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Original article

Vitellogenin in the honey bee midgut

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Abstract – The alimentary canal carries out many functions critical to insect physiology, including digesting and absorbing nutrients and water, housing beneficial gut bacteria, and eliciting immunological responses. The midgut, in particular, is a compartment for digestion and absorption and serves as a first point of contact between ingested pathogens and the insect immune system. Recently, we found the protein vitellogenin (Vg) localized in midgut cells of some honey bee workers. Vg is an important egg-yolk precursor protein in nearly all oviparous animals, but it also has immunological functions shared across many taxa. Additional and unexpected Vg functions have been characterized in honey bees, but none of these functions involve the midgut directly. Therefore, we sought to map out how Vg is localized and expressed in this organ across the two most common worker bee behavioral groups, namely nurses and foragers. We used immunohistochemistry and quantitative reverse transcription PCR to show Vg has different localization patterns in nurses and foragers and limited gene expression. Our study provides a platform for building a more detailed understanding of the possible roles of Vg in insect midgut cells, and it adds to the current knowledge of this fascinating, multi-functional protein.

Honey bee / midgut / vitellogenin / histology / peritrophic membrane

1. INTRODUCTION

The insect alimentary canal has been the subject of intense scientific inquiry. These efforts have led to the discovery and characterization of pluripotent stem cells (Ohlstein and Spradling 2006; Ward et al. 2008), a greater understanding of the molecular pathways activated in response to ingested pathogens (Buchon et al. 2009; Kumar et al. 2010; Lehane and Billingsley 2012), and the classification and functional characterization of gut bacteria (Anderson et al. 2013; Engel and Moran 2013). The insect alimentary canal is compartmentalized into the foregut, midgut, and hindgut, with the foregut and hindgut originating from the ectodermal layer and the midgut originating from the endodermal layer (Snodgrass 2018). In most adult insects, the foregut contains the crop which is used to store food, while the hindgut contains the rectum which absorbs water and ions, as well as the ileum which houses many gut bacteria (Chapman 1998; Martinson et al. 2012). The midgut, located between the foregut and hindgut, is the site of digestion and absorption and plays a critical role in insect immune physiology.

The insect midgut is composed primarily of elongated epithelial cells called enterocytes that protrude into the lumen and which perform several important functions (Snodgrass 2018). They produce and secrete digestive enzymes (Lehane and Billingsley 2012) and also secrete a chitinous

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material called the peritrophic membrane which protects the enterocytes from abrasion and serves as a sieve to prevent pathogens, toxins, and large molecules from contacting the enterocytes (Brandt et al. 1978; Hegedus et al. 2009; Lehane 1997). Moreover, enterocytes produce and secrete an arsenal of immunological defenses when pathogens are detected in the lumen, including antimicrobial peptides (AMPs) and reactive oxygen species (ROS) (Buchon et al. 2009; Dussaubat et al. 2012; Ha et al. 2005; Nakajima et al. 2002).

Recently, we discovered that the protein vitellogenin (Vg) is present in midgut cells of at least some honey bee workers (Harwood et al. 2019). Vg is an egg-yolk precursor protein synthesized in the insect fat body that is used to deliver nutrients to developing eggs in most oviparous animals (Pan et al. 1969; Raikhel and Lea 1983). However, Vg also serves many immunological functions: it binds to and eliminates pathogenic bacterial and fungal cells by recognizing pathogen-associated molecular patterns (PAMPs) (Li et al. 2009, 2008; Salmela et al. 2015; Zhang et al. 2015); it protects host cells from oxidative stress by binding to and neutralizing ROS (Havukainen et al. 2013; Salmela et al. 2016); it binds to damaged host cells and protects them from further injury (Havukainen et al. 2013); and it transports the zinc required to maintain innate immune cell (hemocytes) (Amdam et al. 2004a). None of these functions have been documented to involve midgut tissue directly. Therefore, after finding Vg in worker midgut cells (Harwood et al. 2019), we sought to map out how Vg is localized and expressed in this organ. The rational for this work was to start building a foundation for understanding the possible roles of Vg in the gut of honey bee nurses and foragers.

