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RESEARCH ARTICLE

Social immunity in honey bees: royal jelly as a vehicle in transferring bacterial pathogen fragments between nestmates

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ABSTRACT

Social immunity is a suite of behavioral and physiological traits that allow colony members to protect one another from pathogens, and includes the oral transfer of immunological compounds between nestmates. In honey bees, royal jelly is a glandular secretion produced by a subset of workers that is fed to the queen and young larvae, and which contains many antimicrobial compounds. A related form of social immunity, transgenerational immune priming (TGIP), allows queens to transfer pathogen fragments into their developing eggs, where they are recognized by the embryo's immune system and induce higher pathogen resistance in the new offspring. These pathogen fragments are transported by vitellogenin (Vg), an egg-yolk precursor protein that is also used by nurses to synthesize royal jelly. Therefore, royal jelly may serve as a vehicle to transport pathogen fragments from workers to other nestmates. To investigate this, we recently showed that ingested bacteria are transported to nurses' jelly-producing glands, and here, we show that pathogen fragments are incorporated into the royal jelly. Moreover, we show that consuming pathogen cells induces higher levels of an antimicrobial peptide found in royal jelly, defensin-1.

KEY WORDS: Honey bees, Social immunity, Royal jelly, American foulbrood

INTRODUCTION

Royal jelly has long fascinated biologists because of the key role it plays in caste development in honey bees. This glandular secretion is produced by a subset of the worker caste known as nurses and fed to the queen throughout the duration of her development and adult life, while worker-destined larvae are fed royal jelly for the first 3 days of their lives before being switched to a more pollen-based diet (Johansson, 1955; Haydak, 1970; Townsend and Lucas, 1940). As analytical chemistry methods advanced, scientists began to study the nutritional components of royal jelly and found it contained many pathogen-killing compounds that protect the queen and young larvae from disease (Blum et al., 1959; Fontana et al., 2004; Fujiwara et al., 1990; Okamoto et al., 2003; Romanelli et al., 2011; Sugiyama et al., 2012; Vucevic et al., 2007). The transfer of anti-pathogenic compounds between nestmates is a form of social immunity, a suite of behavioral and physiological traits that help colony members protect one another from pathogens (Cremer et al., 2007). A related form of social immunity, transgenerational immune priming (TGIP), has also garnered much attention in recent years (Sadd et al., 2005; Moret, 2006; Freitak et al., 2009, 2014; Zanchi et al., 2011; López et al., 2014; Knorr et al., 2015; Salmela et al., 2015). Here, female insects that survive a pathogen attack can transfer pathogen fragments to their eggs and produce offspring that are more disease resistant. These pathogen fragments contain pathogen-associated molecular patterns, structural components found in the cell walls of microbial cells, which trigger an immune response in the developing offspring. While TGIP has been demonstrated in honey bees, the studies relied on injecting queens with an inoculum and this presented a problem: how would a queen be inoculated with pathogens under natural conditions given that she feeds exclusively on royal jelly and has no opportunity to consume potentially contaminated nectar and pollen collected by her foragers? A major discovery by our group hinted at a new possible pathway. Queens transfer pathogen fragments into their eggs using vitellogenin (Vg) (Salmela et al., 2015), an egg-yolk precursor protein that is also used by nurses to synthesize royal jelly (Amdam et al., 2003). Thus, honey bees may use royal jelly as a vehicle for transferring pathogen fragments between nurses and queens and larvae as part of a colony-level immune pathway. In a first step to elucidating this pathway, we showed that nurses that ingested bacteria were able to transfer it from their midgut to their jelly-producing glands, and that knocking down *vg* expression impeded this process (Harwood et al., 2019). Now, we seek to determine whether nurses that ingest bacterial pathogens can incorporate them into their royal jelly, and whether ingesting pathogens also increases the concentration of other antimicrobial components in the royal jelly.

Understanding immune pathways in honey bees is important not only for the field of organismal biology but also for human food security. Honey bees are the premier insect pollinator in agriculture, adding at least $15 billion annually to the value of crops in the USA alone (USDA press release, 6 April 2020: https://downloads.usda.library.cornell.edu/usda-esmis/files/rn301137d/f7623q868/ft849239n/hcny0819.pdf). However, high annual colony losses continue to plague American beekeepers (United States Department of Agriculture, 2019), owing to multiple stressors like pesticide exposure and poor nutrition. But bee pests and pathogens are also a major contributor and are often detected in colonies that have perished (United States Department of Agriculture, 2019). These pathogens include a multitude of bacteria, funguses and viruses, many of which have no pharmacological treatments. Understanding how social immunity mediates pathogen infections could allow for more comprehensive treatment regimens and help reduce the disease burden that contributes to colony losses.

It is important to understand how mechanisms of social immunity complement the immunological defenses present in individual bees.

