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RESIN COLLECTION AND SOCIAL IMMUNITY IN HONEY BEES

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Diverse animals have evolved an ability to collect antimicrobial compounds from the environment as a means of reducing infection risk. Honey bees battle an extensive assemblage of pathogens with both individual and "social" defenses. We determined if the collection of resins, complex plant secretions with diverse antimicrobial properties, acts as a colony-level immune defense by honey bees. Exposure to extracts from two sources of honey bee propolis (a mixture of resins and wax) led to a significantly lowered expression of two honey bee immune-related genes (hymenoptaecin and AmEater in Brazilian and Minnesota propolis, respectively) and to lowered bacterial loads in the Minnesota (MN) propolis treated colonies. Differences in immune expression were also found across age groups (third-instar larvae, 1-day-old and 7-day-old adults) irrespective of resin treatment. The finding that resins within the nest decrease investment in immune function of 7-day-old bees may have implications for colony health and productivity. This is the first direct evidence that the honey bee nest environment affects immune-gene expression.

KEY WORDS: Antimicrobial peptides, *Apis mellifera*, ecological immunity, propolis.

Although social living can be of a benefit for many organisms, it also creates the possibility for high costs, particularly due to an increased chance of disease outbreaks and the potential for pathogens and parasites to exploit the high concentration of individuals (Schmid-Hempel 1998). This is certainly the case in social insects, which typically live in large colonies with a constant interaction among individuals. With this in mind, the finding of the Honey Bee Genome Sequencing Consortium (2006) that honey bees (*Apis mellifera*) appear to have a sparse immune-response system was curious (Evans et al. 2006). The authors pointed out that the immune defenses of individual honey bees might be compensated by behavioral and colony-level mechanisms (recently termed social immunity; Cremer et al. 2007). In honey bees, the nest environment itself is often considered to be one aspect of colony-level immunity because stored honey has antimicrobial properties as does royal jelly, the larval food secreted by adult nurse bees (Morse and Flottum 1997). Several behaviors are also known to reduce parasite and pathogen loads. For example, hygienic behavior is a trait by which individual bees are able to detect and remove parasitized pupae or diseased larvae before they reach an infectious stage (Rothenbuhler 1964; Spivak and Gilliam 1998). Colonies that express this behavior are resistant to *Ascosphaera apis* (the fungal agent of chalkbrood disease), *Paenebacillus larvae* (the bacterium that causes the deadly American foulbrood), and the parasitic mite *Varroa destructor* (see Wilson-Rich et al. 2009 for a recent review).

We hypothesize that resin collection and use by honey bees provide an additional colony-level disease response. Various plant species secrete highly antimicrobial resins to protect vegetative apices and young leaves (Langenheim 2003). Several species across the animal kingdom may use these plant-produced resins to reduce effects of parasites and pathogens. One well-described example, *Formica paralugubris*, a Swiss wood ant, mixes resin globules from coniferous trees with nest material, and this resin

decreases the number of total microorganisms in the nest (Christe et al. 2003). Furthermore, laboratory experiments in this species suggest that protective resin leads to increased survival of individuals when exposed to pathogens and a lowered investment of energy in immune function when unchallenged (Chapuisat et al. 2007; Castella et al. 2008a,b). Other more general examples include white-nosed coatis (*Nasua narica*) in Panama that spread resins on their coats as a hypothesized means of protection against parasites (Gompper and Hoylman 1993). The resinous, aromatic leaves used by European starlings for nest construction may decrease parasite loads and boost immunity among developing nestlings (i.e., Gwinner et al. 2000; Gwinner and Berger 2005; Mennerat et al. 2009). Female assassin bugs also harvest resin and then transfer it to eggs as they are being laid, which inhibits ant predation (Choe and Rust 2007). Among the social insects, many bees, especially in the tropics, collect and use resins as a nest-building material (Roubik 1989). It is possible, at least in tropical regions where resinous plants are more abundant, that bees simply exploit this commonly available resource for structural benefits. However, there are other properties of the resin that may benefit all individuals in the nest. This is most likely the case in colonies of some ants and honey bees where a few individuals collect and return to the nest with loads of resin to use in the nest interior.