In honey bees, workers are functionally sterile females that undergo an age-associated task specialization focusing on brood care (nursing) vs. foraging for resources outside the colony. Vg plays a role in regulating transitions between maternal behaviors involving brood care and foraging (Amdam et al. 2004b): Nurse bees have high Vg titers while forager bees have low titers, and reducing Vg titers in young worker bees by means of RNA-interference (RNAi)-mediated *vg* gene knockdown triggers precocious foraging behavior. The transition from nursing to foraging is associated with sweeping changes in worker gene expression, behavior, and physiology, including changes in immunity (Amdam et al. 2004a, 2004b; Bedick et al. 2001; Fluri et al. 1982; Schmid et al. 2008), gut function (Crailsheim et al. 1992), and dietary preferences (Brodschneider and Crailsheim 2010). To decouple behavior-associated patterns from age-associated patterns (foragers are typically older than nurses and thus behavior is naturally confounded by age), we compared nurses and foragers of the same age using single-cohort colonies (see Methods). Here, we asked whether Vg localization in midgut tissue differs between the two behavioral groups using immunohistochemistry, and we tested whether *vg* is expressed in the gut of both honey bee nurses and foragers using quantitative reverse transcription PCR (RT-qPCR). These results allow us to make progress towards understanding Vg's function in the gut, and some of these possible functions are discussed here.

2. METHODS

2.1. Bees

Honey bee colonies were maintained at the Arizona State University Bee Lab in Mesa, Arizona. To obtain nurses and foragers of the same age, we used single-cohort colonies. Two such colonies were established in which newly emerged workers (< 24 h old) were housed with a queen and several frames of honey, pollen, and empty comb. After several days, many of these workers prematurely transition from nurses into foragers to meet the resource needs of the colony. Seven days after establishing the colonies, we paint marked foragers that we observed returning to the nest with pollen. On day 14, we collected 25 marked foragers and an equal number of nurses. This ensured that all nurses and foragers had been performing their given task for at least 7 days. Nurse bees were selected by observing workers that entered brood cells to feed the larvae.

2.2. Immunohistochemistry

Bees were anesthetized on ice before their midguts were dissected. Cold 1X phosphate buffered

saline (PBS) was applied to maintain tissue freshness. Midguts were fixed in 4% paraformaldehyde overnight at 4 °C then washed 3 times for 10 min in cold 1X PBS before being embedded in an agarose medium. The embedded tissue was cut into 150-μm-thick longitudinal sections using a Leica VT1000s vibratome. Our longitudinal sections offered us two views of midgut structure depending on where in the organ the section was made: sections made through the center of the lumen provide a cross section to observe the epithelial cells protruding into the gut lumen (Fig. 1), while sections made at the external surface of the organ allows one to observe how midgut cells are arranged in two-dimensional space when attached to the basement membrane and surrounding muscles (Fig. 2). All samples received the same staining treatment. First, sections were blocked for 10 min with 5% goat serum (Jackson ImmunoResearch #005-000-121) in a solution of 1X PBS and 0.1% Triton X-100 (Sigma #T8787). We then added a 1:1000 dilution of polyclonal rabbit-anti-Vg primary antibodies (raised against 180 kDa honey bee vitellogenin; Pacific Immunology, Ramona, CA) and incubated the tissue overnight at 4 °C. The tissue was then washed 3 times for 10 min and incubated for an additional 3 h at room temperature in 1X PBS with 5% goat serum and 0.1% Triton X-100, this time with a 1:1000 dilution of goat-anti-rabbit secondary antibodies tagged with Alexa Fluor® 488 (Jackson ImmunoResearch #111-545-047) and a 1:500 dilution of rhodamine phalloidin (Invitrogen™ #R415). Tissue was then washed 3 times for 10 min in cold 1X PBS and incubated an additional 15 min at room temperature in 1X PBS with 0.1% Triton X-100 and a 1:30000 dilution of DAPI (4′,6-diamidino-2-phenylindole. Invitrogen™ #D3571) before being washed for a final 5 times for 10 min in 1X PBS. The tissue was then mounted on slides and imaged on a Leica SP5 confocal microscope with a 20X oil-immersed lens. Separate 10 μm z-stack image series were taken sequentially for each fluorophore to avoid crosstalk between excitation and emission spectrums. We imaged tissues from 3 to 6 individuals from each behavioral group. To confirm the binding specificity of the secondary antibodies, we also created antibody-control staining slides. Here, staining procedure was the same except the primary antibodies were excluded from the first incubation step. Control slides were made using the longitudinal sections through the center of the lumen (i.e., cross-sectional images, Fig. 1).

