CHARACTERIZING THE IMMUNE RESPONSE OF THE ALFALFA LEAFCUTTING BEE THROUGHOUT PUPAL DEVELOPMENT

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Sara Elizabeth Cummings

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Characterizing the immune response of the alfalfa leafcutting bee through pupal development

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December 9, 2014
Date

Dr. Wendy Reed
Department Chair

ABSTRACT

Megachile rotundata is a solitary cavity nesting bee that is the primary commercial pollinator of alfalfa in North America. During prepupal to adult development, they may be exposed to fungal, parasitic, and bacterial pathogens. However, little is known about their immune function throughout pupation. We characterized functional immunity of *M. rotundata* across development stages. We injected prepupal, pupal, and adult bees with live *E. coli* and compared mortality across groups. We also developed an assay to measure antimicrobial peptide (AMP) activity in hemolymph for the same age groups. Both pupal and prepupal bees are sensitive to injected *E. coli*, resulting in high mortality, while adult bees survived longer after infection. Pupal bees had significantly less AMP activity compared to prepupae and adults. Understanding immunity of *M. rotundata* will provide context for improving commercial rearing practices, where measuring AMP activity can now serve as a biological marker of bee quality.

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1.1. *Megachile rotundata*

1.1.1. Natural history of *Megachile rotundata*

Megachile rotundata is a solitary, cavity-nesting bee. They are native to Eurasia, but now also inhabit North America, ranging from Canada south to New Mexico (Stephen, 2003). Adult bees emerge in late June and pollinate through July (Klostermeyer and Gerber, 1969). Females mate within a week of emergence (Richards, 1994) and begin pollinating and constructing brood cells, which are capsules constructed from leaves and lined with silk-like adhesive, provisioned with pollen and nectar stores before oviposition. Female bees naturally build nests in existing holes and construct the brood cells in series (Pitts-Singer and Cane, 2011). Each brood cell takes an average of 7.5 hours to complete and provision, and females lay one to two eggs per day (Klostermeyer and Gerber, 1969). Males are positioned closer to the openings of the nest cavity by the mother, as they emerge from their leaf capsules before the females (Pitts-Singer and Cane, 2011). Females are 60% larger (Yocum et al., 2011), because males receive on average of 17% less food provision by mass (Owen and McCorquodale, 1994). Females are able to control the sex of the eggs they lay by either fertilizing them, making them female, or not fertilizing them, making them male (Klostermeyer, 1973).

After hatching, the larvae consume their food provisions, progressing through five larval stages within the leaf capsule (Pitts-Singer and Cane, 2011). Prepupae overwinter in a state of suspended development called diapause. After diapause, the prepupae progress to a stage called post-diapause quiescence, which can last up to eight months. During this stage, development can resume as spring temperatures increase (Richards et al., 1987). Metamorphosis takes about four

to six weeks in a natural environment (Kemp and Bosch, 2000). Bees undergoing metamorphosis exhibit several developmental markers including molting to pupae, acquiring eye-pigmentation, undergoing sclerotization (a darkening and hardening of the cuticle), finally reaching adulthood, and emerging. Trostle & Torchio (1994) observed that pharate adults remain in the brood cell with proboscis and wings fully extended for two to three days before the wings harden. Once development is complete, adult bees chew through their leaf capsule and emerge to mate and begin the next year's cycle. During development and pupation, *M. rotundata* may face immune challenges of *Pteromalus venustus* parasitoid wasps or chalkbrood fungus *Ascosphaera apis*.

1.1.2. Commercial management of the alfalfa leafcutting bee

Management of *M. rotundata* as a key pollinator species for the alfalfa industry has been a large motivating factor in studying this bee. Their natural life cycle, especially their prepupal overwintering phase, has been challenging to deal with in rearing practices, but current protocols aim to balance bee survival with pollination rates. Adult bees that emerge in an appropriate field stay within 100 m of their nest, moving pollen from flower to flower by an average distance of 4 m (Amand et al., 2000). Foraging females collecting nectar and pollen provisions ensure high crop yields, tripping 78% of alfalfa flowers visited (Cane, 2002). Under favorable conditions, a single female accounts for more than a pound of alfalfa seed production (Cane, 2002). Utilization of *M. rotundata* boosts alfalfa seed yields up to 22-fold (Richards, 1993), with 2000 females per hectare increasing yields by 200-400 kg/ha (Free, 1993). Usage of the bee accounted for a yield of 46,000 metric tons of alfalfa in 2004 alone (Pitts-Singer, 2008).