Honey bees collect resins on their hind legs, as they do pollen, and bring it back to the nest where it is mixed with varying amounts of wax and used mainly as a form of cement, called propolis, to seal cracks and holes in the nest architecture. Feral honey bees nesting in tree cavities line the entirety of the interior nest wall with a thin layer of resin, which has been termed the "propolis envelope" (Seeley and Morse 1976). In temperate regions, it is thought that the main sources of resins are *Populus*, *Betula*, and *Alnus* species, although others are used by honey bees less predominately (Ghisalberti 1979; Crane 1990). In tropical areas, bees have been recorded collecting resins from *Clusia* flowers and from woody plants such as *Baccharis dracunculifolia* (i.e., Salatino et al. 2005), among others. The antimicrobial properties of propolis with respect to human health and disease have received much attention (see Bankova 2005). However, few studies have examined the roles played by resins in honey bee colony disease resistance (i.e., Mlagan and Sulimanovic 1982; Garedrew et al. 2002; Bastos et al. 2008), and only one study has been conducted outside the laboratory (Antúnez et al. 2008). Foraging for resins is energetically demanding and provides no clear direct, individual reward, so it is possible that resin use functions as an aspect of social immunity.

Here, we investigated the effect that resins in field colonies of honey bees have on the immune system of an individual honey bee. We hypothesized that the presence of propolis within the colony reduces the amount or diversity of pathogenic and saprophytic microbes within the nest and thus results in a lowered physiological investment in the production of antimicrobial peptides and cellular immunity in bees throughout the colony. Humoral defenses (i.e., antimicrobial peptides) and cellular defenses (i.e., melanization, phagocytosis, and encapsulation) are known to be produced in response to infection and wounding (i.e., Evans 2004). As a chronically high activation of the immune system at the individual level can lead to decreased productivity at the colony level (Evans and Pettis 2005), factors that reduce immune investment could lead to increased productivity. With the recent sequencing of the honey bee genome (HGSC 2006), we are able to more precisely investigate questions concerning immune activity in honey bees. In light of Colony Collapse Disorder (the sudden loss of bees within colonies across the country) and the current issues relating to colony health (Cox-Foster et al. 2007; Higes et al. 2008), it is particularly important to gain a greater understanding of the mechanisms involved in reducing a physiological stress of honey bees.

For this study, we experimentally enriched colonies with either regional propolis from Minnesota or the well-studied Brazilian "green" propolis collected in Minas Gerais, Brazil (i.e., Salatino et al. 2005) to determine the effects of propolis on immune investment and microbial loads in honey bee colonies.

Material and Methods

EXPERIMENTAL DESIGN

Twelve four-frame experimental colonies were treated with resin whereas six control colonies were not treated. Each experimental colony was comprised of a sister queen and a mixture of bees from five "source" colonies to ensure genetic homogeneity. The source colonies had no visible clinical symptoms of disease and had low levels of the parasitic mite, *V. destructor*, as high mite levels have been shown to complicate immune expression in honey bees (Gregory et al. 2005; Yang and Cox-Foster 2005). To obtain this mixture, approximately 18 kg of bees from the source colonies were put into a large, screened box. The bees were mixed in this box, then divided into 18 1-kg ($\approx$ 7000 bees) "packages" (screened boxes built to hold bees and a caged queen). After two days, the bees and queen from the packages were transferred to new "nucleus" boxes (beekeeping equipment built to hold four combs). Each nucleus box was started with two honey frames, one frame of empty comb, and one frame with foundation on which the bees could build a new comb.

