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The role of Vitellogenin in the transfer of immune elicitors from gut to hypopharyngeal glands in honey bees (*Apis mellifera*)

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ABSTRACT

Female insects that survive a pathogen attack can produce more pathogen-resistant offspring in a process called trans-generational immune priming. In the honey bee (*Apis mellifera*), the egg-yolk precursor protein Vitellogenin transports fragments of pathogen cells into the egg, thereby setting the stage for a recruitment of immunological defenses prior to hatching. Honey bees live in complex societies where reproduction and communal tasks are divided between a queen and her sterile female workers. Worker bees metabolize Vitellogenin to synthesize royal jelly, a protein-rich glandular secretion fed to the queen and young larvae. We ask if workers can participate in trans-generational immune priming by transferring pathogen fragments to the queen or larvae via royal jelly. As a first step toward answering this question, we tested whether worker-ingested bacterial fragments can be transported to jelly-producing glands, and what role Vitellogenin plays in this transport. To do this, we fed fluorescently labelled *Escherichia coli* to workers with experimentally manipulated levels of Vitellogenin. We found that bacterial fragments were transported to the glands of control workers, while they were not detected at the glands of workers subjected to RNA interference-mediated Vitellogenin gene knockdown, suggesting that Vitellogenin plays a role in this transport. Our results provide initial evidence that trans-generational immune priming may operate at a colony-wide level in honey bees.

1. Introduction

Efficient anti-pathogen defense mechanisms support the survival of individuals. Various physiological and behavioral mechanisms have evolved to maximize organismal fitness as part of the immune system (Schmid-Hempel, 2001). Immune responses must act quickly and target bacterial and fungal cells, as well as viruses. In vertebrates, a complex antibody-based immunological memory has evolved, which renders individuals and initially also their offspring immune to the same pathogen (Hasselquist and Nilsson, 2009). Invertebrates, although lacking antibody-based immunological memory, can prime both themselves (Roth et al., 2009; Sadd and Schmid-Hempel, 2006; Tidbury et al., 2011) and offspring (Freitak et al., 2009; Little et al., 2003; López et al., 2014; Moret, 2006; Sadd et al., 2005; Tidbury et al., 2011) against pathogens. The phenomenon is called trans-generational immune priming. Trans-generational immune priming occurs by females transferring ingested pathogen fragments to their developing eggs, where they elicit an immune response in the developing embryo (Freitak et al., 2014).

We found that Vitellogenin (Vg) may facilitate trans-generational immune priming in honey bees, as this protein transports immune elicitors into developing eggs (Salmela et al., 2015). Vg is an egg yolk precursor protein, essential in delivering nutrients into the eggs of most oviparous species. Moreover, it can bind pathogen-associated molecular patterns (PAMPs) (Li et al., 2009, 2008; Liu et al., 2009; Salmela et al., 2015). PAMPs include molecular motifs of bacterial and fungal cells like lipopolysaccharides and peptidoglycans. The ability of Vg to bind PAMPs, and the protein's transport into eggs, suggests a central role for Vg in immune priming.

Honey bee Vg has evolved to have important social functions (Amdam et al., 2003a; Nelson et al., 2007). Colonies have a division of labor, with the queen responsible for reproduction and sterile workers responsible for colony maintenance. Workers further undergo an age-associated behavioral maturation that depends on titers of Vg in their

Abbreviations: PAMPs, pathogen-associated molecular patterns; PBS, phosphate-buffered saline; RNAi, RNA interference; Vg, vitellogenin

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blood (haemolymph) (Amdam and Omholt, 2003; Guidugli et al., 2005; Nelson et al., 2007). Young workers remain in the nest as nurses to clean cells, rear brood, and feed the queen, and have high titers of Vg that peak around age 5–15 days (Engels et al., 1990; Fluri et al., 1982). A sharp drop in Vg titers and a concomitant increase in juvenile hormone titers prompt workers to transition into foragers and leave the nest to collect nectar and pollen (Amdam and Omholt, 2003; Nelson et al., 2007). Additionally, Vg is used by the nurse bees as an amino acid donor for producing royal jelly (Amdam et al., 2003a), a protein-rich food synthesized in their hypopharyngeal (head) glands that they feed orally to the queen and young larvae (Snodgrass, 1956). The hypopharyngeal glands express Vg receptors, presumably to allow uptake of Vg circulating in the haemolymph (Guidugli-Lazzarini et al., 2008).

The immune elicitors used to initiate trans-generational immune priming may come from environmental pathogens encountered and ingested by a female. However, honey bee queens have limited exposure to environmental pathogens since they reside entirely in the nest except for their mating flight (or flights) early in life and possible swarming flight later in life (Michener, 1969). For trans-generational immune priming to be effective in honey bees, a queen must prime her offspring against pathogens that her workers encounter. As we suggested previously (Salmela et al., 2015), queen exposure may occur via consuming contaminated food. As queens feed exclusively on worker-produced royal jelly (Haydak, 1970), this potential pathway would require workers to transfer ingested pathogen fragments to their hypopharyngeal glands. Here, we test if that requirement can be met by observing whether bacterial fragments fed to workers are transferred to their hypopharyngeal glands. In this study, we fed heat-killed fluorescently-labelled *Escherichia coli* particles to worker honey bees and used immunohistochemistry to localize Vg and bacterial particles in three tissues: the midgut, the fat body, and the hypopharyngeal glands. The midgut is where ingested pathogen cells are broken down and potentially absorbed through the epithelium (Buchon et al., 2013). The fat body regulates the metabolic and immunological state of the organism and plays a central role in immune response (Bulet and Stöcklin, 2005; Stokes et al., 2015). It is also the primary site of Vg synthesis and storage (Bownes, 1986; Isaac and Bownes, 1982; Pan et al., 1969; Raikhel and Lea, 1983). We contrasted control workers and RNA interference (RNAi)-mediated Vg-knockdown workers to examine what role Vg plays in this pathway. We also measured how Vg RNAi affected expression of two other control genes that either share similar sequence homology or function as Vg: *Vitellogenin-like-C* (*vg-like-C,* Salmela et al., 2016) and *apolipophorin-III* (*apoLp-III,* Weers and Ryan, 2006; Whitten et al., 2004), respectively. These controls are used to validate the specificity of the double-stranded RNA (dsRNA) used in the RNAi protocol, and to ensure that any difference between treatments in *E. coli* tissue localization is due to Vg-knockdown and not due to inadvertent effects to other genes that share similar immunological functions as Vg.