Adult females readily lay eggs in artificial nesting sites in the fields, taking cues from

previously used nesting sites (Stanley and Pitts-Singer, 2011). The leaf capsules can be collected and stored for adult emergence the following season. To augment collection of leaf capsules in the field, farmers buy them by the gallon in fall or winter as diapausing prepupae, and their prices over the years have ranged from approximately $20 to $85 per gallon (Hodgson, 2011). Collected bees are stored in large bins at low temperatures until spring (Richards, 1984). Farmers keep brood cells at 6°C until their fields are ready for pollination. To ensure high pollination yields, about 5 weeks from the predicted 50% bloom of the alfalfa fields, post-diapause bees are incubated at 29°C to mimic warming spring temperatures (Pitts-Singer and Cane, 2011). Adults emerge within that 5 weeks, and pollinate as they collect leaf pieces, as well as the nectar and pollen provisions for the next generation of eggs.

Recent research suggests that the current protocol for commercial storage of these bees can be greatly improved (Bennett et al., 2013; Rinehart et al. 2011; Yocum et al., 2011). Pitts-Singer (2008) confirmed that Canadian populations of *M. rotundata* have higher reproductive success compared to those at lower latitudes, though there were regional and annual differences in fecundity, which were attributed to climatic conditions. Current rearing protocols used by USDA scientists recommend keeping developing bees at 29°C, which compared to outside storage methods decreases mortality from 35% to 21% and speeds up emergence time by two to five weeks (Kemp and Bosch, 2000).

One issue with optimizing rearing protocols is quality control. For example, how does one assess the fitness of surviving bees under various storage regimes? Quantitative measurements of the quality of the bees would allow a cost-benefit analysis for improvements in rearing methods. Identifying sublethal effects of other stressors like pesticides would also be useful for ensuring quality of emerging adults. One possible marker of bee quality is immune

function. Bees in storage may be attacked by fungi and parasites. Understanding immune system physiology for *M. rotundata* may allow use of immunological markers as indices of bee quality when investigating improved commercial rearing practices.

1.2. Immunity

1.2.1. Insect immunity

While insect immune systems are thought to be less complex than those of vertebrates, insects have many immune responses that are specific in action, allowing them to survive infection. Even though insects lack adaptive immunity, they have robust innate immune systems, including immunological memory (Eleftherianos et al., 2006; Rodrigues et al., 2010) and immune priming (Roth et al., 2010). In addition, induced immune responses are specific to type of infection, with a different suite of genes expressed depending on the pathogen (Riddell, 2009).

Innate immune responses can be broadly characterized as cell-mediated or humoral. Cell-mediated immune responses, such as phagocytosis, encapsulation and nodule formation are carried out by an insect's immune cells, called hemocytes (Lackie, 1988; Strand and Pech, 1995). Humoral immune responses are seen in the hemolymph and fat body and include the production of antimicrobial peptides (AMPs) and activation of the phenoloxidase (PO) cascade. The PO cascade generates cytotoxic molecules and causes melanization (Hancock and Diamond, 2000; Hoffmann, 1995; Zasloff, 2002). Although ostensibly helpful, categorizing immune responses as either cell-mediated or as humoral may be misleading and is somewhat arbitrary. Indeed, many humoral processes affect hemocyte function, and many important molecular components of

humoral responses are generated or mediated by hemocytes (Hoffmann, 1995; Hoffmann and Reichhart, 1997; Leonard et al., 1985; Strand, 2008a).

Another way to characterize immune functions is by whether the response is constitutive or inducible. Constitutive responses are always present as a non-specific, but immediately effective, defense against pathogens (Cerenius and Soderhall, 2004). In contrast, inducible responses can only be observed and measured after challenge with a pathogen (Haine et al., 2008). PO, lysozymes and phagocytosis are considered to be constitutive, while nodulation/encapsulation and AMP production are inducible and can take several hours to appear (Cerenius and Soderhall, 2004; Uttenweiler-Joseph et al., 1998). Again, although categorizing immune responses is useful, the classification is not always clear-cut. For example, elements of the PO cascade are constitutively expressed, but there may also be some PO that is induced upon a challenge. Any breach in the insect's cuticle will cause a clotting and coagulation response that includes hemocytes expressing PO activity, and melanization to prevent additional hemolymph loss (Muta and Iwanaga, 1996). These overlapping defense systems provide a comprehensive immune protection for insects, and the response mechanisms have been adapted for each insect's life history and ecological niche.