2.3. Gene expression

A total of 16 nurses and 16 foragers were used for gene expression comparison. Midguts were dissected from anesthetized bees, and RNA was extracted via phenol-extraction using TRIzol® reagent (Invitrogen #15596018). RNA was diluted to a concentration of 200 ng/μl, and DNA was removed via DNase I treatment (Thermo Scientific™ #EN0525). One-step RT-qPCR was performed using a QuantiTect SYBR® Green RT-PCR kit (Qiagen #204245) and performed on an ABI Prism 7500 (Applied Biosystems). *Actin* was used as a reference gene, as it is stably expressed across honey bee tissues and commonly used in honey bee research (Lourenço et al. 2008; Scharlaken et al. 2008). Each sample was prepared in duplicate for both *actin* and *vg*. The PCR program began with a reverse transcription step at 50°C for 30 min, followed by an activation step at 95° for 15 min. It then repeated 40 cycles of 94 °C for 15 s, 52 °C for 31 s, and 72 °C for 32 s. RT-qPCR primer sequences have been used previously (Harwood et al. 2019) and were as follows:

Vg Forward: 5’ – GTTGGAGAGCAACA TGCAGA - 3’
Vg Reverse: 5’ – TCGATCCATTCCTT GATGGT – 3’
Actin Forward: 5’ – TGCCAACACTGTCC TTTCTG – 3’
Actin Reverse: 5’ – AGAATTGACCCACC AATCCA – 3’

2.4. Statistics and analysis

We assessed differences in Vg tissue localization visually between nurses and foragers. The aim was to look for general patterns relating to Vg localization in different regions of the midgut or within different regions of enterocytes. Relative gene expression levels were calculated using the ΔΔCt method (Schmittgen and Livak 2008).