We found that *E. coli* particles were transferred to the hypopharyngeal glands of control workers, but not to the glands of Vg-knockdown workers. This observation confirms that ingested pathogen fragments can be transported to the production-site of royal jelly, and that Vg plays a role in this process in worker bees. As an additional finding, we detected Vg protein immunoreactivity in the midgut and hypopharyngeal glands of both controls and Vg-knockdowns. This result suggests that these organs can maintain Vg stores after the rate of *de novo* synthesis has been suppressed by RNAi in the fat body.

2. Methods

2.1. Bees

Stock colonies of honey bees were maintained at the Arizona State University (ASU) Bee Research Facility in Mesa, Arizona. Frames of sealed brood from two hives were placed overnight in a 34 °C incubator with 80% relative humidity. Newly emerged workers (up to 24 h old) were then secured to a wax-filled dissecting dish with two crossed needles and immobilized in a refrigerator. Following oft-used protocols first established in Amdam et al. (2003b), bees were next subjected to one of three injection treatments: i) a 1 µl injection of 10 ng/µl double-stranded RNA (dsRNA) of the *vitellogenin* (*vg*) gene to achieve RNAi (N = 89); ii) a 1 µl sham injection of nuclease-free water (vehicle, Ambion #AM9938) to serve as an injection control (N = 85); or iii) no injection to serve as a handling control (N = 139). For injection controls, injecting the vehicle is a thoroughly established procedure that is frequently performed and widely accepted (e.g., Amdam et al., 2003b; Ament et al., 2011; Antonio et al., 2008; Guidugli et al., 2005; Ihle et al., 2015, 2010; Seehuus et al., 2006; Wheeler et al., 2013). Injections were made between for 5th and 6th abdominal segment using a 10 µl Hamilton syringe with a G30 needle (BD). Bees that showed signs of bleeding after an injection were discarded and omitted from the experiment. The remaining bees were paint marked according to injection treatment and placed in two established host colonies at the main ASU campus in Tempe, Arizona. After 7 days we collected 158 bees (32 Vg dsRNA-injected, 41 sham-injected, 85 control-handled) and placed them into feeding cages (13 × 8 × 7 cm). The cages received 1 of 2 feeding treatments: either 30% sucrose in distilled water (control feeding), or the same food but with 0.5 mg/mL of *E. coli* (K – 12 strain) BioParticles® with Texas Red® conjugate (Molecular Probes #E2863) (bacteria feeding). Food was provided in 10 mL aliquots via a 30 mL syringe, which was replenished daily. Cages were also provided with water and the bees remained caged in the 34 °C incubator with 80% humidity for 48 h before being dissected.

2.2. Immunohistochemistry

Bees were anesthetized on ice and pinned to a wax-filled dissecting dish. The midgut, hypopharyngeal glands, and dorsal fat body were dissected and fixed separately in 4% paraformaldehyde for 48 h at 4 °C, while the ventral fat body was prepared separately to confirm RNAi-mediated gene knockdown. Tissues were washed three times in 1X phosphate-buffered saline (PBS) before the midgut and hypopharyngeal glands were embedded in agarose gel and sectioned into 100 µm sections using a Leica VT1000s vibratome. The fat body was not embedded in agarose, but instead remained attached to the cuticle for the staining and washing procedures before being dissected from the cuticle and whole-mounted onto a slide. This was for practical purposes, as the fat body is a 1-cell thick sheet of tissue that does not require sectioning and would likely be damaged with other processing protocols. All tissue samples were incubated overnight at 4 °C in 1X PBS containing 1:1000 polyclonal rabbit-anti-Vg 1° antibodies (raised against 180 kDa honey bee vitellogenin; Pacific Immunology, Ramona, CA), 5% goat serum (Jackson ImmunoResearch #005-000-121), and 0.1% Triton X-100 (Sigma #T8787). Antibody specificity has been tested and confirmed in previous studies (Seehuus et al., 2007). Samples were then washed five times before being incubated at room temperature for 3 h in 1X PBS containing 1:1000 goat-anti-rabbit 2° antibodies conjugated with Alexa Fluor® 488 (Jackson ImmunoResearch #111-545-047), 5% goat serum, and 0.1% Triton X-100. The samples were washed three more times before being incubated for 15 min at room temperature in 1X PBS containing 1:30000 DAPI (Molecular Probes®D1306) and 0.1% Triton X-100. After a final five washes the samples were mounted in glycerol on glass slides. We also performed negative staining controls on all tissue-types to again confirm the specificity of the 1° antibodies and to look for any issues of autofluorescence that may occur in the range of wavelengths covered by Alexa Fluor® 488. For this, tissues were prepared and stained exactly as described above, except that in the first incubation the tissue was bathed in PBS with goat serum and triton but no 1° antibodies. Slides were imaged on a Leica TCS SP5 confocal microscope using a 40X oil-immersion objective. We imaged tissues from 22 bees, representing 3–4 individuals for all 6 treatments (3 injections treatments × 2 feeding treatments).

2.3. Preparation of dsRNA

Double-stranded RNA was prepared as previously described (Amdam et al., 2006, 2003b; Antonio et al., 2008; Guidugli et al., 2005; Ihle et al., 2015, 2010; Nelson et al., 2007). Primers were designed against the honey bee *vitellogenin* gene (GenBank number: AJ517411) cDNA clone *AP4a5* and fused with the T7 promotor sequence (underlined).

Forward: 5′ – TAATACGACTCACTATAGGGCGAACGACTCGACCAA CGACTT – 3′.

Reverse: 5′ – TAATACGACTCACTATAGGGCGAAACGAAAGGAACG GTCAATTCC – 3′

PCR amplification was performed under normal conditions using Illustra™ PuReTaq Ready-to-Go™ PCR beads (GE Healthcare # 27955701) and the *AP4a5* clone as a template. PCR produced a product (excluding the T7 promotor) with a size of 504 bp. The product was purified using a QIAquick PCR purification kit (Qiagen #28104) and the RNA was prepared using a RiboMAX™ Large Scale RNA Production System (Promega #P1300). The RNA was then extracted via phenol-chloroform extraction using TRIzol® LS reagent (Invitrogen #10296028) and re-suspended in nuclease-free water to a final concentration of 10 ng/μl.