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Honey bees have several layers of defense, starting with structural barriers like a water-tight cuticle that block pathogen entry (Moret and Moreau, 2012). Pathogens that are ingested will end up in the midgut, the organ responsible for digestion and absorption, and here they face another physical barrier, the peritrophic matrix (Brandt et al., 1978; Lehane, 1997; Hegedus et al., 2009). This is a chitinous substance secreted from the midgut epithelial cells that acts as a sieve to block out large particles like pathogen cells from being absorbed. If pathogens breach these physical barriers, they are detected by pathogen pattern recognition receptors that activate several cellular and humoral immune responses. Cellular defenses can include phagocytosis by hemocytes (Evans and Spivak, 2010; Marringa et al., 2014; Lavine and Strand, 2002), while humoral responses can include the production of melanin to encapsulate foreign particles (González-Santoyo and Córdoba-Aguilar, 2012) and the production of antimicrobial peptides (AMPs) that directly kill pathogens (Bulet and Stöcklin, 2005; Bulet et al., 1999; Evans et al., 2006). The melanization response is induced when bee venom serine protease (Bi-VSP) activates the phenoloxidase cascade that leads to the production of melanin (Choo et al., 2010). AMPs, such as lysozyme and defensin-1, are induced following activation of the Toll or immune deficiency (IMD) pathways (De Gregorio et al., 2002).

Despite a robust anti-pathogen arsenal, honey bees actually possess fewer immune related genes than solitary bee species (Evans et al., 2006; Claudianos et al., 2006), thanks largely to the evolution of social immunity. Royal jelly contains many of the enzymes and peptides just discussed, including Bi-VSP, lysozyme and defensin-1, as well as glucose oxidase, which produces hydrogen peroxide that also kills pathogens (Fontana et al., 2004; Fujiwara et al., 1990; Romanelli et al., 2011; Furusawa et al., 2008; Han et al., 2011; Fujita et al., 2012). This fortified food is particularly important for the most vulnerable colony members, the young larvae, whose immune defenses are still developing. For example, young larvae have lower levels of pro-phenoloxidase (Chan and Foster, 2008; Chan et al., 2009) (important in the melanization cascade) and AMPs (Chan and Foster, 2008), and their still-maturing midgut and peritrophic matrix leaves their midgut epithelium vulnerable to breach (Yue et al., 2008; Garcia-Gonzalez and Genersch, 2013; Riessberger-Gallé et al., 2016). As a result, larvae are susceptible to some diseases that are fairly innocuous for adults. This includes American foulbrood, caused by the spore-forming Gram-positive bacteria *Paenibacillus larvae* (Genersch, 2010; Hansen and Brødsgaard, 1999). American foulbrood infection is not only lethal for colonies but also costly for beekeepers because spores are resilient and remain viable for years, meaning beekeepers are forced to destroy infected hives. Thus, larvae stand to benefit from food supplemented with compounds that kill pathogens, or with pathogen particles that can elicit immune priming.

In this study, our aim was twofold: first, to determine whether nurses that consume American foulbrood vegetative cells could incorporate pathogen fragments into their royal jelly, and second, to ascertain whether this induces higher levels of immune proteins found in royal jelly. To this end, we fed nurses with fluorescently labeled and heat-killed *P. larvae* cells and examined the royal jelly they produced thereafter. We used fluorescence microscopy to confirm the presence of bacteria cell fragments in the royal jelly, and mass spectrometry to compare the proteomic profile of royal jelly from challenged and control colonies. In particular, we tested the hypothesis that royal jelly from challenged colonies would be higher in the immune proteins glucose oxidase, Bi-VSP, lysozyme and defensin-1.

MATERIALS AND METHODS

Culturing pathogenic bacteria

Vegetative cells of *P. larvae* were obtained from the Göteburg University culture collection and cultured under normal bacteria growing conditions. Briefly, cells were suspended in MYPG broth and kept at 37°C overnight in a shaking incubator, before being plated on MYPG agar and left to incubate for 1 week at 37°C. After that, vegetative cells were harvested and suspended in 1× phosphate-buffered saline (PBS), before being heat-killed in an autoclave. The resulting non-viable bacteria cells were then conjugated to a fluorescent dye using pHrodo™ Red Phagocytosis Particle Labeling Kit (Invitrogen #a10026) following the manufacturer's instructions. Once dyed, *P. larvae* cells were suspended in 1× PBS to a concentration of 10 mg ml^{-1} and stored at 4°C until ready for use.