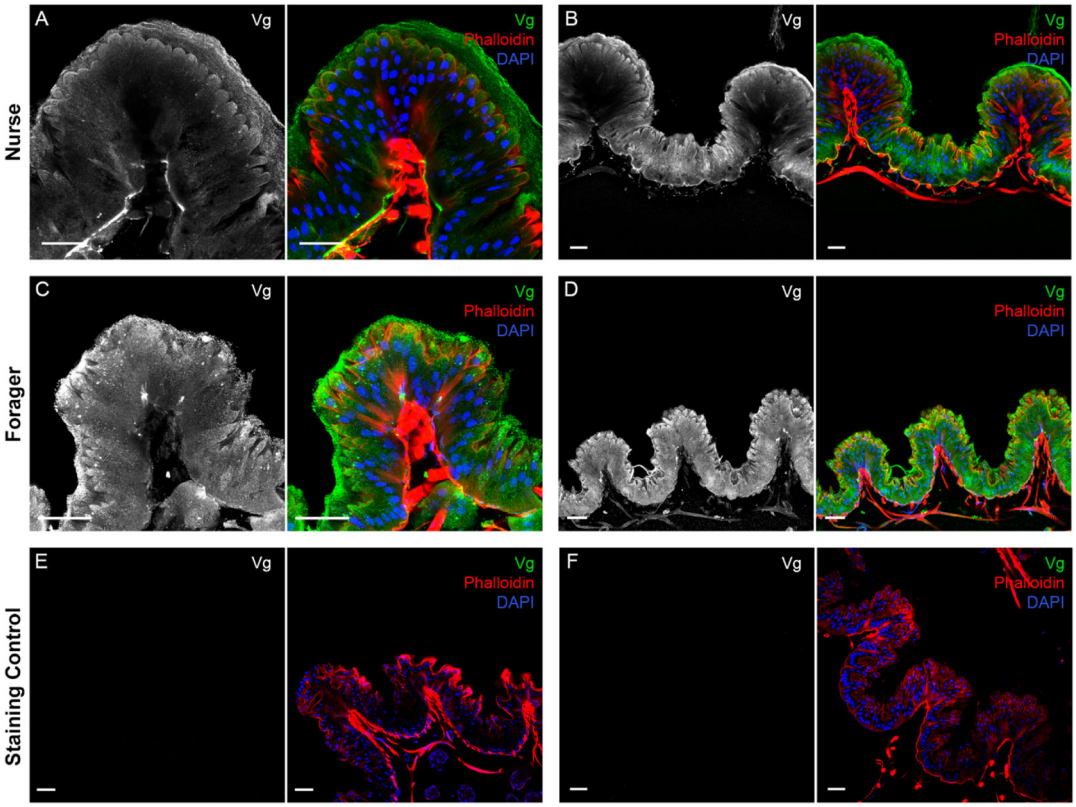

Fig. 1 Confocal micrographs showing longitudinal cross sections of the honey bee worker midgut. The protein Vg is stained with Alexa Fluor 488 (green), nuclei are stained with DAPI (blue), and F-actin is stained with rhodamine phalloidin (red). In each sample, images are oriented with the midgut lumen towards the top and the midgut exterior towards the bottom. Scale bars represent 50 μm. Positive signal for Vg is observed in both the nurses (**A**–**B**) and foragers (**C**–**D**), but not in the Vg control stained samples (**E**–**F**). Vg is seen not only in the gut epithelial cells, but also in the peritrophic membrane that shields the epithelial cells in the lumen. The peritrophic membrane is more prominent in nurses (**A**–**B**) than foragers (**C**–**D**). These are representative images of observations made on N = 3 and N = 6 nurses and foragers, respectively

Differences between nurses and foragers were determined using a t-test on log10-transformed data. All statistical analysis was performed in R (3.5.2).

3. RESULTS

3.1. General observations of structure

In the longitudinal cross sections, we observed enterocytes protruding into the lumen that measure approximately 100–200 μm in length (Fig. 1). Along the length of the organ, the midgut folds inward to increase surface area. These folds can be seen in Fig. 1B, D, E, and F, with a closeup of the folds seen in Fig. 1A and C. Enterocytes are connected to one another at their base via a basement membrane, and in order to facilitate gut motility the exterior of the midgut is wrapped in a lattice of longitudinal and circular muscles. These muscles are clearly visible with the phalloidin stain in Fig. 1B and D, which appear as bright red lines and dots running along the bottom of the images. The peritrophic membrane is visible in the lumen above the enterocytes in Fig. 1A and B, and to a lesser extent in Fig. 1C, and reaches a maximum thickness of approximately 50 μm in our samples.