Six of the nucleus boxes were treated with green propolis from Brazil (BR); six were treated with propolis from Minnesota (MN); and six were used as controls. To treat with propolis, the inside surfaces of the box were painted with 300 mL propolis extract (13% propolis in 70% ethanol, following the extraction procedure of previous experiments with the exception that crude

propolis was extracted in 70% ethanol; Gekker et al. 2005; Bastos et al. 2008) simulating a propolis envelope around the brood nest. The six control boxes were painted only with 70% ethanol, the solvent used to make the propolis extract. In addition, the frame of empty comb was sprayed with 100 mL of a 7% propolis extract for the BR-treated boxes, a 6% propolis extract for the MN-treated boxes, and 70% ethanol for the controls. The colonies were inspected weekly to insure the queen was laying eggs, there were no clinical symptoms of disease, and that the colonies were functioning normally.

Seven-day-old were collected for subsequent analysis of gene transcripts. To obtain these known-aged bees, combs containing pupae ready to emerge as adult bees were removed from each of the original five source colonies and placed in cages in an incubator (34°C, 70% relative humidity). When the bees emerged, they were color marked on the thorax to indicate their source colony and were introduced into each of the 18 colonies over the course of two days (i.e., approximately 300 marked bees/source were introduced per colony). Samples of 12 bees of each color (60 bees per colony) were collected from each colony after one week. These bees were frozen at −80°C for subsequent analysis.

For analysis of age-related differences in gene expression, third-instar larvae and 1-day and 7-day-old bees that had fully developed from egg to adult within the nucleus colonies, and thus progeny of sister queens, were also collected and frozen at −80°C.

GENE TRANSCRIPT ANALYSIS

RNA was extracted from whole individual adult bees and third-instar larvae using a standard RNA extraction protocol (RNAqueous, Ambion, Austin, TX). DNA was removed from this extract using DNAse I (37°C for 1 h, 75°C for 10 min). First-strand cDNA was then synthesized by incubating 8 μg total RNA per bee in a 96-well plate with 3.9 μL of a master mix containing 50 U Superscript II (Invitrogen, Carlsbad, CA), 2 nmol DNTP mix, 2 nmol poly(dT)$_{18}$, and 0.1 nmol poly(dT)$_{(12–18)}$ at 42°C for 50 min followed by 15 min at 70°C as described in Evans (2006). Transcript abundances for cDNA were assayed by real-time PCR using primer pairs that amplify 120–300 bp sections of the target genes (Table 1). Genes encoding five antimicrobial peptides (abaecin, apidaecin, defensin1, and hymenoptaecin) and a candidate for cellular immunity (the EGF-family protein member AmEater) were used. Gene transcript levels of vitellogenin were analyzed as an indicator of general robustness (see Amdam et al. 2005). Lastly transcripts of eubacterial 16s RNA were measured by real-time PCR and "generic" primers to assess bacterial loads of the colonies. Gene transcripts were normalized relative to expression levels for the gene encoding actin, a gene with a consistent expression in honey bees.

Reactions to amplify the DNA products were conducted in 96-well plates using a Bio-Rad Icycler (Bio-Rad Corp., Hercules, CA). A total of 50 ng cDNA from each of the tested bees was used as a template for PCR reactions driven by 1 U *Taq* with proscribed 1× buffer (Roche Applied Sciences, Indianapolis, IN) and final concentrations of 1 mM dNTP mix, 2 mM additional $MgCl_2$, 0.2 μM of specific primers (one bee or pathogen gene assayed/reaction), 1× concentration SYBR-Green I dye (Applied Biosystems, Foster City, CA), and 10 nM fluorescein in a 25 μL reaction volume. The reactions were conducted under a fixed thermal protocol consisting of 5 min at 95°C, followed by 40 cycles of a four-step protocol that involves 94°C for 20 sec, 60°C for 30 sec, 72°C for 1 min, and 78° for 20 sec. Fluorescence measurements were taken repeatedly during the 78°C step. This

Table 1. Oligonucleotide primers and sequence identification for real-time PCR.