Bees and feeding experiment

Naturally mated European honey bees, *Apis mellifera* Linnaeus 1758, were maintained in standard Langstroth hive boxes at the University of Helsinki on the Vikki campus in Finland. These standard Langstroth hives served as donors for smaller queenless colonies housed in mini mating hives. To establish these smaller queenless colonies, a section of brood comb containing 1 day old larvae and measuring 12 cm×12 cm was excised from the donor hive and transferred into a mini mating Nuc box. Roughly 100–200 nurses were also transferred from the same donor hive and placed in the mini mating Nuc box. Nurses were identified as those individuals that were seen entering brood cells and feeding larvae in the donor hive. In total, N=6 small queenless colonies were established, each from a separate donor hive. The colonies were made queenless so as to stimulate nurses to produce more royal jelly in an effort to make a new queen (Sahinler and Kaftanoglu, 1997). The nurses and young larvae were sealed in the mini mating Nuc boxes for 3 days and provided with a 30% sucrose solution in a syringe-feeder suspended from the ceiling. N=3 colonies received a control diet, while N=3 had fluorescently labeled *P. larvae* added to their sucrose to a final pathogen concentration of 0.6 mg ml^{-1}. Control and pathogen-laced diets were replenished with fresh food daily. After the 3 days, newly deposited royal jelly was harvested from the worker brood combs of each colony, and divided into one of two workflows: fluorescence microscopy or proteomics analysis.

Fluorescence microscopy

Royal jelly from each comb was transferred into 200 µl reaction tubes. To estimate the volume of jelly obtained from each hive, we pre-filled 30 reaction tubes with increasing volumes of water ranging from 1 to 30 µl and had two observers independently and blindly assess which water volume matched the royal jelly volume. In all 6 cases, each royal jelly sample received the same volume estimate from the two observers. Next, each royal jelly sample was diluted with 1× PBS (pH 7.4) to a final concentration of 10% by volume and vortexed. Samples were then applied to a hemocytometer and examined under a Leica DM6000 fluorescence microscope. On the hemocytometer, the field of view is evenly divided into 9 sections, and we made observations on 5 sections in total: the 4 corner sections and the center section. This allowed us to calculate the concentration of fluorescent particles per microliter of royal jelly (C), using the following formula:

$$C = \left(\frac{P}{S \times V}\right) \times D, \quad (1)$$

where P is the total number of positive fluorescent particles observed in each sample, S is the total number of squares observed

on the hemocytometer (N=5), V is the volume of each square observed on the hemocytometer (0.1 µl) and D is the dilution factor of the royal jelly (10× dilution). All samples were observed under identical microscope settings.

The camera was set at 16 bit resolution (Waters, 2009), which yields a brightness intensity value ranging from 0 to 65,535. To identify positive fluorescence signals in our pathogen-diet samples, we first needed to establish a baseline level of background noise, above which we could determine whether a signal was real fluorescence or merely an artefact. To do this, we examined the intensity values from each image of our control-diet samples, and in each we subtracted the minimum intensity value from the maximum intensity value to obtain a range of background noise (Waters, 2009). We then compared all of our control-diet sample images to obtain a mean and standard deviation of the range of background noise. Thereafter, we deemed any signal we observed in our pathogen-diet samples as being a positive fluorescence signal above background noise if it was at least 3 standard deviations (P≤0.01) above the mean background intensity range. The mean (s.d.) background intensity range was 329±272, giving us a positive fluorescence intensity threshold of 1147.

Additional positive and negative controls

To confirm that labelled bacteria can fluoresce in royal jelly, we collected royal jelly samples from multiple European and North American regions (Austria, Norway, and Arizona and Illinois, USA), diluted them to 10% in PBS buffer, and added labeled bacteria (1% by volume) directly to the samples. We used PBS buffer at standard pH 7.4 (as used above), or at reduced pH 4.0 that matches the acidity of royal jelly to determine whether the acidity of the environment affects the level of fluorescence, as stated by the labeling kit manufacturer. Moreover, we also performed an additional negative control (pH 4.0 PBS, no labelled bacteria) to confirm that observations of fluorescence were not due to microscopy artefacts or autofluorescence of material components. We found that royal jelly samples from all geographic regions showed fluorescence when labeled bacteria were added, regardless of the pH of the buffer used (Fig. S1). This microscopy work was performed on a Zeiss AxioZoom V16 using an Axiocam HRm monochrome camera.

Sample preparation for proteomics analysis

The royal jelly samples were diluted by addition of 150 µl 8 mol l^{-1} urea and sonicated in a water bath. The protein concentration was adjusted to 2.1 mg ml^{-1} using the Bradford assay with final volume of 100 µl. Cysteine bonds were reduced with 45 mmol l^{-1} dithiothreitol (#D0632, Sigma-Aldrich) for 20 min at 37°C and alkylated with 0.1 mol l^{-1} iodoacetamide (#57670 Fluka, Sigma-Aldrich) at room temperature. Samples were digested by adding 0.75 µg trypsin (Sequencing Grade Modified Trypsin, V5111, Promega) overnight at 37°C. After digestion, peptides were purified with C18 microspin columns (Harvard Apparatus) according to the manufacturer's protocol. The dried peptides were reconstituted in buffer A (30 µl 0.1% trifluoroacetic acid in 1% acetonitrile).