In the sections made at the external surface of the midgut, we observed that cells attach to the basement membrane in a cluster formation and

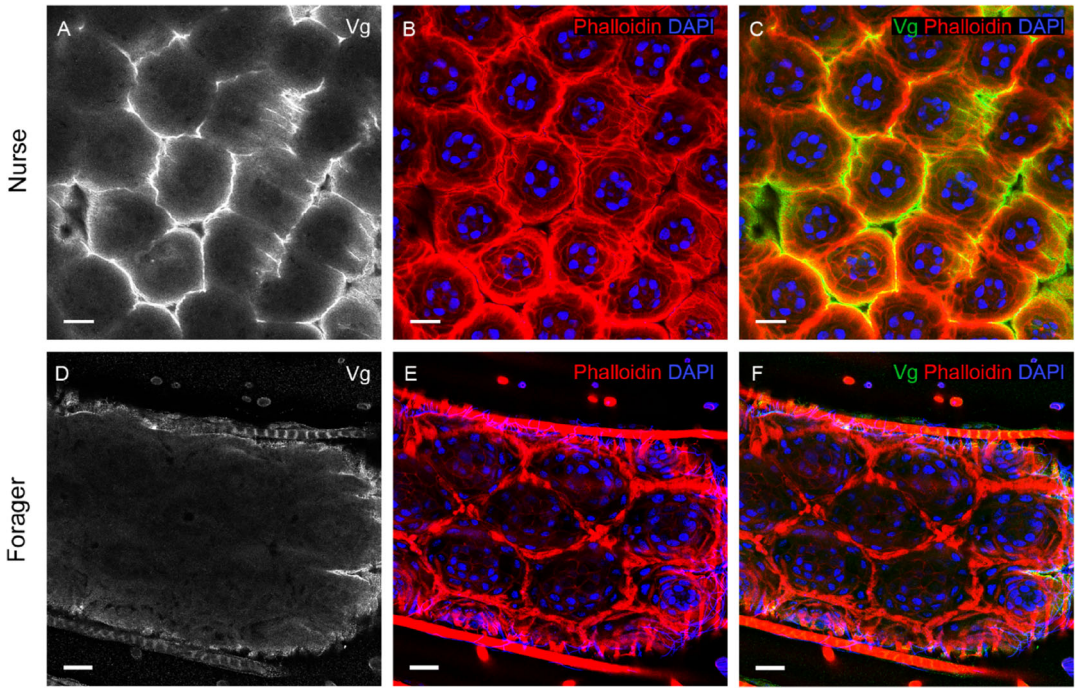

Fig. 2 Confocal micrographs looking at the outside surface of the honey bee worker midgut. Vg is stained with Alexa Fluor 488 (green), nuclei are stained with DAPI (blue), and F-actin is stained with rhodamine phalloidin (red). Scale bars represent 25 μm. Cells are arranged in cluster formations, with cells at the center of the clusters having their nuclei closer to the basement membrane. Vg appears around these clusters in nurse bees (**A–C**), but not in foragers (**D–F**).

that cells towards the centers of the clusters have their nuclei placed closer to the basement membrane (Fig. 2B, E). Based on their structure, we interpret these clusters as being regenerative crypts (Raes et al. 1994; Illa-Bochaca and Montuenga 2006; Park et al. 2009), with stem cells towards the center of the clusters.

3.2. Vg localization

We found that Vg protein was localized throughout the enterocytes of same-aged nurses and forages (Fig. 1). For both groups, Vg does not appear to be restricted to certain regions of the midgut or compartmentalized within the enterocytes: it is found throughout the cytoplasm from the base of the cells to the apex. However, when observing clusters of regenerative cells (Fig. 2), Vg appears to surround these clusters in nurses but the pattern is not observed in foragers. This Vg may be deposited extracellularly around these cell clusters, or this phenomenon could be due to microscope edge effects if cells towards the outside of the cluster contain a higher abundance of Vg than cells towards the center (Fig. 2A, B). Alternatively, this pattern may be due to a greater deposition of Vg near the basement membrane in nurses than in foragers (Fig. 1A, C). Nevertheless, Vg does appear to be secreted out of enterocytes and into the peritrophic membrane along the luminal edge of the enterocytes (Fig. 1A, B). We found that the peritrophic membrane was consistently present in all nurse samples imaged but was absent or greatly reduced in all forager samples (Fig. 1).