Primer name	Sequence (5′ to 3′)	GenBank entry
Abaecin.f	CAGCATTCGCATACGTACCA	NP_001011617
Abaecin.r	GACCAGGAAACGTTGGAAAC	NP_001011617
Actin.f	TTGTATGCCAACACTGTCCTTT	NC_007076
Actin.r	TGGCGCGATGATCTTAATTT	NC_007076
AmEater.f	CATTTGCCAACCTGTTTGT	XP_001120277
AmEater.r	ATCCATTGGTGCAATTTGG	XP_001120277
ApidNT.f	TTTTGCCTTAGCAATTCTTGTTG	NP_001011613
ApidNT.r	GTAGGTCGAGTAGGCGGATCT	NP_001011613
Defensin1.f	TGCGCTGCTAACTGTCTCAG	NP_001011616
Defensin1.r	AATGGCACTTAACCGAAACG	NP_001011616
Hymenopt.f	CTCTTCTGTGCCGTTGCATA	NP_001011615
Hymenopt.r	GCGTCTCCTGTCATTCCATT	NP_001011615
VgMC.f	AGTTCCGACCGACGACGA	NP_001011578
VgMC.r	TTCCCTCCCACGGAGTCC	NP_001011578
Bact774.f	GTAGTCCACGCTGTAAACGATG	Stackebrandt et al. 1994
Bact1391.r	GACGGGCGGTGTGTRCA	Stackebrandt et al. 1994

procedure was followed by a melt-curve dissociation analysis to confirm product size.

DATA ANALYSIS

Threshold cycles for real-time PCR were defined as the point when well fluorescence became greater than 10 times the mean standard deviation across all samples. Threshold values for each target gene were subtracted from the actin threshold for each sample to get a measure of relative cDNA abundance.

We compared these normalized levels of the gene transcripts in 7-day-old bees from resin-treated and resin-untreated colonies using a nested analysis of variance (ANOVA) with the original source of the bee being nested within treatment. Post hoc Tukey's HSD tests were used when treatment effects showed significance.

To examine possible effects of a resin-rich environment at different ages of development, only bees that developed fully in the experimental colonies were analyzed. Age effects were determined by a nested-ANOVA with treatment being nested within age-group. Post hoc Tukey's HSD tests were used when age effects showed significance.

Results

EFFECT OF RESIN TREATMENT

Normalized gene transcript levels were analyzed for 7-day-old bees from MN-propolis treated, BR-propolis treated, and control colonies. Six colony replicates were done for each of the three treatments with an average of 24 bees of the total 60 bees collected from each colony being analyzed for a gene expression for this portion of the study. Across the treatments, there were significant differences in expression of two immune-related genes. For the antimicrobial peptide hymenoptaecin, 7-day-old bees collected from the MN-propolis-treated colonies had significantly lower relative transcript abundances than those collected from control colonies whereas bees from the BR-propolis-treated colonies had intermediate levels, they were not significantly different from either (Fig. 1A; $F_{2,10} = 3.72$, $P = 0.025$). Similarly for eater, 7-day-old bees collected from BR-propolis-treated colonies had significantly fewer transcripts than those from control colonies with bees collected from MN-propolis-treated colonies intermediate but not significantly different from either (Fig. 1B; $F_{2,10} = 5.31$, $P = 0.005$).

In addition, the eubacterial load differed significantly across treatments. Seven-day-old bees from control colonies had significantly higher bacterial transcript abundances than bees from colonies treated with MN-propolis, with the bees from BR-propolis-treated colonies intermediate but not significantly different from either (Fig. 1C; $F_{2,10} = 3.19$, $P = 0.042$).