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

The analysis was carried out on an EASY-nLC1000 liquid chromatograph (Thermo Fisher Scientific) connected to a Velos Pro-Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific) with a nano electrospray ion source (Thermo Fisher Scientific). The LC-MS/MS samples were separated using a two-column setup consisting of a 2 cm C18-Pepmap trap column (Thermo Fisher Scientific), followed by a 15 cm C18-Pepmap analytical column (Thermo Fisher Scientific). The linear separation gradient consisted of 5% buffer B (0.1% trifluoroacetic acid in 98% acetonitrile) for 5 min, 35% buffer B for 60 min, 80% buffer B for 5 min and 100% buffer B for 10 min at a flow rate of 0.3 µl min^{-1}. For each LC-MS/MS run, 6 µl of sample was injected and analyzed. A full MS scan was acquired with a resolution of 60,000 at normal mass range in the orbitrap analyzer and followed with CID-MS2 top 20 most intense precursor ions with in ion trap (energy 35). Data were acquired using LTQ Tune software.

Protein identification

In a shotgun proteomics approach, proteins are enzymatically digested into smaller peptides, and these peptides are then matched to proteins in an annotated genome. Some of these peptide sequences may be shared by several proteins, while some are unique to a single protein in the proteome. Our analysis yielded a total of 496 protein hits containing at least 1 peptide spectrum match (PSM) and 1 unique peptide. To further rule out false positives, we only considered proteins with at least 2 unique peptides as being reliable protein hits, as has been done in other royal jelly proteomics studies (Hu et al., 2019; Zhang et al., 2014), and in part because experimental variation in sample preparation steps affects most greatly the least abundant proteins in mass-spectrometric samples (Zhang et al., 2010). Proteins were annotated to the *Amel_4.5* honey bee genome, and we used UniProt and GenBank to confirm their identities and functions. We then compared the abundance of 4 immune-related proteins in control-diet and pathogen-diet royal jelly: glucose oxidase, Bi-VSP, lysozyme and defensin-1.

Statistical analysis

To evaluate the abundance of immune-related proteins between control-diet and pathogen-diet royal jelly, we compared the PSM values of our chosen proteins. We used non-parametric Wilcoxon rank sum tests to address our limited sample size (N=3 per treatment). We used one-tailed comparisons as our planned hypotheses predicted that these select immune proteins would be upregulated in royal jelly from pathogen-fed colonies. Fold-changes in protein abundance were calculated as $(PSM_{pathogen}-PSM_{control})/PSM_{control}$. All analyses were performed in R (version 3.5.2).

RESULTS

Pathogen fragments in royal jelly

We found that royal jelly samples from all pathogen-diet colonies contained particles that fluoresced at significantly higher intensity than our established background fluorescence threshold (see Materials and Methods), suggesting that the bacterial fragments were incorporated into the royal jelly. No control-diet royal jelly samples contained particles that surpassed this threshold (Fig. 1). On average, pathogen-diet colonies had 66.67±28.87 (mean±s.d.) fluorescent particles per microliter of royal jelly (N=3) while control fed colonies had 0 fluorescent particles per microliter of royal jelly (N=3) (Fig. 1).

Proteomics

We found a total of 496 protein hits, and of these, 44 had at least 2 unique peptides assigned to them (Table 1). The most abundant proteins in all samples were major royal jelly proteins, as was expected. In general, the proteins we observed in our samples were quite consistent with other recent proteomics studies of royal jelly

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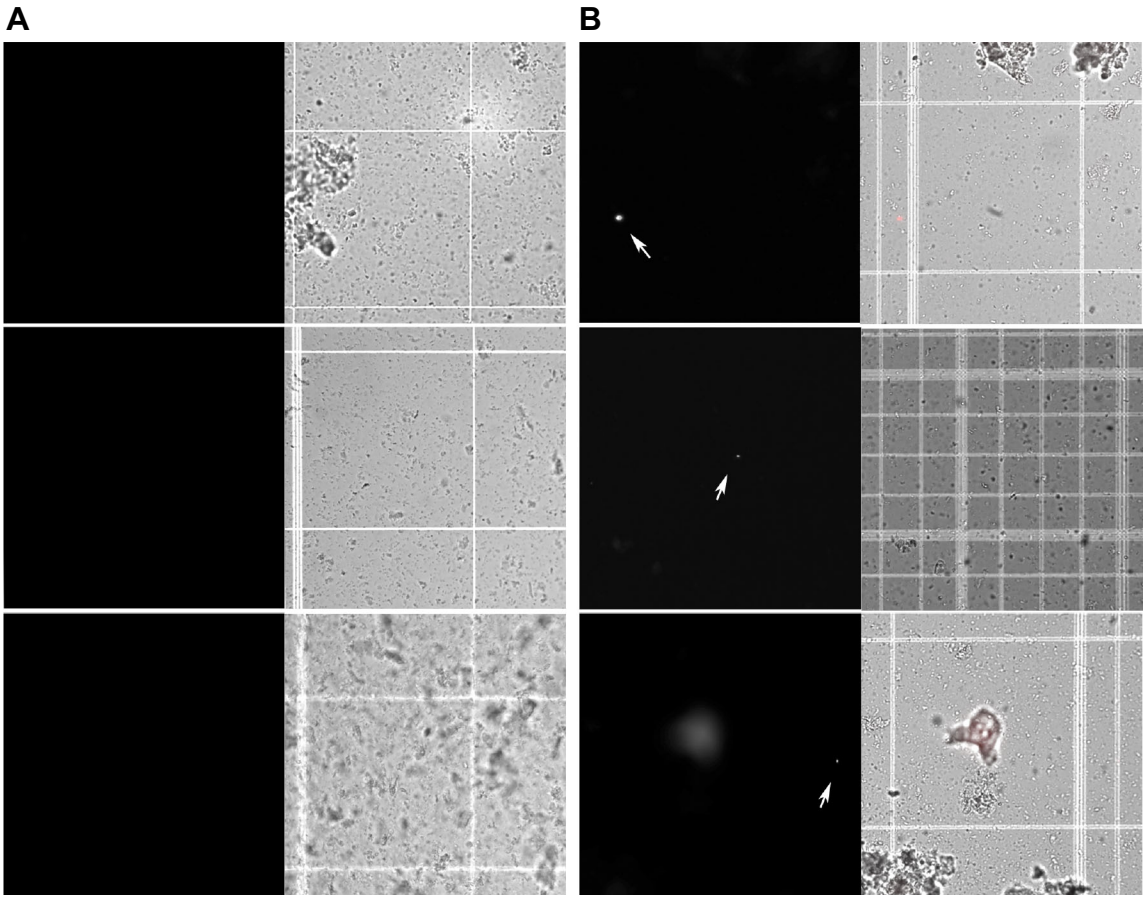