2.4. RNAi knockdown validation

Gene expression was compared using fat body tissue, as this is the primary site of *vg* expression (Bownes, 1986; Isaac and Bownes, 1982; Pan et al., 1969; Raikhel and Lea, 1983) and the target tissue for *vg* RNAi in honey bees (Amdam et al., 2006, 2003b; Antonio et al., 2008; Guidugli et al., 2005; Ihle et al., 2015, 2010; Nelson et al., 2007). RNA from the ventral fat body was extracted with TRIzol® LS reagent and re-suspended in nuclease-free water to a concentration of 100 ng/μl. A 1-step RT-qPCR was performed in triplicate with an ABI Prism 7500 (AppliedBiosystems) using a Quantitech SYBR® Green RT-PCR kit (Qiagen #204243). Actin was used as a housekeeping gene because it is stably expressed across honey bee tissues (Lourenço et al., 2008; Scharlaken et al., 2008) and is commonly used for knockdown validation (Amdam et al., 2004; de Azevedo and Hartfelder, 2008; Ihle et al., 2015; Wang et al., 2013, 2012). Data were analyzed using the ΔΔCT method (Schmittgen and Livak, 2008). The effect of treatment on *vg* expression was determined using a one-way ANOVA, and differences between individual treatment groups were calculated with Tukey's honest significant difference test. Data were log-transformed to achieve normality. Analyses were performed in R (v3.3.2). Negative controls (no template) were used to rule out DNA contamination. Primer sequences used for RT-qPCR reactions were as follows:

Vg Forward: 5′ – GTTGGAGAGCAACATGCAGA – 3′
Vg Reverse: 5′ – TCGATCCATTCCTTGATGGT – 3′
Actin Forward: 5′ – TGCCAACACTGTCCTTTCTG – 3′
Actin Reverse: 5′ – AGAATTGACCCACCAATCCA – 3′

2.5. Effect of Vg RNAi on expression of control genes

RNAi can have unintended consequences by affecting expression of off-target genes, thereby confounding the interpretation of results. In these cases, it becomes unclear whether the phenomena observed are due to successful knockdown of the target gene, or altered expression of some off-target genes. For example, injected dsRNA is broken down into small interfering RNAs (siRNAs) that target transcripts with complementary sequences, but if the dsRNA lacks sufficient specificity then the resulting siRNAs can also knockdown other genes with a similar complementary sequences (Jackson and Linsley, 2010). To validate the specificity of our *vg* dsRNA we measured expression of *vg-like-C*, as this *vg* homolog shares a similar sequence as *vg* and would likely be knocked down if our dsRNA were insufficiently specific (Morandin et al., 2014; Salmela et al., 2016). We can validate the specificity of our dsRNA if *vg-like-C* is equally expressed in individuals across all three injection and control treatments. Additionally, confounding results in RNAi can arise by the unintended activation of the immune system. Injected dsRNA activates the antiviral state in honey bees and alters expression of hundreds of genes (Flenniken and Andino, 2013). If expression is altered in an immune gene with similar functions as *vg*, then any differing pattern of *E. coli* tissue localization observed between treatment groups could be due to this immune response to dsRNA and not due to the Vg-knockdown. *ApoLp-III* is a good candidate for an off-target immune-related control gene because of its functional similarity to *vg*: it performs several innate immunity functions including binding many PAMPs (Weers and Ryan, 2006; Whitten et al., 2004), it circulates in the haemolymph (Kawooya et al., 1984), it is expressed in the fat body (Cole and Wells, 1990) and in the hypopharyngeal glands (Corby-Harris et al., 2016), and it is present in royal jelly (Han et al., 2011). We contrasted *apoLp-III* expression in individuals injected with nuclease-free water (sham) and those injected with *vg* dsRNA to confirm that observed patterns of *E. coli* tissue localization were due to the effect of Vg-knockdown and not due to an immunological response to dsRNA. We measured *vg-like-C* and *apoLp-III* expression using the same procedure described above, and used honey bee primer sequences that were previously used and published by other (Lourenço et al., 2009; Salmela et al., 2016)

Vg-like-C Forward: 5′ – AACGCGATCACATCAGTCGT – 3′
Vg-like-C Reverse: 5′ – CGTGCCGCCAACAGATATGG – 3′
ApoLp-III Forward: 5′ – TCTGACAAAGCTGCGAAATC – 3′
ApoLp-III Reverse: 5′ – AGTTGCGGCAGTTTGAAGTT – 3′

3. Results

3.1. Gene expression

Individuals injected with *vg* dsRNA (hereafter referred to as Vg-knockdown bees) showed successful *vg* gene expression knockdown compared with control-handled and sham-injected bees (Fig. 1A). Treatment groups differed significantly in *vg* expression (1-way ANOVA, $F_{2,\ 45} = 16.00$, $p = 5.6E-6$). Control-handled bees had significantly higher *vg* expression than both the sham-injected ($p = 0.026$) and Vg-knockdown ($p < 0.001$) bees, while sham-injected bees had significantly higher *vg* expression than Vg-knockdown bees ($p = 0.014$) (Fig. 1A). Injection treatments did not affect expression of the first control gene, *vg-like-C*, as there was no significant difference in expression among bees from any treatment (1-way ANOVA, $F_{2,\ 44} = 1.28$, $P = 0.288$) (Fig. 1B). For the second control gene, *apoLp-III,* there was no difference in expression between individuals injected with nuclease-free water (sham) and those injected with *vg* dsRNA ($P = 0.151$), nor between sham-injected and control-handled individuals ($P = 0.830$) (Fig. 1C). There were small but significant differences in expression between Vg-knockdown and control-handled bees ($P = 0.040$) (1-way ANOVA, $F_{2,\ 44} = 3.47$, $P = 0.040$).

3.2. Histology

Despite this effect of RNAi-mediated *vg* knockdown, bees from all treatments showed positive signal for Vg protein in their midguts (Fig. 2). Moreover, bees from all treatments that were fed fluorescently labelled *E. coli* particles showed uptake of label into their midgut. Bees subjected to the control feeding (Fig. 2M-N) did not show this signal. The Vg and *E. coli* particles appeared in close proximity or even overlapped in a few instances, but mostly the two signals were separate.

As expected, fat body from control-handled and sham-injected bees showed positive Vg signal while that from Vg-knockdown bees showed little to no signal (Fig. 3). Honey bee fat body contains two cell types, trophocytes and oenocytes, with the former the site of Vg synthesis and the latter performing lipid metabolism functions (see Section 4)). Trophocytes can be identified by their large irregularly-shaped nuclei,

