Staufenbiel R, Gruner M, Zeippert L, Pieper B, Koelling-Speer I, Speer K (2012) Floral markers of cornflower (*Centaurea cyanus*) honey and its peroxide antibacterial activity for an alternative treatment of digital dermatitis. J Agric Food Chem 60:11811–11820. doi:10.1021/jf303699t
Parker BJ, Barribeau SM, Laughton AM, de Roode JC, Gerardo NM (2011) Non-immunological defense in an evolutionary framework. Trends Ecol Evol 26:242–248. doi:10.1016/j.tree.2011.02.005
Pirk CWW, Human H, Crewe RM, vanEngelsdorp D (2014) A survey of managed honey bee colony losses in the Republic of South Africa—2009 to 2011. J Apicult Res 53:35–42. doi:10.3896/IBRA.1.53.1.03
Ratcliffe NA, Mello CB, Garcia ES, Butt TM, Azambuja P (2011) Insect natural products and processes: new treatments for human disease. Insect Biochem Mol Biol 41:747–769. doi:10.1016/j.ibmb.2011.05.007
Schmid-Hempel P (1998) Parasites in social insects. Princeton University Press, Princeton
Seeley TD (1997) Honey bee colonies are group-level adaptive units. Am Nat 150(Suppl 1):S22–S41. doi:10.1086/286048
Seeley TD (2010) Honeybee democracy. Princeton University Press, Princeton
Seeley TD, Morse RA (1976) The nest of the honey bee (*Apis mellifera* L.). Insect Soc 23:495–512. doi:10.1007/BF02223477
Simone M, Evans JD, Spivak M (2009) Resin collection and social immunity in honey bees. Evolution 63:3016–3022. doi:10.1111/j.1558-5646.2009.00772.x
Simone-Finstrom M, Spivak M (2010) Propolis and bee health: the natural history and significance of resin use by honey bees. Apidologie 41:295–311. doi:10.1051/apido/2010016
Simone-Finstrom MD, Spivak M (2012) Increased resin collection after parasite challenge: a case of self-medication in honey bees? PLoS One 7:e34601. doi:10.1371/journal.pone.0034601
Strassmann J (2001) The rarity of multiple mating by females in the social hymenoptera. Insect Soc 48:01–13. doi:10.1007/PL00001737
Viuda-Martos M, Ruiz-Navajas Y, Fernández-López J, Pérez-Alvarez JA (2008) Functional properties of honey, propolis, and royal jelly. J Food Sci 73:R117–124. doi:10.1111/j.1750-3841.2008.00966.x
Walker P, Crane E (1987) Constituents of propolis. Apidologie 18:327–334. doi:10.1051/apido:19870404
White JW Jr, Subers MH, Schepartz AI (1963) The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system. Biochim Biophys Acta 73:57–70. doi:10.1016/0926-6569(63)90108-1
Wilson-Rich N, Spivak M, Fefferman NH, Starks PT (2009) Genetic, individual, and group facilitation of disease resistance in insect societies. Annu Rev Entomol 54:405–423. doi:10.1146/annurev.ento.53.103106.093301

Supplementary Material

Table S1: Characteristic sugar profiles for each honey type, determined following a HPLC-RID method (Dezmirean et al. 2012).

Sugar (%)	Black locust	Linden	Sunflower	Honeydew
Glucose	27.99	33.56	38.43	26.70
Fructose	44.01	33.95	39.00	30.12
Saccharose	0.78	0.00	0.07	0.83
Turanose	2.53	1.52	1.08	1.40
Maltose	4.04	2.18	1.53	2.59
Trehalose	0.72	0.64	0.35	0.62
Isomaltose	0.50	0.68	0.22	0.69
Erlose	2.58	0.29	0.10	2.51
Melezitose	0.00	0.10	0.00	3.80
Total sugars	**83.15**	**72.92**	**80.78**	**69.26**

Table S2: Bacterial growth inhibition of each honey type tested with several honey concentrations. Values in the same column with different superscripts (a-c) are significantly different (ANOVA and post hoc Scheffé test were used).

	50%	25%	10%	5%
Linden	n.d.	69.79 ^{a} ± 7.20	10.29 ^{b} ± 5.28	16.63 ^{a} ± 2.93
Sunflower	n.d.	59.16 ^{b} ± 5.39	17.28 ^{a} 4.55	18.18 ^{a} ± 2.72
Black locust	n.d.	56.68 ^{b} ± 6.99	2.85 ^{c} 5.27	-0.87 ^{c} ± 2.32
Honeydew	n.d.	57.75 ^{b} ± 4.36	0.49 ^{c} ± 4.71	7.75 ^{b} ± 3.46

n.d., not detectable as all bacteria were killed