No significant differences were noted for gene transcript levels of abaecin, apidaecin, defensin1, or vitellogenin ($P > 0.05$ for treatment affects for each). The average gene transcript

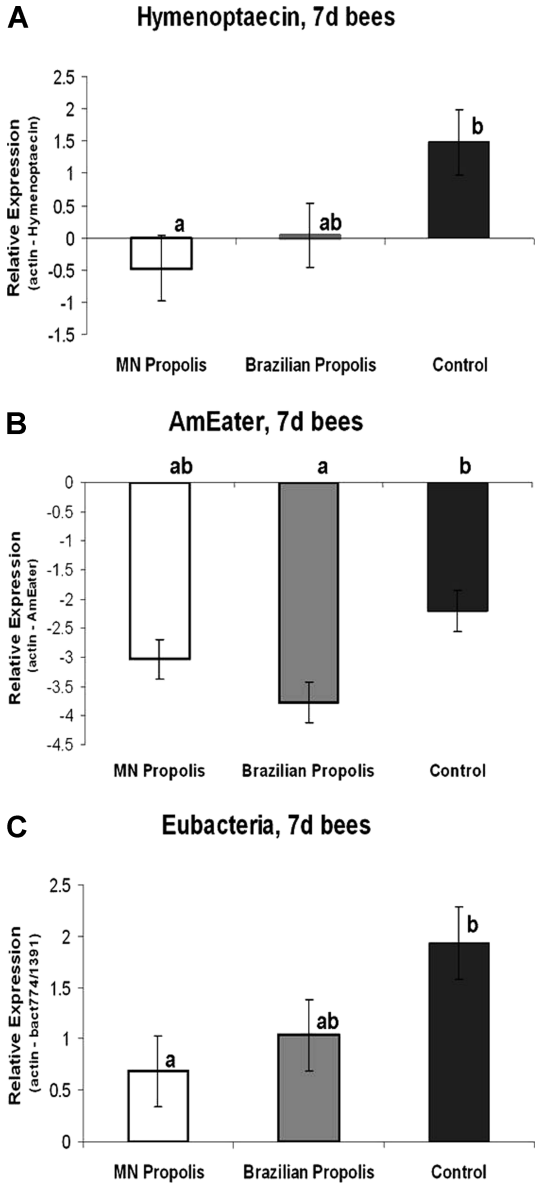

Figure 1. Gene transcript levels for (A) eubacteria (Bact 774/1391), (B) AmEater (a gene involved in cellular immunity), and (C) the antimicrobial peptide hymenoptaecin normalized to the housekeeping gene actin. Six colony replicates were done for each of the three treatments with an average of 24 bees from each colony being analyzed for a gene expression. Significant differences due to a treatment determined by nested-ANOVA followed by post-hoc Tukey's HSD are indicated by the letters over each bar (A) $F_{2,10} = 3.19$, $P = 0.042$; (B) $F_{2,10} = 5.31$, $P = 0.005$; (C) $F_{2,10} = 3.72$, $P = 0.025$.

levels (±SE) for abaecin showed a nonsignificant trend for reduced levels in bees from the MN-propolis-treated colonies (MN: 0.486 ± 0.48, BR: 1.377 ± 0.34, C: 1.048 ± 0.33; $F_{2,10} = 1.89$, $P = 0.152$). There were no clear trends for apidaecin (MN: 0.091 ± 0.32, BR: 0.260 ± 0.31, C: 0.461 ± 0.34; $F_{2,10} = 0.322$, $P = 0.725$), defensin1 (MN: 5.758 ± 0.38, BR: 6.106 ± 0.37, C: 5.986 ± 0.42; $F_{2,10} = 0.201$, $P = 0.818$), or vitellogenin

(MN: −0.576 ± 0.27, BR: −0.895 ± 0.27, C: −0.379 ± 0.28; $F_{2,10} = 0.931$, $P = 0.395$).

EFFECT OF AGE

Third-instar larvae and 1- and 7-day-old bees that developed in the experimental colonies were analyzed for gene transcript levels of several immune-related genes and for the presence of eubacteria (refer to Table 2 for normalized means and standard errors). Six colony replicates were done for the BR-propolis treatment, five for the MN-propolis treatment, and five control colonies. An average of four bees was analyzed per age group per colony.