1.2.2. Cell-mediated immunity

Hemocyte types vary in structure and function and across species. They are classified using morphological, histological, functional characteristics, as well as antigenic and molecular markers (Gardiner and Strand, 1999; Jung et al., 2005; Lanot et al., 2001). The naming conventions are not universal for hemocytes, and the well-studied hemocyte morphology of *Drosophila melanogaster* does not necessarily translate to other species (Table 1).

Table 1. Hemocyte classification. Cell types, morphological descriptions, and major functions of various insect hemocytes

Cell type	Crystal cell	Lamellocyte	Spherulocyte	Oenocyte	Plasmatocyte	Granulocyte	Prohemocyte
Description	proPO containing, adhesive cells	Large, flat, adhesive cells that differentiate from prohemocytes quickly in response to an immune challenge	Cytoplasm full of large vesicles	Non-adhesive, contain cytoplasmic PO components, do not proliferate	Adhesive defensive cells	Adhesive defensive cells, constitutively expressed. Similar cells: Spherulocytes	Progenitor (stem) cells
Function	Melanize nodule /encapsulation targets, aid in hemplymph clotting	Encapsulate large targets, such as parasitoid eggs	Transport cuticular components	Release proPO in response to an immune challenge, melanize targets of nodulation/ encapsulation.	Encapsulate large targets, responsible for phagocytosis in *Drosophila*	Phagocytose foreign bodies	Differentiate into all other hemocyte types
Taxa	*Drosophila*	*Drosophila*	*P. includes*	*P. includens*, mosquitoes	*Drosophila*, *P. includens*, mosquitoes	*Pseudoplusia includens* (Lepidopteran), mosquitoes	All
References	(Lanot et al., 2001)	(Lanot et al., 2001; Lavine and Strand, 2002)	(Gardiner and Strand, 1999)	(Castillo et al., 2006; Gardiner and Strand, 1999; Wang et al., 2011)	(Gardiner and Strand, 1999; Wang et al., 2011)	(Castillo et al., 2006; Gardiner and Strand, 1999; Wang et al., 2011)	(Lavine and Strand, 2002; Ratcliffe et al., 1985; Strand, 2008a; Wang et al., 2011)

All cell types differentiate from stem-cell-like progenitor cells called prohemocytes (Lackie, 1988; Ratcliffe et al., 1985). Hematopoiesis, or the creation of hemocytes, occurs at embryogenesis and throughout larval/nymphal stages (Akai and Sato, 1971; Ratcliffe et al., 1985). During embryogenesis of *Drosophila*, prohemocytes and hematopoietic organs originate from dorsal mesoderm (Holz et al., 2003; Jung et al., 2005). Hematopoietic organs, called lymph glands in *Drosophila*, are a source of prohemocytes as the insect develops. Cells in circulation are also continually able to proliferate, with the exception of oenocytes (Gardiner and Strand, 2000). Continued division of hemocytes in circulation in the hemolymph is critical for maintaining cell populations throughout development, so that cellular immunity can provide protection from pathogens (Gardiner and Strand, 2000). Throughout the larval stages, holometabolous insects exhibit a pattern of increasing hemocyte production from hematopoietic organs, with the most cells produced in the final instar before metamorphosis (Beetz et al., 2008; Gardiner and Strand, 2000; Lanot et al., 2001; Nakahara et al., 2003). Hemocyte proliferation halts, and a drop in total hemocyte count is seen in adult honey bees (Schmid et al., 2008) and adult mosquitoes (Hillyer et al., 2005), possibly indicating an alternative immune response strategy for the adult life stage.

In addition to their role in immunity, hemocytes are also important during metamorphosis. Hemocytes have been shown to proliferate and differentiate into a macrophage-like cell to play a crucial role in tissue remodeling during metamorphosis (Lanot et al., 2001; Thomas and Rudolf, 2010). These cells use lysozyme activity to digest doomed larval tissue in the hemocoel (Lanot et al., 2001). Hemocytes play this dual role in many insects, present in high numbers during pupation in honey bees (Wilson-Rich et al., 2008), ground beetles (Giglio and Giulianini, 2013), and tobacco hornworms (Eleftherianos et al., 2008).