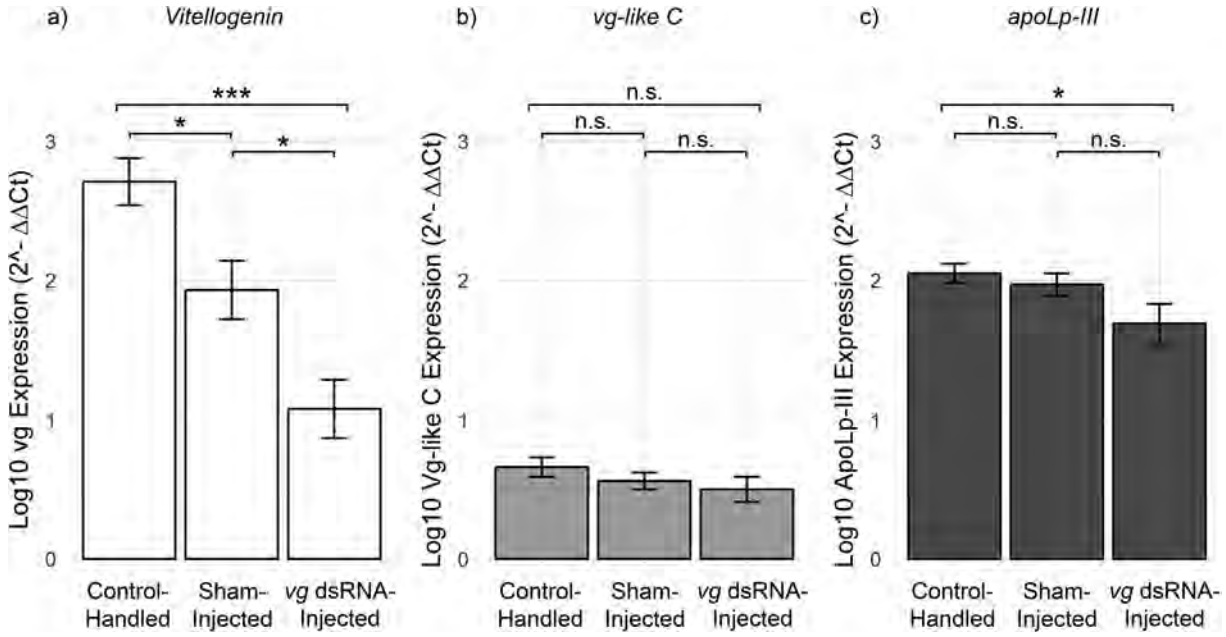

Fig. 1. Relative expression levels of three genes following *vg* dsRNA-injection, sham-injection, or control-handling. Bar heights represent the mean $\log_{10}$ gene expression level of N = 16 individuals sampled, while error bars represent ± 1 standard error. Expression levels were determined using the ΔΔCt method. Data were normalized via log-transformation prior to analysis. Pairs of treatment groups with stars above were deemed significantly different from one another via a Tukey HSD test. A: Relative *vg* expression differed significantly between all treatment groups (1-way ANOVA: $F_{2, 45} = 16$, $p < 0.001$). B: Relative *vg-like-C* expression did not differ between treatment groups (1-way ANOVA, $F_{2, 44} = 1.28$, $P = 0.288$). C: Relative *apoLp-III* expression significantly differed between treatment groups (1-way ANOVA, $F_{2, 44} = 3.47$, $P = 0.040$), but this difference was only observed between control-handled and vg dsRNA-injected individuals ($P = 0.040$).

while oenocytes have smooth round nuclei. Positive signal for Vg was restricted to trophocytes and absent in oenocytes. Bees from all treatment groups that were fed *E. coli* particles showed positive fluorescent *E. coli* signal in their fat body. The particles appeared in both the trophocytes and oenocytes, located on the cell membrane or in the cytoplasm (Fig. 3). We observed small granular structures in the oenocyte cytoplasm that faintly autofluoresce in the same Texas Red channel as the *E. coli* , but these spots were substantially dimmer that the positive *E. coli* signal. These granules are likely fat droplets that are known to autofluoresce (Clokey and Jacobson, 1986; Fletcher et al., 1973; Le et al., 2010) (see Section 4).

Bees from all treatments showed positive signal for Vg in their hypopharyngeal glands, even Vg-knockdown bees. *E. coli* particles, however, were observed in the glands of control-handled and sham-injected bees, but not in the glands of Vg-knockdown bees (Fig. 4). The *E. coli* signal was mostly confined to the surface of the glands but was also observed in the collecting duct leading away from the glands (Fig. 4C-D). In several instances, *E. coli* and Vg appear to co-localize in the glands, which resolve as yellow pixels in the micrographs (e.g., Fig. 4 B, D, & F).

4. Discussion

This experiment has demonstrated two aspects of honey bee worker physiology not previously known. First, ingested bacterial fragments are transported to workers' hypopharyngeal glands, and second, this phenomenon is absent or greatly diminished when *vg* expression is down-regulated with RNAi (Fig. 4). Hypopharyngeal glands are an intriguing destination for bacterial fragments because of the important social function the glands play in food production by workers (Amdam et al., 2003a; Patel et al., 1960; Snodgrass, 1956). The hypopharyngeal glands absorb Vg in the haemolymph and appear to metabolize the protein into amino acids that are used during the synthesis of royal jelly (Amdam et al., 2003a; Seehuus et al., 2007). Workers feed this protein-rich jelly to the queen and young larvae (Snodgrass, 1956). In this way, the hypopharyngeal glands use Vg much the same way that some other insects use Vg to produce trophic eggs (Amdam et al., 2003a), which are non-viable eggs laid by females to nourish young offspring or to boost overall reproductive output (Brian, 2012; Engels et al., 1990). Converting Vg into royal jelly instead of trophic eggs is a novel adaptation of honey bees and may have allowed for more efficient brood rearing (Amdam et al., 2003a). Our current work further suggests that this route of social nutrient transfer incorporating immune elicitors could facilitate more efficient protection of honey bee brood via mechanisms of trans-generational immune priming.

The bacterial fragments that we observe at the glands of control-handled and sham-injected workers may be part of a trans-generational immune priming pathway whereby nestmates share immunological signals via food secretions. This could occur in a couple of ways. Firstly, bacterial fragments may be transported with Vg across the gland membrane and incorporated into royal jelly and then orally transferred directly to larvae or to the queen. As we suggested previously (Salmela et al., 2015), one potential source of pathogen exposure for queens is through ingesting contaminated food. It is well documented that female insects that ingest bacteria transport the pathogens from their midgut to their ovaries for trans-generational immune priming (Freitak et al., 2009; Knorr et al., 2015). The same pathway would operate for honey bee queens, except their food (with bacterial fragments) would come from worker-produced royal jelly. Alternatively, the bacterial fragments may remain along the gland membrane and initiate a molecular signal cascade that produces an immune response, perhaps influencing specific products secreted in the royal jelly. Royal jelly contains several components with immunological functions, including *defensin1* and major royal jelly protein 3 (Blum et al., 1959; Bucekova et al., 2017; Fontana et al., 2004; Fujiwara et al., 1990; Klaudiny et al., 2012; Okamoto et al., 2003; Alessandra et al., 2011; Sugiyama et al., 2012; Vucevic et al., 2007). Possibly, the presence of bound bacterial fragments on the gland surface can induce more of such immunomodulators to be incorporated into the jelly. New experiments are required to determine whether bacterial fragments that bind to honey bee hypopharyngeal glands are incorporated into jelly and/or induce changes to jelly composition.