Fig. 1. Fluorescence and brightfield micrographs of royal jelly samples from control-diet and pathogen-diet treatments observed on a hemocytometer. All samples (A, control-diet treatment; B, pathogen-diet treatment) were prepared simultaneously using identical protocols and observed under identical microscope settings using a Leica DM6000. Dark images in the left-hand columns were taken with a TRITC laser to identify fluorescence in the Texas Red wavelength, while images in the right-hand columns are brightfield images of the same area to show the deposition of royal jelly on the hemocytometer. Positive fluorescence spots (white arrows) were only observed in the pathogen-diet samples (B) and not in the control-diet samples (A).

(Furusawa et al., 2008; Han et al., 2011; Fujita et al., 2012, 2013; Hu et al., 2019; Zhang et al., 2014), but there are a few proteins identified in this study that have not been reported elsewhere. These include: Transferrin, an iron transport molecule (Kucharski and Maleszka, 2003) that is also found in honey bee venom gland tissue (Peiren et al., 2008); Serpin-5, a serine protease inhibitor known to regulate prophenoloxidase activation in other insects (Li et al., 2016); Artichoke, a chemosensory protein with functions in larval locomotion in *Drosophila melanogaster* (Andrés et al., 2014); and NPC2-like, a cholesterol transporter with implied alloparental functions in honey bees (Thompson et al., 2006). We also found 3 additional proteins in our pathogen-diet royal jelly samples: Glutathione peroxidase, Peptidyl-prolylcis-trans isomerase and an uncharacterized protein (LOC725202). These proteins were also found in our control-diet samples, but they failed to meet our criteria of having 2 unique peptides (Table 1).

When comparing control-diet and pathogen-diet samples, we observed very little change in relative protein abundance within the royal jelly (Table 1). Among our focal immune proteins, we found no significant difference in protein levels between treatments for glucose oxidase (U=7, P=0.9), Bi-VSP (U=1.5, P=0.134) or lysozyme (U=2, P=0.184). However, we did find significantly more of the AMP defensin-1 in pathogen-diet samples than in control-diet samples (U=0, P=0.05) (Fig. 2). It had a fold-change of +0.68.

DISCUSSION

This study shows that nurses that ingest cells of the pathogen *P. larvae* appear to incorporate fragments of the cells into the royal jelly they produce (Fig. 1). We have shown recently that worker-ingested bacteria are transported to the hypopharyngeal glands, the site of royal jelly synthesis (Harwood et al., 2019), and here we show that they are incorporated into the royal jelly. This finding represents an overlooked social immunity pathway that can allow nestmates to share immunological memory. Our results also suggest that royal jelly composition may be sensitive to foreign material circulating systemically in the hemolymph. In a similar recent study, researchers found that nurses fed with double-stranded RNA (dsRNA) were able to incorporate these molecules into the royal jelly and transfer them to larvae, where the dsRNA remained biologically active (Maori et al., 2019). dsRNA activates the antiviral RNA-interference (RNAi) pathway that elicits sequence-specific gene silencing, and synthetic dsRNA is frequently used for targeted gene knockdown studies (Fire et al., 1998). Furthermore, another study showed that nurses fed with pesticides can also incorporate trace amounts of these agrochemicals into their royal jelly (Böhme et al., 2018), although the emphasis here was on how well the hypopharyngeal glands prevent systemic pesticides from entering the royal jelly. Nevertheless, these results indicate that royal jelly composition may be sensitive to the condition of nurse bees. These findings imply that royal jelly can serve as a conduit for