Phagocytosis in insects is carried out by adhesive hemocytes, usually characterized as granulocytes and/or plasmatocytes, and phagocytic activity is enhanced by the activation of the PO cascade (Leonard et al., 1985). Hemocytes engulf targets marked for destruction ranging from bacteria or yeast cells to synthetic beads or particles of India ink (Hernandez et al., 1999; Yokoo et al., 1995). A single granulocyte in an *Aedes aegypti* mosquito has been recorded to phagocytose 1500 cells of *E. coli* (Hillyer et al., 2005). Although phagocytic activity is generally correlated with total hemocyte counts, Kurtz (2002) observed relatively constant phagocytosis in *Panorpa vulgaris* (scorpionfly) larvae, in spite of dropping hemocyte numbers as they approached pupation. Different hemocyte types may play multiple roles in cell-mediated immunity or may perform specific functions, depending on the insect.

Encapsulation and nodulation are essentially the same process, by which hemocytes adhere in layers to an invading object, forming a melanized physical barrier. The difference between encapsulation and nodulation is the size of the adhesion target. Nodules are formed around small targets (e.g. bacteria), while large targets (e.g. parasite eggs, or nylon monofilaments used in assays) are encapsulated (Rantala and Roff, 2007; Smilanich et al., 2009; Strand, 2008b). In *Drosophila*, encapsulation is carried out by lamellocytes, which quickly proliferate from prohemocytes upon an immune challenge such as a parasitoid infestation (Sorrentino et al., 2002; Wertheim et al., 2005). In the mosquitoes *Anopheles gambiae* and *A. aegypti*, however, no specialized capsule-forming hemocytes are found, and nodulation responses are carried out by granulocytes, their most common cell type (Castillo et al., 2006). Phagocytic activity is enhanced by activation of the PO cascade (Leonard et al., 1985), and melanization occurs in conjunction with a nodulation/encapsulation response, because the hemocytes involved are producing PO. However, Pinera et al. (2013) found in crickets *Acheta*

domesticus that encapsulation capacity is independent from total PO activity. These types of cellular immune responses are also involved in hemolymph clotting and wound healing (Muta and Iwanaga, 1996), and have homologous systems in vertebrates (Vilmos and Kurucz, 1998).

1.2.3. Humoral defense

One of the main components of the humoral immune system is the production of antimicrobial peptides (AMP). AMPs are present as part of every eukaryotic organism's immune system (Vilmos and Kurucz, 1998). They are characterized as small proteins (<25 kDa) that execute a broad scope of activities against bacteria and/or fungi (Imler and Bulet, 2005). There are large variations in overall peptide sequence of AMPs expressed across even closely-related species, but there are areas of conserved sequences with respect to the precursor protein, signaling pathways, and mechanisms of bacterial destruction (Zasloff, 2002). Despite the ancient evolutionary lineage of AMPs, prokaryotic cells generally do not have resistance to these kinds of molecules, because the AMPs attack mechanism targets the bacterial cell membrane (Matsuzaki, 1999). On a prokaryotic cell, the outer layer of lipid bi-layer contains negatively charged phospholipid head-groups, compared to eukaryotic cells, whose membranes are generally neutral in charge (Matsuzaki, 1999; Zasloff, 2002). The structure of AMPs allows them to breach the charged membranes of bacteria, allowing access to intracellular targets or simply disrupting membrane function, ultimately killing the prokaryotic cells (Matsuzaki, 1999; Yang et al., 2000). There exists a hard-wired signal-transduction pathway that links microbe infection and activation of genes to produce corresponding AMPs (Zasloff, 2002). AMP also act as a longer term prophylactic from infection, persisting in the hemocoel and providing a survival benefit in the case of subsequent infections (Moret and Siva-Jothy, 2003). Uttenweiler-Joseph et

al. (1998) examined the immune response of *Drosophila melanogaster* over several hours after an experimental infection and showed that the peak volume and variety of AMPs were present after 24 hours, with some remaining stable for up to three weeks.

Some varieties of AMPs can be constitutively expressed as a local innate immune response in tissues that are not immune-functioning but may need to defend against bacterial exposure, like the epithelial tissue in the cuticle, gut, or tracheal system (Lemaitre and Hoffmann, 2007). In the silk worm *Bombyx mori*, the silk and cocoon have shown constitutive expression of antimicrobial proteins as protection during the immobile pupal stage (Pandiarajan et al., 2011). The expression of these constitutive, local AMPs are not upregulated during infection (Lemaitre and Hoffmann, 2007), unlike the AMPs produced mostly from the fat body as part of an immune response.