No significant effects due to the resin treatments were found through this analysis. In general, however, the antimicrobial peptides tested showed a general increase in abundance with age (abaecin: $F_{2,10} = 16.74$, $P < 0.0001$; apidaecin: $F_{2,10} = 7.24$, $P = 0.008$). The one gene involved in cellular immunity (eater) was lower in larvae ($F_{2,10} = 30.93$, $P < 0.0001$), but did not increase across 1- and 7-day-old bees. In addition, normalized gene transcript levels coding for general eubacteria did show a significant increase between 1- and 7-day-old bees ($F_{1,5} = 4.75$, $P = 0.031$). For vitellogenin, transcript levels increased between the larval and adult stages ($F_{2,10} = 62.08$, $P < 0.0001$) but there were no significant differences between 1- and 7-day-old bees, although there was a trend for an overall increase with age.

Gene transcript levels for hymenoptaecin were negligible for third-instar larvae and 1-day-old bees, and so were not included. Levels for apidaecin were also negligible for third-instar larvae. Defensin1 was not assayed for these samples.

Discussion

This is the first report that a component of the nest environment alone can influence immune expression in honey bees. Our findings indicate that individual bees in resin-enriched colonies in the field are able to invest less energy on immune function for two divergent immune-related genes, and that this effect is conceivably due to decreased bacterial loads. This decreased investment or downregulation in immune function is the first clear evidence that the use of resins by honey bees may have implications for colony health and productivity. These field results support laboratory studies done with *F. paralugubris*, a resin-collecting ant species, which have shown that nest material enriched with resin has fewer overall microorganisms compared to resin-poor nest material (Christe et al. 2003) leading to a reduction in general immune activity (Castella et al. 2008b).

Resin foragers comprise a small percentage of the total numbers of foragers in a honey bee colony. Typically a total of 5–15 foragers will continuously collect resin during a single day (Meyer 1956; Nakamura and Seeley 2006), whereas in a 5-min period 150 foraging bees can return to the hive with pollen or nectar (i.e., Weidenmüller and Tautz 2002). Additionally, foraging for the sticky resins is a demanding process, highlighted by the unloading process, which typically takes 30 min but can take several hours (Meyer 1956; Nakamura and Seeley 2006) versus an 11-min average unloading time for a pollen forager (Nakamura and Seeley 2006). Because a very small proportion of colony members partake in the difficult task of resin foraging, the energy expended to collect propolis is likely minimal at the colony level

Table 2. Average gene transcript abundances normalized to the housekeeping gene actin with standard errors of each gene for the different age groups tested. Six colony replicates were done for the BR-propolis treatment, and five each were done for the MN-propolis and control treatments. An average of four bees was analyzed per age group per colony. Significant differences for each gene across age and irrespective of treatment are indicated by letters after the age group ($P<0.05$).

Gene	Age	Treatment		
		Control	MN	Brazil
Eubacteria	Third instar	–	–	–
(Bact774/1391)	1 day[a]	0.454±0.70	2.314±0.75	1.069±0.72
	7 day[b]	3.350±0.72	3.092±0.69	1.608±0.72
Abaecin	Third instar[a]	−2.552±0.54	−2.002±0.48	−1.766±0.54
	1 day[b]	−0.725±0.45	−0.429±0.52	−0.296±0.48
	7 day[b]	0.306±0.33	1.495±0.76	0.369±0.86
Apidaecin	Third instar	–	–	–
(ApidNT)	1 day[a]	−1.155±0.26	−0.116±0.34	−0.509±0.306
	7 day[b]	0.462±0.77	0.856±0.72	0.325±0.88
Eater	Third instar[a]	−5.105±0.52	−5.009±0.48	−4.277±0.58
(AmEater)	1 day[b]	−1.929±0.35	−2.078±0.41	−1.839±0.41
	7 day[b]	−1.787±0.77	−1.571±0.74	−1.582±0.93
Vitellogenin	Third instar[a]	−3.547±0.51	−3.600±0.51	−2.443±0.53
(VgMC)	1 day[b]	0.109±0.26	0.468±0.30	0.203±0.29
	7 day[b]	1.033±0.46	0.781±0.40	1.114±0.61