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Table 1. List of proteins found in royal jelly of control-diet and pathogen-diet samples

Accession no.	Control-diet PSM	Pathogen-diet PSM	Fold-change	Control-diet unique peptides	Pathogen-diet unique peptides	Protein name
GB55205	1115.3±227.1	905.7±99.4	−0.19	39.00	34.33	Major Royal Jelly Protein 1
GB55204	526.7±147.3	441.7±40.3	−0.16	23.33	20.33	Major Royal Jelly Protein 3
GB55212	304.3±77.5	257.7±43.4	−0.15	32.67	30.00	Major Royal Jelly Protein 2 precursor
GB55206	170.7±62.9	238.3±37.5	0.40	27.00	26.67	Major Royal Jelly Protein 4
GB45796	293.3±124.1	213.0±14.2	−0.27	2.67	2.33	Uncharacterized protein (LOC727045)
GB44549	169.7±11.3	158.7±9.0	−0.06	30.00	29.00	Glucose oxidase
GB55208	160.0±25.2	134.0±10.0	−0.16	18.00	15.67	Major Royal Jelly Protein 5 precursor
GB55213	134.0±20.9	125.0±6.5	−0.07	25.00	23.33	Major Royal Jelly Protein 7 precursor
GB43247	120.0±29.2	99.0±50.1	−0.18	31.33	27.33	Alpha-glucosidase
GB41428	48.0±17.7	80.7±6.9	0.68	3.67	3.33	Defensin-1
GB53578	44.0±9.4	54.3±7.7	0.23	19.67	20.00	Glucosylceramidase
GB55207	52.0±5.7	52.0±5.4	0.00	11.00	10.67	Major Royal Jelly Protein 6 precursor
GB55209	42.0±5.7	41.7±6.8	−0.01	8.67	9.00	Uncharacterized protein
GB43908	49.7±2.4	41.3±3.3	−0.17	40.67	32.33	Aldehyde oxidase/Xanthine dehydrogenase
GB44548	37.3±5.0	38.3±6.1	0.03	20.33	19.33	Glucose dehydrogenase isoform X1
GB48020	15.3±1.2	22.0±8.8	0.43	10.33	8.67	Uncharacterized protein
GB48969	13.7±0.9	19.3±6.2	0.41	5.67	5.67	Uncharacterized protein (LOC408608)
GB50226	20.3±3.3	18.3±3.3	−0.10	17.67	16.00	Transferrin
GB41326	18.0±5.0	18.0±2.2	0.00	12.00	12.67	Venom acid phosphatase Acph1
GB49854	18.0±5.7	18.0±13.5	0.00	13.67	11.00	Alpha amylase
GB41777	13.7±1.7	17.3±2.9	0.27	5.67	5.67	Uncharacterized protein
GB44223	11.0±5.4	16.3±5.2	0.48	10.33	15.33	Lysosomal alpha-mannosidase
GB51783	14.0±2.2	15.7±1.7	0.12	8.33	9.67	Carboxylpeptidase Q
GB55452	11.3±0.5	15.3±1.7	0.35	6.67	7.67	Apolipophorin III-like
GB53579	15.0±2.9	15.0±2.4	0.00	11.67	11.33	Putative glucosylceramidase 4
GB54611	10.7±3.9	13.7±0.5	0.28	8.67	10.00	Serpin-5 (serine protease inhibitor)
GB41776	9.7±0.5	11.7±2.1	0.21	4.00	3.67	Uncharacterized protein
GB43708	10.7±0.9	9.3±1.7	−0.13	7.67	6.67	Ferritin heavy polypeptide-like 17
GB49552	4.7±2.5	8.7±3.7	0.86	4.67	6.67	Venom serine protease Bi-VSP
GB51613	4.7±2.4	8.3±1.9	0.79	4.00	7.00	Uncharacterized protein (LOC408570)
GB53830	4.3±0.9	7.0±0.8	0.62	3.67	6.00	Protein Artichoke
GB50012	6.3±0.5	7.0±2.2	0.11	5.33	5.00	Uncharacterized protein (LOC726323)
GB42800	6.0±1.4	6.7±0.9	0.11	6.00	6.00	Protein Takeout-like
GB40759	6.7±0.9	6.3±1.9	−0.05	4.67	4.33	Icarapin-like
GB43731	8.0±1.4	5.7±2.9	−0.29	5.33	5.50	Ferritin subunit
GB44533	4.0±1.2	5.0±0	0.15	4.33	5.00	Uncharacterized protein (LOC408851)
GB40758	4.0±0.8	5.0±0	0.25	2.00	2.00	Icarapin-like precursor
GB47104	3.0±0.8	4.0±0.8	0.33	3.00	4.00	Lysozyme
GB50115	4.0±0	4.0±0.8	0.00		2.33	Uncharacterized protein (LOC725202)
GB48634	2.7±0.9	3.3±0.5	0.25		2.67	Glutathione peroxidase
GB43823	6.0±1.6	3.3±1.2	−0.44	4.00	2.33	Chemosensory Protein 1 precursor
GB44564	4.0±1.4	3.0±0.8	−0.25	4.00	3.00	Protein NPC2-like
GB55451	3.0±0	2.7±0.5	−0.11	3.00	2.67	Uncharacterized protein
GB46652	2.3±0.5	2.7±0.5	0.14		2.67	Peptidyl-prolyl cis-trans isomerase