In *D. melanogaster*, two specific AMPs have been identified in response to different infections. Gram-negative bacteria result in the activation of the Toll signaling pathway, and upregulation of diptericin (Imler & Bulet, 2005), while fungus, yeast, or gram-positive bacteria cause an upregulation of drosomycin, via the imd signaling pathway (Imler and Bulet, 2005; Lemaitre and Hoffmann, 2007). Further specification in parasite-host interactions has been seen in *Bombus terrestris*, in which different strains of the gut parasite *Crithida bombi* elicit different patterns in AMP gene expression (Riddell et al., 2009). These examples of specificity in immune response indicate that insect immunity is capable of being more functionally 'adaptive,' especially in the context of the evolutionary arms race that embodies pathogen-host interactions.

Another major component of the humoral immune defense is the production of phenoloxidase (PO). The PO cascade, also known as the melanization reaction, involves the release and activation of the zymogen proPO from oenocytoid hemocytes (or crystal cells in

Drosophila) and other immune cells and tissues (Castillo et al., 2006; Lanot et al., 2001). Upon infection, serine proteases cleave proPO producing catalytically active PO (an oxidoreductase), cytotoxic compounds like quinones, other short-lived chemical intermediates, and finally melanin (Cerenius et al., 2008; Cerenius and Soderhall, 2004). Melanin darkens and hardens into a physical barrier and is involved in hemolymph clotting (Muta and Iwanaga, 1996), as well as the encapsulation/nodulation response (Ling and Yu, 2005). The PO cascade is activated upon recognition of non-self, via pathogen-associated molecular patterns (PAMPs) like peptidoglycans and liposaccharides from bacteria, or β1,3-glucans from fungi (Cerenius et al., 2008). Because the PO reaction is so volatile, its activation is carefully regulated spatially and temporally to protect from self-harm (Cerenius and Soderhall, 2004). It is also the fastest acting immune response, as proPO is constitutively expressed in a variety of tissues and cell types. Higher PO capacity has been linked to darker, more melanized cuticle, which translates to greater pathogen resistance (Barnes and Siva-Jothy, 2000; Giglio and Giulianini, 2013; Wilson et al., 2001).

1.3. Immunity interactions

Immunity in insects, as with all organisms, is not a stand-alone process. Physiological processes that affect and are affected by immune function are constantly in flux as an insect develops, interacts with its environment, and struggles to survive and reproduce. Many of these interactions have been studied across species, and it is important to be able to assess them on a broader scale to draw conclusions about immunity as a whole, in the full context of development.

1.3.1. Immunity through development

Immunity is commonly studied in development within specific life stages, such as in the adult or certain larval instars due to the ease of study or specific interests. For example, *Manduca sexta*, the tobacco hornworm, is commonly studied during its 5th and final larval instar, because of its large size and hemolymph volume. Late within its 5th instar, *M. sexta* larvae succumb more quickly to infection and show decreasing humoral and cellular immune responses (Eleftherianos et al., 2008). *M. sexta* also showed more than a 50% reduction in nodulation, PO capacity, and AMP activity between a day zero and day five larvae (Eleftherianos et al., 2008). However, Beetz et al. (2008) also saw a three- to five-fold increase in hemocyte counts in caterpillars just preparing for pupation (wanderers) compared to the caterpillars freshly molted into their 5th instars, with the most lysozyme activity in wanderers. This is consistent with observations in the wax moth *Galleria mellonella*, which was found to have the greatest hemolymph PO activity at day five of the final larval phase before pupation, and again at late pupal phase (Benesova et al., 2009). In adult insects, *A. aegypti* mosquitoes exhibit an age-associated mortality increase in response to an *E. coli* immune challenge (Hillyer et al., 2005). This mortality was attributed to a decrease in hemocyte count by 24% in day zero versus day five post-adult emergence, which supports the finding that adult mosquitos are not be able to produce more hemocytes after metamorphosis (Akai and Sato, 1971). Insects may have increased or altered immune function at the critical phases of metamorphosis, when the greatest morphological changes are seen. The pupal stage of the ground beetle *Carabus lefebvrei* exhibits an additional defense outside the normally described immune system, with secretions of volatile gaseous chemicals, called monoterpenes, into the subterranean pupal cell as protection against microbial and fungal infection (Giglio et al., 2009). While immunity has been studied in

juvenile and adult insects, comprehensive characterizations of the immune response across stages of development on species of insects are uncommon. Due to changing metabolic demands during development or different life history strategies, immune function may vary in speed, mechanism, or specificity, depending on the insect's life cycle.