3.3. Gene expression

We found that *vg* was expressed in the midguts of both nurses and foragers. For both groups, *vg* transcript amplification occurred at later cycles (PCR cycles 27.9 and 28.5, respectively), indicating low transcript abundance. The relative

expression did not differ significantly between nurses and foragers when compared via their 2^-ΔΔCt values (t = − 0.71, df = 30, p = 0.48) (Fig. 3).

4. DISCUSSION

This research yielded several intriguing results that expand our understanding of honey bee physiology. First, Vg is abundant throughout the midguts of nurses and foragers, but different patterns of localization are observed. Compared to foragers, honey bee nurses appear to have a greater concentration of Vg near the basement membrane (Fig 1A, C), around the regenerative crypts (Fig 2), and extracellularly in the peritrophic membrane (Fig 1A–D). Additionally, we show that Vg is likely synthesized in the honey bee midgut (Fig. 3), consistent with previous findings of *vg* transcripts in this organ (Mao et al. 2013). However, as we observed a high degree of Vg immunostaining but low *vg* transcript abundance, one questions whether Vg is imported into this tissue from the hemolymph. Other studies have detected Vg receptors in the midgut, albeit at very low levels (Guidugli-Lazzarini et al. 2008). Further studies using fluorescent in situ hybridization will help resolve this matter and indicate where within the midgut *vg* is expressed. As with other insects, honey bees primarily synthesize Vg in their fat body tissue (Tufail and Takeda 2008), but the protein can also be synthesized in queen ovaries (Guidugli et al. 2005). In some blood-feeding arthropods like ticks, *vg* is expressed in the midgut to activate the ovaries and transport required nutrients from the blood meal (Boldbaatar et al. 2010; Thompson et al. 2007), while in mosquitos a blood meal stimulates fat body production of Vg to facilitate egg production (Bonizzoni et al. 2011). In these examples, Vg appears to be carrying out its typical reproductive function in response to a nutrient stimulus, but roles of Vg in the honey bee worker

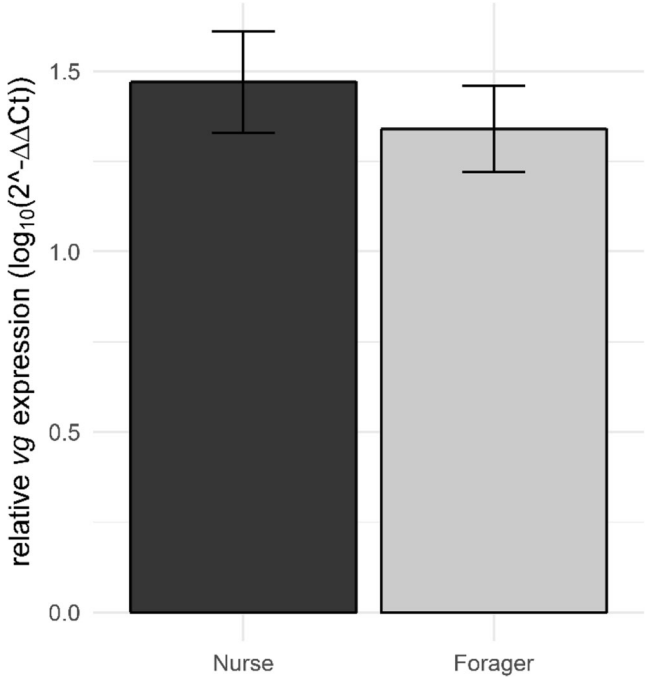

Fig. 3 *vg* expression in n = 16 midguts each from same-aged nurses and foragers. Bar heights represent the mean log10 *vg* expression in the two groups, as calculated by the ΔΔ-Ct method, with error bars representing ± 1 standard error. Statistical comparison between the two groups was performed via a Student's t-test on log10-transformed data. Expression levels were found to not significantly differ between the two behavioral groups (t = − 0.71, df = 30, p = 0.48)

midgut may be less clear, since these workers are functionally sterile and only rarely activate their ovaries. In honey bees, worker reproduction primarily occurs after irreversible loss of the dominant reproductive queen, with some exceptions (see Barron et al. 2001 for review).