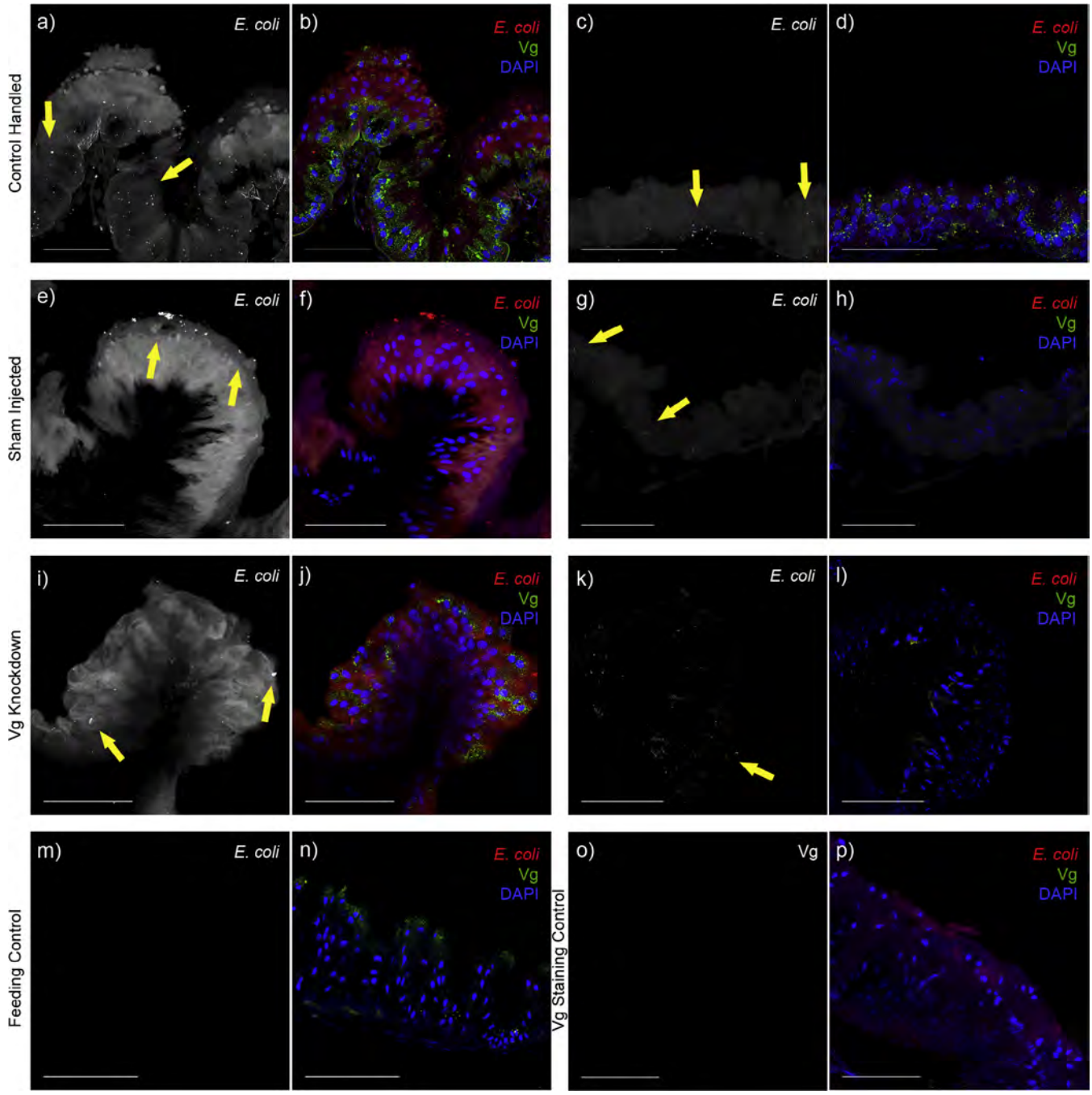

Fig. 2. Midguts of *E. coli*-fed and control-fed workers. The organs were sectioned longitudinally, and each set of images shows a collection of epithelial cells protruding into the lumen. Cell nuclei are stained blue with DAPI, *E. coli* particles are stained red with Texas Red, and Vitellogenin protein is stained green with Alexa 488. White scalebars are 100 μm in length. Yellow arrows indicate examples of positive *E. coli* signal. A-D: representative samples of control-handled bees fed with *E. coli*; E-H: representative samples of sham-injected individuals fed with *E. coli*; I-L: representative samples of Vg-knockdown individuals fed with *E. coli*; M-N: representative sample of a control-handled individual fed with a control diet; O-P: representative sample of Vg control staining (incubated with Alexa 488, but without Vg 1° antibody). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The second key finding of this study is that *E. coli* is present at the glands of control-handled and sham-injected bees, but not detected at glands of Vg-knockdown bees. This finding suggests that Vg may be an *E. coli* transporter in this pathway. That Vg binds to *E. coli* is well established (Li et al., 2008; Salmela et al., 2015; Shi et al., 2006; Tong et al., 2010), and we have shown previously that Vg is necessary and sufficient to transport *E. coli* into the ovaries (Salmela et al., 2015). Vg binds to a substantial array of different ligands, including both gram-negative and gram-positive bacteria (Li et al., 2009; Salmela et al., 2015; Shi et al., 2006; Tong et al., 2010; Zhang et al., 2011), fungal cells (Li et al., 2008), viruses (Huo et al., 2014; Whitfield et al., 2015), damaged and apoptotic host cells (Havukainen et al., 2013), reactive oxygen species (Havukainen et al., 2013; Nakamura et al., 1999; Seehuus et al., 2006), and zinc (Amdam et al., 2004). Moreover, these binding abilities are conserved in Vitellogenins across disparate taxa, including insects (for review, see Salmela and Sundström, 2017), fish (for review, see Sun and Zhang, 2015, Zhang et al., 2015), and corals (Du et al., 2017), suggesting that a variety of binding abilities evolved early in Vg's evolutionary history. Strong binding ability stems from Vg's molecular structure, and includes a positively charged region in