1.3.2. Metabolic demands during development

Metabolic rate is a measure of how much energy is being expended by an organism. As ATP (energy) is produced by an animal, oxygen is consumed, and carbon dioxide and water are produced. Oxygen consumption and carbon dioxide production can be measured indirectly using respirometry, quantifying how much ATP is being produced and used (Lighton, 1996).

Metabolic rates in insects are known to vary throughout development. The Colorado potato beetle, *Leptinotarsa decemlineata*, has an increase in resting metabolic rate during the first 2 days after adult emergence, which then gradually decreases with age and is independent of mass (Piiroinen et al., 2010). Adult tsetse flies, *Glossina pallidipes,* also show a rise in metabolic rates in the days just after emergence, thought to be due to the development of flight muscles (Terblanche et al., 2004). During pupation and metamorphosis, however, holometabolous insects like *M. rotundata* experience large changes in functional morphology, including to the respiratory system structures. The giant silk moth *Samia cynthia* has a high metabolic rate as a caterpillar, using more than 14 spiracles and exhibiting continuous gas exchange patterns (Hetz, 2007). As a prepupa and pupa, however, *S. cyntnia* uses discontinuous gas exchange, has a much lower metabolic rate, and uses between eight and ten spiracles at any given time (Hetz, 2007). This is consistent with early findings in *Cecropia* silk worms that exhibit high metabolic demands as caterpillars entering pupation (>1000 mm^3 O_2 g^{-1} hr^{-1}),

minimal O_2 consumption as pupae, and increases up to 300 mm^3 O_2 g^{-1} hr^{-1} just prior to adult emergence (Schneiderman and Williams, 1953). Kemp et al. (2004) found that *M. rotundata* have respiration rates of less than 0.025 O_2 ml $g^{-1}hr^{-1}$ during pupal stages (seven days after incubation at 29°C) that increase almost ten-fold as adults. This suggests that *M. rotundata* may have a similar pattern of high metabolic demands at early and late pupation, with lower rates seen in mid-pupation and rising again in the pharate and adult stages.

1.3.3. Metabolic cost of immunity

Infection causes an increase in metabolic demand across many species (Hoffmann A.A., 1991). In both hemi- and holometabolous insects, an induced encapsulation response elicited an increase of 25-28% in adult metabolic rates compared to naïve controls (Ardia et al., 2012). Diapausing pupae of the white cabbage butterfly *Pieris brassicae* showed a metabolic increase of 8% during an encapsulation experiment (Freitak et al., 2003). If there is a selective pressure, like a high rate of parasitism in *Drosophila*, over several generations a lower baseline metabolic rate will evolve (Fellowes and Godfray, 2000). Generally, a change in metabolic rate during an immune challenge indicates necessary energy expenditures resulting from the immune response, which could result in trade-offs in other areas of the organism's physiology.

While increased cost may be a common occurrence, the mechanisms by which insects compensate vary. Armitage et al. (2003) looked at the cost of an initial constitutive investment in PO, by comparing darker *T. molitor* beetles (ones with higher melanization in their cuticle) to lighter ones, and found no trade-off in longevity or fecundity. However, they found a cost to the induced immune response which resulted in reduced longevity. In *Spodoptera littoralis*, genetic strains that have higher melanization in the cuticle show a tradeoff in lower PO activity, but

strains with higher PO activity develop more slowly, indicating that investment in melanin is less costly than having higher PO defense (Cotter, 2008).

Another indicator of metabolic demand can be observed in food consumption. For example, *Apis mellifera* showed an increase in sugar-feeding after an immune challenge, thought to compensate for the increased metabolic demands of fighting an infection (Martin-Hernandez et al., 2011). In contrast, induced anorexia is seen in the cricket *Gryllus texensis* in response to an infection and is thought to reduce trade-offs between demands from digestion and demands from immune response on metabolism (Adamo et al., 2010).

When measuring the effect of immune challenges on metabolic rates, it is important to consider the other confounding factors that affect the measured costs. Often the administration of an infection requires a wound (an injection) and possible blood loss. Ardia et. al. (2012) observed four insects: crickets, mealworms, cockroaches, and June beetles and investigated their immune responses to various challenges. Removal of hemolymph incurred a cost across all 4 species, as they noted 10% greater CO_2 production compared to wounding alone. Under stressful environmental conditions, such as starvation, tradeoffs in energy allocation have been shown to favor immunocompetence over reproduction in *B. terrestris*. The bees showed no drop off in encapsulation capabilities in reduced food resource experiments, but did suffer a cost to reproduction (Schmid-Hempel and Schmid-Hempel, 1998). These types of tradeoffs are particularly important when considering a developing insect with a fixed energy budget, such as *M. rotundata*.