We observed that *vg* is expressed at similar levels for same-age nurses and foragers (Fig. 1). This result was unexpected, given that *vg* expression in the fat body and Vg titers in the hemolymph are substantially different between nurses and foragers. It has been well-established over decades of research that worker behavior and *vg* expression are intricately linked: nurses have high titers of circulating Vg and foragers have low titers (Nelson et al. 2007), and conversely, knocking down Vg via RNAi in young adults causes nurses to prematurely transition into foragers (Amdam and Omholt 2003; Antonio et al. 2008; reviewed in Harwood et al. 2016). In the midgut, however, behavioral maturation and *vg* expression appear to have been decoupled. The exact reason for this decoupling remains unclear, but it could be that the midgut maintains steady (albeit low) expression of *vg* in order to carry out some function(s) that are important for both nurses and foragers. Alternatively, midgut *vg* expression may still vary with age as it does with fat body expression and circulating titers of the protein (Nelson et al. 2007), but our use of same-age nurses and foragers will not reveal this pattern. This latter point will require further investigation.

Finally, we found that the peritrophic membrane is consistently present in nurses but is absent or greatly reduced in foragers (Fig. 2). This may be due to dietary and physiological changes that coincide with the transition from nurse to forager, as nurses feed primarily on protein-rich pollen in order to produce royal jelly to feed the larvae and queen, while foragers feed on carbohydrate-rich honey (Brodschneider and Crailsheim 2010). Pollen is much more abrasive than honey and may require a more robust peritrophic membrane to protect midgut cells. In ants, carnivorous species are observed with larger peritrophic membranes than their nectar- or honeydew-feeding counterparts (Cook and Davidson 2006). Different diets also require different digestive machinery. The peritrophic membrane contains many digestive enzymes secreted from the enterocytes (Brandt et al. 1978; Hegedus et al. 2009; Lehane 1997), and nurses have higher enzymatic activity in their midgut than foragers (Jimenez and Gilliam 1989; Moritz and Crailsheim 1987). The level of enzymatic activity is directly related to the amount of protein consumed (Jimenez and Gilliam 1989), and foragers have a reduced ability to digest protein (Crailsheim et al. 1992). Alternatively, these results could simply be an artifact of our samples, wherein the nurses had more recently consumed food to trigger the secretion of a peritrophic membrane. However, given the consistent pattern across multiple samples, this explanation seems unlikely. Intriguingly, we found that Vg is secreted out of the enterocytes and into the peritrophic membrane, which suggests that it is intended to interact with the ingested materials and/or microorganisms in the gut lumen.

The evidence gathered in this and previous studies points to an as-yet-unknown function of Vg in the midgut: Vg is stably expressed in nurses and foragers, it is secreted into the peritrophic membrane, it is resilient against systemic *vg* knockdown via RNAi (Harwood et al. 2019), and the Vg receptor is expressed in midgut tissue at low levels (Guidugli-Lazzarini et al. 2008). The precise nature of Vg's role in the midgut remains unclear, but its other non-reproductive functions may give a clue. First, we propose that Vg can play a role in antimicrobial activity and cellular maintenance in the insect midgut. For example, Vg may be functioning as part of the anti-microbial defense mechanism against ingested pathogens. The midgut is a key immunological organ (Chapman 1998), and enterocytes produce AMPs to kill pathogenic cells. Likewise, Vg can bind PAMPs from Gram-negative and Gram-positive bacterial pathogens (Li et al. 2009; Salmela et al. 2015), either destroying such cells directly (Li et al. 2009) or opsonizing them to recruit other components of the innate immune system to destroy them (Li et al. 2008). Interestingly, the AMPs produced by enterocytes activate the immune deficiency (IMD) pathway which combats Gram-negative bacteria, but it is believed that enterocytes do not respond to Gram-positive bacteria (Chapman 1998). Several deadly honey bee pathogens like American foulbrood (*Paenibacillus larvae*) and European foulbrood (*Melissococcus plutonius*) are Gram-positive bacteria, and their point of entry is the larval gut. We do not yet have data on Vg localization in