Columns for control-diet and pathogen-diet peptide spectrum match (PSM) values display the mean across 3 samples, ±1 s.d. Fold-change is the relative change in pathogen-diet PSM compared with control-diet PSM: (pathogen−control)/control. Columns for the number of unique peptides show the mean across 3 samples.

sharing immune elicitors like pathogen fragments, dsRNA and other foreign substances between colony members.

The incorporation of pathogen fragments into royal jelly suggests that trans-generational immune priming may operate at the colony level in honey bees. Unlike females from solitary species, honey bee queens do not normally venture outside their nest (Maeterlinck, 1901), nor do they ingest food collected from their environment (Haydak, 1970), so they have fewer opportunities to accumulate immune elicitors from pathogens that currently threaten the colony. Pathogen fragments in royal jelly may help queens inoculate their offspring against a wider array of immune elicitors, and hence protect them from pathogens they are likely to encounter as adults. This colony-level pathway may be particularly suited to tackling pathogens like American foulbrood, as larvae are highly susceptible to the disease but adult workers are immune. Larvae only need to consume as few as 10 American foulbrood spores for an infection to set in (Shimanuki, 1997; Brødsgaard et al., 1998), but spores fail to germinate in the digestive tract of adults (Wilson, 1971). Nurses may also be able to directly inoculate larvae with royal jelly, meaning this pathway between nestmates would operate both vertically between offspring and parent, and horizontally between siblings. While our results point to a possible colony-level immune pathway, we caution that discovering a transport mechanism for a single honey bee pathogen is merely a first step and much more work is needed to support this.

This study also showed that exposing nurses to American foulbrood does not induce large changes in the proteomic profile of the royal jelly they produce. Between samples from control-diet and pathogen-diet colonies, there was minimal change in the relative abundance of most proteins, including immunological proteins like glucose oxidase, Bi-VSP and lysozyme (Table 1). However, the AMP defensin-1 appears to be an exception, with a modest relative fold-change of +0.68 in royal jelly from pathogen-diet colonies compared with control-diet colonies. This finding raises two

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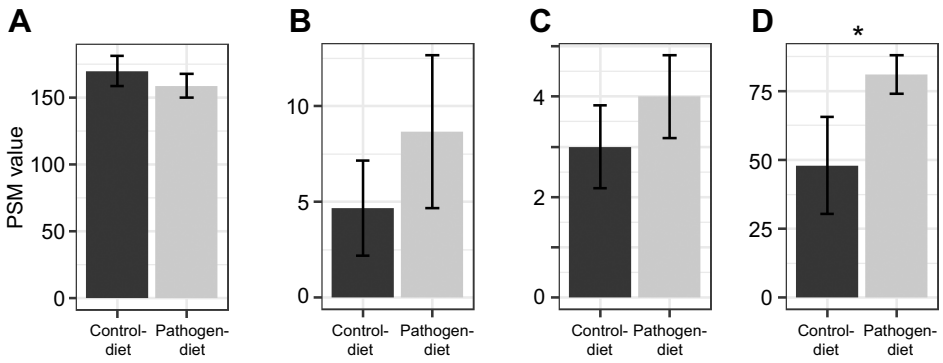

Fig. 2. The abundance of select immune proteins in control-diet and pathogen-diet royal jelly samples. (A) Glucose oxidase; (B) bee venom serine protease (Bi-VSP); (C) lysozyme; and (D) defensin-1. Bar heights represent the mean peptide spectrum match values over 3 samples, with error bars denoting ±1 s.d. Of the 4 proteins compared, only the antimicrobial peptide defensin-1 was significantly upregulated in pathogen-diet samples (Wilcoxon W=0, n=6, *P=0.05). PSM, peptide serum match.

immediate questions. First, how does exposure to a pathogen lead to nurses producing elevated levels of defensin-1 in the royal jelly? One possibility is that *P. larvae* fragments bind to pathogen pattern recognition receptors in the Toll pathway (for Gram positive bacteria) and induce greater gene transcription of defensin-1, but the evidence for this is somewhat inconsistent. Studies have shown that adult workers exposed to bacteria, including American foulbrood, will increase expression of defensin-1 (Evans et al., 2006; Casteels-Josson et al., 1994), but a recent study failed to observe significant upregulation defensin-1 expression in nurse head tissue after exposure to American foulbrood (López-Uribe et al., 2017). Nevertheless, insect AMPs like defensin-1 can be observed in hemolymph for weeks after pathogen exposure (Casteels, 1998), even as transcription of such AMP genes subsides during that time (Uttenweiler-Joseph et al., 1998), so only measuring gene expression may miss important information about the availability of the final peptide products. Alternatively, pathogen exposure may upregulate the translation of defensin-1 mRNA into peptides, or increase the rate of post-translational modifications used to activate the peptide (Casteels-Josson et al., 1994), but this will have to be studied further.