compared to the potential energy expended by many individuals to maintain elevated expression of immune-related genes in the absence of propolis. The costs of an elevated immune system has been well-documented across bee species and include impaired learning ability at the individual level (i.e., Mallon et al. 2003; Alghamdi et al. 2008), reduced life span under stressful conditions (i.e., Moret and Schmid-Hempel 2000), and lowered colony productivity (Evans and Pettis 2005). Therefore, honey bees harvest this antimicrobial substance and incorporate it into nest architecture, which can benefit, on balance, the "social" immune system of honey bees. More generally, this is a rare example of an environmental compound that can modulate immune function. It will be interesting to see whether that modulation is indirect (i.e., via decreased microbial loads as suggested here and by Castella et al. 2008b) or direct.

An important aspect of this experiment is that these colonies were not challenged with pathogens or parasites. The changes in immune expression seen here were changes in what are essentially baseline levels of immunity in field colonies. It is possible that when colonies are challenged, greater differences or differences among more immune-related genes would arise. This idea is supported by the laboratory study done with *F. paralugubris* that showed that when individual ants housed in a Petri dish with resin were challenged with a pathogen, they had higher survival rates than those without resin (Chapuisat et al. 2007). Based on this, it appears as though the presence of resin does not suppress the immune system, but merely allows for it to be downregulated, because a pathogen challenge can still cause an upregulation of immune proteins (see Chapuisat et al. 2007; Castella et al. 2008b).

It is clear that among the antimicrobial peptides different signaling pathways can be involved in regulating their expression, which could account for the fact that differences were seen across some genes and not others in response to the resin treatment. For example Relish, a transcription factor of the Imd pathway, appears to regulate abaecin and hymenoptaecin but not defensin1 (Schlüns and Crozier 2007). There are also likely negative and positive feedback loops within the signaling pathways of the immune system, which could also influence differences across genes (Feldhaar and Gross 2008). Furthermore, individual variation in immune expression, even within closely related individuals, is extremely high, adding complexity to the relationship between the regulating pathways and the immune-related genes (Decanini et al. 2007). We had no a priori hypotheses about which genes might show differential expression in response to resin treatment. The fact that differences in transcript abundances were found for two different immune-related genes despite all of this inherent variation highlights the significance of these results.

This is the first known report to analyze gene expression across adults and larvae from the same genetic and environmental background under no pathogen challenge. The results presented here showing a relative increase in expression from larvae to 1-day and 7-day-old adults are supported by other evidence indicating that immune function is reduced in larvae as compared to young adults using more standard tests of immunocompetence (Wilson-Rich et al. 2008). Bees older than seven days were not analyzed as part of this study because as bees age investment in immune function becomes extremely variable as individuals change physiological state and behavioral task and begin to immunosenesce (i.e., Amdam et al. 2005). This is particularly the case at the onset of foraging, which typically ranges from 10-day to 30-day old among nest-mates (Winston 1987).

The sample sizes were likely too low for the third-instar larvae and 1-day-old bees to detect differences due to exposure to a resin-rich environment. However, it is possible that larvae would be unaffected by the propolis on the nest walls because they are surrounded by other antimicrobial substances (i.e., royal jelly, Morse and Flottum 1997). Additionally 1-day-old bees, just emerging from their cells, have possibly not yet been exposed enough to the various microbes in the nest to cause a full upregulation of their immune systems. Future work should be done on this front, as little research has examined general differences in immune-gene expression across larvae and adults in colonies with little pathogen or parasite pressure. Additionally, the results of this study indicate that not all immune-related genes are expressed at detectable levels at all ages. Age effects on baseline levels of immune expression need to be studied further at the genetic level.

Because the honey bee genome is now fully sequenced (HGSC 2006), we have a unique opportunity to study more subtle effects of the immune system at the individual level and then follow that to the colony level. The present study is only a first step in research concerning the importance of resins in their use as propolis as a form of social immunity by honey bees.

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