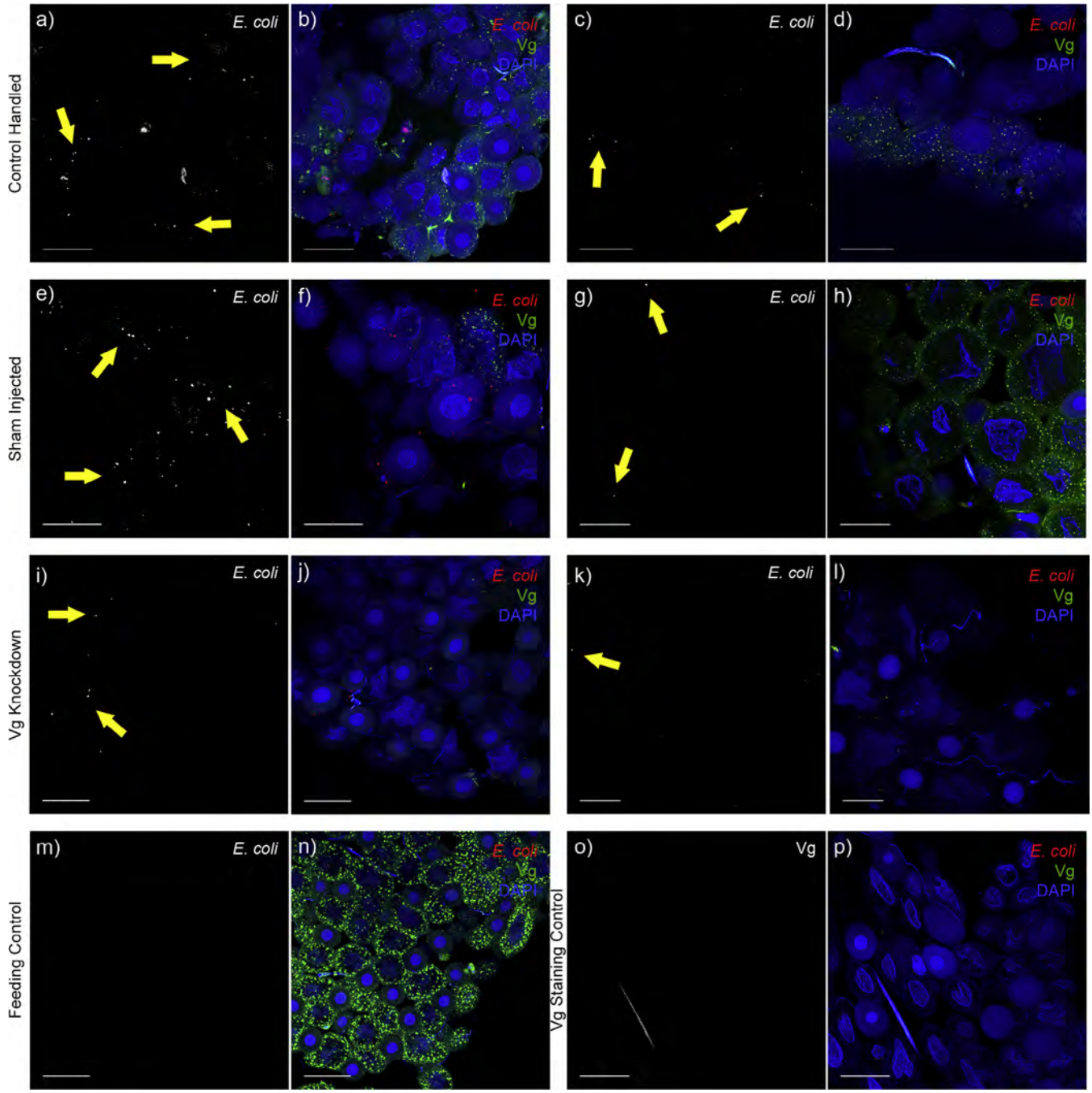

Fig. 3. Fat body cells of *E. coli*-fed and control-fed workers. Each sample shows a collection of the two cell-types found in the honey bee fat body, the trophocytes (with large, irregularly shaped nuclei) and the oenocytes (with rounded, smooth nuclei). Cell nuclei are stained blue with DAPI, *E. coli* particles are stained red with Texas Red, and Vitellogenin protein is stained green with Alexa 488. White scalebars are 50 μm in length. Yellow arrows indicate examples of positive *E. coli* signal. A-D: representative samples of control-handled bees fed with *E. coli*; E-H: representative samples of sham-injected individuals fed with *E. coli*; I-L: representative samples of Vg-knockdown individuals fed with *E. coli*; M-N: representative sample of a control-handled individual fed with a control diet; O-P: representative sample of Vg control staining (incubated with Alexa 488, but without Vg 1° antibody). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the protein's α-helical domain (Havukainen et al., 2013). In nurse-age bees like those used in this experiment, Vg makes up 30–50% of all haemolymph proteins (Engels and Fahrenhorst, 1974; Fluri et al., 1982), which would provide ample binding-opportunity for *E. coli* that is digested and enters the haemolymph.

That *E. coli* is not detected at the glands of Vg-knockdown bees is likely due, at least partly, to a reduction in Vg molecules in the haemolymph available to bind *E. coli.* However, this is likely an incomplete explanation, as Vg titers are *reduced* but not eliminated by RNAi: In a previous study by our group using the same Vg-knockdown and control protocols, 10-day old Vg-knockdowns had an 80% reduction in median Vg titers compared with nurse controls (1.89 μg/μL and 9.24 μg/μL, respectively) (Nelson et al., 2007). An additional contributing factor to *E. coli* absence at the glands of Vg-knockdowns is likely Vg's lower binding affinity to *E. coli* than other ligands. As we showed previously, honey bee Vg has a lower binding affinity to *E. coli* than to the honey bee pathogen *Paenibaccilus larvae*, a gram-positive bacteria responsible for American foulbrood disease (Salmela et al., 2015). Vg also has lower