The second question raised by these results is whether a relative fold-change of +0.68 in defensin-1 is biologically relevant. Conventionally, many human proteomics studies opt for a +1.2 threshold for significant upregulation (Serang et al., 2013; Keenan et al., 2009), so defensin-1 would not meet this criterion. However, even modest increases in defensin-1 concentration in royal jelly may improve larval resistance to American foulbrood. Not only has defensin-1 been shown to directly inhibit American foulbrood (Bachanová et al., 2002; Bíliková et al., 2001) but also it is effective against Gram positive bacteria at concentrations as low as low as 1 µmol l^{-1} (Fujiwara et al., 1990). Furthermore, several components of the innate immune system are known to work synergistically, whereby two components working together have greater pathogen inhibitory effects than the sum of each component working independently. For example, bacterial growth inhibition assays have shown that AMPs and lysozyme work together to greatly enhance one another's effectiveness (Chalk et al., 1994). Lysozyme has also been shown to combat pathogens synergistically with apolipophorin-III (ApoLp-III) (Zdybicka-Barabas et al., 2013), a lipid-transporting protein with anti-pathogenic functions in insects (Whitten et al., 2004; Kim and Jin, 2015) that is found in royal jelly (Furusawa et al., 2008; Han et al., 2011; Zhang et al., 2014; Fujita et al., 2013). Finally, young honey bee larvae's maturing immune systems may struggle to produce their own defensin-1 when attacked by American foulbrood, as studies have shown that such a challenge induces little to no defensin-1 expression in larvae (Romanelli et al., 2011 Cremer et al., 2007; Serang et al., 2013; Keenan et al., 2009, although see Bachanová et al., 2002 for exception). Thus, additional defensin-1 from exposed nurses may supplement the larvae's dearth of this crucial AMP. Taken together, these findings suggest that even modest increases in defensin-1 in royal jelly may be biologically relevant in protecting the colony's most vulnerable members.

This study also further demonstrated the critical role that the protein Vg plays in honey bee immunity. Owing to its role as a pathogen pattern recognition receptor, which has been documented in a broad range of animal taxa (Salmela et al., 2015; Du et al., 2017; Garcia et al., 2010; Knight, 2019; Li et al., 2008, 2009; Liu et al., 2009; Zhang et al., 2011), as well as its role in egg formation, Vg has been identified as the carrier protein that transports immune elicitors into the queen's eggs (Salmela et al., 2015). Likewise, in nurses, recent findings suggest that Vg plays a key role in transporting ingested pathogens to the hypopharyngeal glands (Harwood et al., 2019). Vg is taken up by the nurses' hypopharyngeal glands to be used as an amino acid donor in the production of royal jelly (Amdam et al., 2003), and it is possible that Vg transports bacteria into the glands to be incorporated into the jelly. Thus, Vg may play a role in all stages of colony-level TGIP in honey bees, from first inoculation and incorporation into the royal jelly, to delivery to the queen or larvae, and finally in the transport into the queen's ovaries.

This study has shown that royal jelly composition can be altered by a pathogen challenge, both by incorporation of pathogen fragments and through increased levels of the AMP defensin-1. The ability to transfer pathogen fragments in royal jelly means that trans-generational immune priming may function at a colony-wide level in honey bees. In addition to a vertical transfer of immune elicitors from a reproductive female to her offspring, honey bees may have a more complex pathway that goes from adult offspring (nurses) to reproductive female to future offspring, as well as horizontal transmission from adult offspring to immature offspring. The benefit here would be that larvae are inoculated with a larger repertoire of immune elicitors accumulated from the collective immunological experience of workers that gather resources from outside the nest, making larvae more resistant against pathogens they are likely to encounter when they themselves venture out of the nest.

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Competing interests

G.H. and G.A. declare no competing or financial interests. H.S. and D.F. are listed as inventors on a patent (WO2017017313A1) for an edible bee vaccine. This patent is assigned to Dalan Animal Health Inc., where D.F. serves as Chief Scientific Officer. The research presented in this paper was conducted independently, with no

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Author contributions

Conceptualization: G.H., H.S., G.A.; Methodology: G.H., H.S.; Validation: G.H.; Formal analysis: G.H., H.S.; Investigation: G.H., H.S.; Resources: H.S., D.F., G.A.; Data curation: G.H.; Writing - original draft: G.H.; Writing - review & editing: G.H., H.S., D.F., G.A.; Visualization: G.H.; Supervision: G.A.; Project administration: D.F., G.A.; Funding acquisition: G.H., D.F., G.A.

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Supplementary information

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