the larval gut of the honey bee, but larvae are known to express the *vg* gene with functions currently unknown (Guidugli et al. 2005). Vg may be deployed to combat Gram-positive bacterial pathogens.

Second, we propose that Vg can protect enterocytes from the host's own immune defenses. Enterocytes secrete ROS to kill pathogens detected in the midgut (Buchon et al. 2009, 2013; Kumar et al. 2010), but ROS can also inflict cellular damage on the host's cells. Vg is an antioxidant (Havukainen et al. 2013; Seehuus et al. 2006) and so may act as a buffer to protect the host's cells from autotoxicity. Moreover, Vg can recognize damaged host cells and protect them against further injury and ROS damage (Havukainen et al. 2013). With abrasive damage from ingested food and ROS damage from immune responses, the insect midgut is a harsh environment that requires pluripotent stem cells to regenerate the population of enterocytes (Ward et al. 2008). Cellular turnover of enterocytes is very quick (about 1–2 weeks in drosophila (Chapman 1998)), and Vg may help to protect damaged enterocytes and extend the life of midgut cells.

Further studies are required to properly elucidate Vg's function in the midgut, but this will require novel experimental approaches to overcome existing challenges. As noted, Vg in the midgut appears to be resilient against standard systemic injections of double-stranded RNA (dsRNA) used to elicit RNAi gene knockdown (Harwood et al. 2019), making it difficult to compare control individuals with gene-knockdown individuals. One can administer *vg* dsRNA orally (e.g., Nunes and Simões 2009), but this introduces issues of inconsistent dosing since individuals consume variable amounts of food (Araujo et al. 2006). Furthermore, ingested dsRNA will be excreted or absorbed through the midgut and into the haemocoel, but the dsRNA that transiently passes through the midgut epithelial cells may have limited efficacy against target transcripts therein. Other studies that tried to target gut-specific genes with orally administered dsRNA failed to knockdown the target gene (Rajagopal et al. 2002). Thus, without an adequate experimental reduction of Vg in the midgut, it is challenging to test for functional roles of this protein.

Nevertheless, the need to understand Vg's role in this important organ remains. This study has expanded our understanding of Vg tissue localization and genetic expression in honey bee workers and provided important new findings. We have shown that *vg* is not only expressed in honey bee workers, but also that expression is steady between same-age nurses and foragers, and that Vg is secreted from enterocytes into the peritrophic membrane. Further functional experiments will be required to ascertain Vg's precise role in this organ.

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AUTHOR CONTRIBUTIONS

Conceptualization and design (Gyan Harwood); methodology (Gyan Harwood and Gro Amdam); formal analysis and investigation (Gyan Harwood); writing, original draft composition (Gyan Harwood); writing, review and editing (Gyan Harwood and Gro Amdam); funding acquisition (Gyan Harwood and Gro Amdam); resources (Gro Amdam); supervision (Gro Amdam).

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DATA AVAILABILITY

All data are included in the text. Raw RT-qPCR data available upon request.

DECLARATIONS

Ethics approval Bees were handled and sacrificed humanely to prevent undue suffering. No specific permits were required for the described studies.

Competing interests The authors declare no competing interests.

Expression de la vitellogénine dans l'intestin moyen de l'abeille domestique.

abeille domestique / intestin moyen / vitellogénine / histologie / membrane péritrophique.

Expression von Vitellogenin im Mitteldarm der Honigbiene.

Honigbiene / Mitteldarm / Vitellogenin / Histologie / Peritrophische Membran.

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