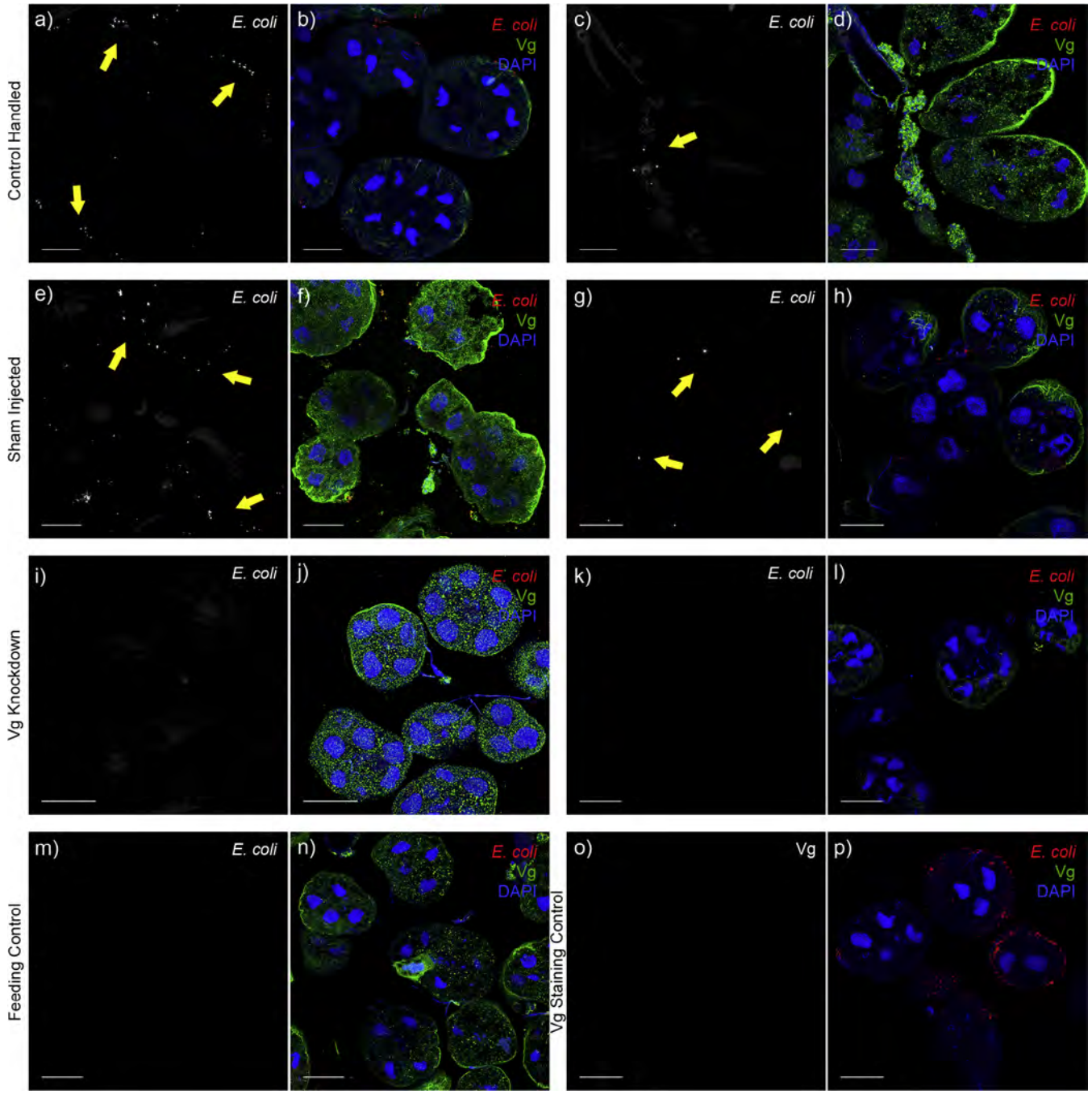

Fig. 4. The hypopharyngeal glands of *E. coli*-fed and control-fed workers. Within each acinus, cell nuclei are stained blue with DAPI, *E. coli* particles are stained red using Texas Red, and Vitellogenin protein is stained green with Alexa 488. White scalebars are 50 μm in length. Individuals from the control handling and sham-injection treatments showed positive signal for *E. coli* in their glands (A-H) but individuals that received an injection of Vg dsRNA lacked *E. coli* signal in their glands (I-L). A-D: representative samples of control-handled bees fed with *E. coli*; E-H: representative samples of sham-injected individuals fed with *E. coli*; I-L: representative samples of Vg-knockdown individuals fed with *E. coli*; M-N: representative sample of a control-handled individual fed with a control diet; O-P: representative sample of Vg control staining (incubated with Alexa 488, but without Vg 1° antibody). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

binding affinity to lipopolysaccharide than to peptidoglycan (Salmela et al., 2015). Lipopolysaccharide comprises the outer membrane of gram-negative bacteria like *E. coli* (Beveridge, 1999) and is absent in gram-positive bacteria, while peptidoglycan is present in both bacterial types but is substantially more abundant in gram-positive bacteria (Salton and Kim, 1996). Vg may have higher binding affinity to peptidoglycan because it is common to both bacterial types and thus represents a more general bacteria marker. Alternatively, this higher binding affinity to peptidoglycan may result from selective pressures on Vg to combat specific gram-positive bacterial diseases such as American foulbrood and European foulbrood (*Melissococcus plutonius*). These diseases are not only deadly (Morse and Flottum, 1997), but they are also the only widespread bacterial pathogens that target developing honey bee larvae (Evans and Schwarz, 2011). In contrast, *E. coli* is not a honey bee pathogen. It was used as a model in this study because it is can be reliably labeled. Thus, it is not expected that selection has acted on Vg to confer effective binding to this bacterium. Vg's lower binding affinity for *E. coli* coupled with reduced Vg abundance in the

haemolymph after *vg* RNAi may explain why *E. coli* is not detected in the glands of Vg knockdowns. For control-handled and sham-injected bees, Vg is available in abundance, allowing it to still effectively transport the labeled bacterial particles to the glands.

Alternatively, *E. coli*'s lack of detection at glands of Vg-knockdown workers may reflect changes in worker physiology. Typically, Vg-knockdown results in workers prematurely transitioning from nurses to foragers (Antonio et al., 2008; Nelson et al., 2007). Foragers no longer need to produce royal jelly to feed larvae, so their hypopharyngeal glands begin to atrophy (Amdam et al., 2005; Huang and Robinson, 1996; Milojevic, 1940). This process may result in fewer Vg receptors, or receptors capable of binding bacterial fragments, being present along the gland membrane. This altered membrane interface, with fewer transport- or binding-opportunities for *E. coli*, could explain why we do not see *E. coli* particles at the glands of Vg knockdowns. This alternative interpretation implies that there can still be sufficient Vg to transport labeled bacterial fragments to the glands of Vg knockdowns. Future molecular studies of the hypopharygeal glands are required to test this possibility.