1.4. Immunity in *Megachile rotundata*

There is a limited amount of research on physiology of *M. rotundata*; historically there are more studies that explore their lifecycle, behavioral traits and commercial management (Richards et. al. 1994, Stanley and Pitts-Singer 2011, Pitts-Singer et. al. 2009). James and Xu (2009) investigated the differences in gene expression between infected *M. rotundata* and healthy individuals. Their work showed that this bee shares several conserved genes that are unregulated during infection and influence immune responses across insect species. Inglis et. al. (1993) explored the detrimental relationship of *M. rotundata* larvae ingesting spores of *Ascosphera aggregata* fungus, which cause chalk brood syndrome in overwintered prepupae. More research is required to understand the specifics of this bee's immune function.

1.5. Conclusion and significance

Studying immune responses in insects is beneficial because, while insects have only innate immunity, the responses are highly evolutionarily conserved. This removes the confounding effects of adaptive immune functions, allowing us to draw clearer conclusions about the functions of the innate immune response. With antibiotic resistance becoming a problem across medical and agricultural fields, insights from naturally occurring antimicrobial defenses could be the key to the next generation of antibiotic drugs. Additionally, ecoimmunology, a field that seeks to understand how and why environmental factors contribute to natural variation in immunity, has become increasingly important. By studying proximate mechanisms of immunity between and within species, we can gain some perspective on immunity's role in the evolution of life-history traits across taxa. We may also link variations in

individual immune responses to physiological trade-offs, environmental variation, and ecological and/or behavioral traits.

Effective pollinators like *M. rotundata* affect both natural ecosystems and agricultural economics. Constructing an overview of the immune function of *M. rotundata* throughout its development improves our ability to manage this important pollinator. When optimizing experimental rearing practices, it is important to develop markers of bee quality. Examining the immune system of *M. rotundata* may allow us to use immune system parameters to as a marker of bee quality. Information about developmentally induced periods of immunological vulnerability helps managers better protect stored bees from potential pathogens. Future exploration of this bee's immunity, such as encapsulation experiments, may better inform us of their relationship with the parasitoid wasp, *Pteromalus venustus*.

CHAPTER 2. CHARACTERIZING THE IMMUNE SYSTEM OF *MEGACHILE ROTUNDATA*

2.1. Introduction

2.1.1. *Megachile rotundata* natural history, commercial use, value for study

The alfalfa leafcutting bee, *M. rotundata,* is a gregarious, but solitary, cavity-nesting bee native to Eurasia but used to great success as a pollinator of alfalfa across North America (Pitts-Singer and Cane, 2011; Richards et al., 1987). Males are generally smaller and faster to develop, and mate with emerging females within the first week after emergence (Pitts-Singer and Bosch, 2010). Female bees then spend two to four weeks constructing individual brood cells out of cut leaves for each egg she lays, provisioning each capsule with pollen and nectar for the developing larvae. After hatching, the bees consume the provision in their brood cells and proceed through five larval instars before the prepupal bees enter diapause, a state of quiescence in which they will spend the winter until development to adulthood initiated by warm temperatures in May and June. This life cycle strategy is particularly interesting, as the bees spend their entire development time within this protected brood cell, only emerging as adults. Leaf capsules can also be harvested and commercially managed by alfalfa farmers and bee growers (Pitts-Singer, 2008; Richards, 1984).

Understanding the physiology of this pollinating species has been of increasing importance, and the lifecycle and pollinating behavior of *M. rotundata* has been of interest in research (Cane, 2002; Klostermeyer and Gerber, 1969), as well as on improvements of rearing methods (Kemp and Bosch, 2000; Rinehart et al., 2011; Yocum et al., 2010). Studies on the bee's immune system have focused on its vulnerability to a common fungal disease, chalkbrood (*Ascosphaera aggregate*)(Inglis et al., 1993; Xu and James, 2009). However, few studies have

focused on the immune function of *M. rotundata* throughout its development, and during the critical phase of pupation (Goerzen, 1990).