Another alternative explanation is that *E. coli*'s presence or absence at the glands is not due to Vg-knockdown, per se, but instead due to off-target effects of injecting dsRNA. These off-target effects might arise from using insufficiently specific *vg* dsRNA that knocks down other genes with sequence homology to *vg*, or from an immunological response to dsRNA that alters expression of other genes which may facilitate *E. coli* transport. However, these alternative explanations seem unlikely based on the data presented here. First, the *vg* dsRNA appears to be highly specific, as the homologous control gene *vg-like-C* showed no difference in expression among all treatment groups. This further reduces the likelihood that genes analogous to *vg* would be subject to off-target RNAi effects. Second, there was no difference in expression of *apoLp-III* between bees that received a sham injection (nuclease-free water) and those that received a *vg* dsRNA injection, suggesting that exposure to dsRNA per se does not alter expression of this functional control gene. *ApoLp-III* circulates in the haemolymph and can bind to the PAMPs of *E. coli* cell walls (Weers and Ryan, 2006), and so could theoretically transport *E. coli* to the hypopharyngeal glands. However, *E. coli* is only visible at the glands of sham-injected individuals and not *vg* dsRNA-injected individuals, despite these treatment groups expressing similar levels of *apoLp-III*. Therefore, it is unlikely that *apoLp-III* is involved in *E. coli* transport to the glands. Finally, Vg and *E. coli* appear to co-localize in some instances at the glands, where the red pixels of *E. coli* and the green pixels of Vg overlap to resolve into yellow pixels (Fig. 4 B, D, & F). This may indicate the Vg-bacteria complex interacting with Vg receptors on the gland surface, and lends further support to the argument that Vg plays a central role in bacteria transport to the glands.

In addition to the main findings, this study also made several intriguing observations that warrant further research. While we were not able to determine the role of Vg in transporting labeled *E. coli* particles from gut lumen to hemocoel, Vg was observed in the epithelial cells lining the midgut interior of all the treatments (Fig. 2). These are the most predominant cells in the insect midgut and are responsible for digestion and absorption (Buchon et al., 2013; Dow, 1987). They extend from the basal lamina in towards the lumen and secrete digestive enzymes and the peritrophic membrane, a chitinous material that protects the cells from abrasion and damage from ingested food (Brandt et al., 1978; Hegedus et al., 2009; Lehane and Billingsley, 2012; Lehane, 1997). They also produce and secrete antimicrobial peptides (AMPs) and reactive oxygen species (ROS) (Buchon et al., 2013, 2009; Ha et al., 2005; Kumar et al., 2010) into the lumen in response to ingested pathogens. However, an excessive immune response damages the epithelial cells, which are quickly replaced by midgut stem cells that proliferate and differentiate (Buchon et al., 2013; Micchelli and Perrimon, 2006; Ward et al., 2008). Given what we know about Vg's properties, there may be several functions it performs in this organ. For example, Vg may intercept and destroy pathogen cells in the midgut, owing to its ability to bind to many pathogens (Li et al., 2009, 2008; Liu et al., 2009; Salmela et al., 2015) and its bactericidal properties (Li et al., 2009; Tong et al., 2010; Zhang et al., 2011). It may also act as an opsonin when bound to pathogens and recruit other immunological factors to destroy the pathogen (Li et al., 2009, 2008; Liu et al., 2009; Zhang et al., 2011). Additionally, Vg is an antioxidant, and it has ability to recognize and bind to damaged host cells and protect them from further ROS damage (Havukainen et al., 2013). In this capacity, Vg may serve to protect epithelial cells from the host's own immunological arsenal and prolong their life until they can be replaced by the differentiating stem cells. Midgut cells express *vg* (Harwood & Amdam, unpublished) and more Vg is likely absorbed from the haemolymph, as the midgut expresses low levels of the Vg receptor (Guidugli-Lazzarini et al., 2008).

In the fat body, bees from all bacteria feeding treatments showed positive *E. coli* signal. Again, we were unable to link presence or absence of immune elicitors to Vg, as we did not account for Vg circulating in the haemolymph, and high levels of Vg in fat body may be insensitive to RNAi treatments in some individuals. The fat body regulates the metabolic and immunological status of the organism by monitoring the haemolymph (Arrese and Soulages, 2010; Chapman, 1998; de Oliveira and Cruz-Landim 2006). It lines the abdominal cuticle surrounding the digestive tract and is well-positioned to detect digested pathogens that enter the haemolymph. It stores and releases lipids, proteins, and carbohydrates (Chapman 1998, reviewed in de Oliveira and Cruz-Landim 2006), and plays a key role in immunity by producing many AMPs (Bulet and Stöcklin, 2005; Stokes et al., 2015). Our data show *E. coli* particles to be associated with both of the two honey bee fat body cell types: trophocytes and oenocytes. Trophocytes possess large irregularly shaped nuclei and regulate metabolism (Chapman, 1998), and are also the primary site of Vg synthesis (Bownes, 1986; Isaac and Bownes, 1982; Pan et al., 1969; Raikhel and Lea, 1983). Oenocytes possess rounded nuclei and contain highly developed smooth endoplasmic reticulum in their cytoplasm (Martins et al., 2011; Martins and Romalho-Ortigao, 2012). They perform functions in lipid metabolism (Gutierrez et al., 2007), and as such, contain lipid droplets (Brasaemle and Wolins, 2012; Gutierrez et al., 2007; Kühnlein, 2011; Makki et al., 2014). They are also involved in cuticle formation (Wigglesworth, 1988), hormone production (Wicker-Thomas et al., 2009), and detoxification of xenobiotics (Lycett et al., 2006; Martins et al., 2011). In this latter function, they express many key immune genes, including cytochrome p450, dehydrogenase, catalase and lysosome P (Martins et al., 2011). Our results do not explain the role of effects of *E. coli* binding to the fat body cells in honey bees, but it may be interacting with pattern recognition receptors as part of the innate immune response to trigger the production of AMPs and other defenses.

We also found Vg inside the hypopharyngeal glands, consistent with previous findings (Seehuus et al., 2007). A novel finding here is that Vg remains in the hypopharyngeal glands even after Vg knockdown (Fig. 4), and the same pattern is observed in the midgut (Fig. 2). The reason for this pattern remains unclear. The Vg receptors in both the hypopharyngeal glands and midgut may be very efficient at extracting Vg from the haemolymph, even after RNAi knocks down the Vg titer to a low level. Alternatively, Vg may only be slowly metabolized in the hypopharyngeal glands of Vg-knockdown workers, which are known to transition from nursing to foraging activities (Antonio et al., 2008; Nelson et al., 2007). Foragers do not synthesize royal jelly, and an associated reduction in the consumption of Vg could lead to a reduced turnover and prolonged presence of Vg in the glands.

5. Conclusion

Overall, this study demonstrates that ingested bacterial fragments are transported to the hypopharyngeal glands of worker honey bees, and that Vg likely plays a role in this transport. We cannot conclude whether bacterial fragments are secreted directly into jelly or whether

they only bind to the gland surface to elicit an immune-related response by the glands. But either mechanism could result in trans-generational immune priming aimed at protecting the brood. This protection could occur by workers feeding bioactive components (bacteria fragments or immune-related molecules) to the queen, or potentially also via direct feeding of the brood, which receive jelly during the first days of larval life (Haydak, 1970). Both mechanisms can be interpreted as an adaptation for a social insect colony to respond to pathogen threats in real-time.

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