2.1.2. Insect immunity and AMP activity throughout development

The immune systems of insects can be characterized as innate, rather than adaptive, as they lack the ability to produce antibodies seen in vertebrates (Hoffmann, 1995). Insect immunity can be described as cell-mediated or humoral. Cell-mediated defenses are carried out by hemocytes, and include phagocytosis, nodulation, and encapsulation (Lavine and Strand, 2002; Strand, 2008a). Humoral immune responses include the activation of the phenoloxidase cascade (PO), and the production of antimicrobial peptides (AMPs). The precursor of PO (proPO) is constitutively expressed and readily available from hemocytes and can be rapidly activated, resulting in melanization (Cerenius and Soderhall, 2004), but AMP production by the fat body is under genetic control, meaning there is a gene activation response that must be biochemically induced over several hours or days, depending on the insect (Zasloff, 2002). Once activated, AMPs can effectively eliminate a wide variety of bacterial and fungal immune challenges (Lemaitre and Hoffmann, 2007), and some kinds of AMPs can persist in the hemolymph for up to two to three weeks post-infection (Uttenweiler-Joseph, 1998). Complex physiological interactions govern these humoral immune responses, and changes in expression and function should be expected as insects develop and grow through various life stages.

The physiology of immune responses is strongly evolutionarily conserved across other taxa, so when we improve our overall understanding of immune responses in insects, we also gain understanding of more complex immune systems of other organisms. By studying the immune function of *M. rotundata*, we will expand on the base of knowledge of insect physiology

and will be able to make broader, more clearly defined inferences about the evolution of immune function, as well as adding context to ecoimmunological studies that investigate evolutionary and ecological interactions with the innate immune system. *M. rotundata* is especially interesting from a development standpoint, as its lifecycle includes the protection of the leaf capsule for the span of its development to adulthood. Understanding basic mechanisms of this insect's immunity will help us better protect beneficial insects from pathogens.

Development of immune systems in insects is commonly studied within specific life stages, such as in the adult or certain larval instars, and usually using model insect species (Ardia, 2012, Eleftherianos, 2008, Schmid, 2008). Although insect innate immunity is evolutionarily conserved, we expect to see variation in response and specificity of immune function across development, and correlation between the risk of infection for each life stage, life expectancy and biological function (Giglio and Giulianini, 2013). The role hemocytes, the fat body, and other contributors to insect immune function play during larval development may differ dramatically across life stages, because the morphological and physiological changes that occur from larva to pupae to adult are profound. Furthermore, while immunity has been studied in juvenile and adult insects, comprehensive characterization of the immune response across stages of metamorphosis in insects are relatively uncommon.

Table 2. Insect immunity through development. A summary of the literature reviewed for how immune function has been shown to change throughout development in different insect species.

Insect	Development stages	Change in immune response	Citation
Manduca sexta (tobacco hornworm)	Larvae (caterpillars) approaching pupation – early to late 5th instar	Decreased AMP, PO and nodulation activity, but increase in total hemocyte counts as they approach pupation.	Eleftherianos (2008), Beetz (2008)
Bombyx mori (silkworm)	Early to late larval instars	Increasing size and proliferation of haemopoietic organs, which are a source of various hemocytes	Akai and Sato (1971)
Galleria mellonella (greater wax moth)	Larvae approaching pupation, pupae, and adulthood	Greatest hemolymph PO activity just prior to pupation, and again just prior to adult emergence – but a lull during pupation.	Benesova (2009)
Apis mellifera (honey bee)	Larvae, adults and pupae of workers and drones	Pupae had highest mortality rate with no AMP activity or clearance of live bacteria upon exposure.	Gaetschenberger (2013)
Aedes aegypti (yellow fever mosquito)	Adult, day 0 to day 5 post emergence	Decreasing hemocyte counts with age, increased mortality in response to an immune challenge	Hillyer (2005),

Insects have developmental and metabolic demands that may affect immune function at the critical phases of metamorphosis, when the greatest morphological changes are seen. This may be confounded by energy demands from the immune response, especially as the pupal phase is non-feeding and thus energy is limited. Table 2 shows some trends seen across development in other holometabolous insects. Though cellular immune indicators such as total hemocyte counts increase during pupation compared to larva and adult stages (Eleftherianos et al., 2008; Giglio and Giulianini, 2013; Wilson-Rich et al., 2008), this may have more to do with the hemocyte's role in metamorphosis and tissue remodeling than immunity (Lanot et al., 2001; Thomas and Rudolf, 2010). In the honey bee *Apis mellifera*, a recent study showed that insects in the pupal phase were more likely to die from infection than either larva or adults, and showed the lowest AMP activity (Gaetschenberger, 2013). Perhaps pupal insects devote so much of their limited resources into metamorphosis they are ill equipped to support the costly humoral immune responses needed to effectively fight off infection. In *M. rotundata*, the pupal and larval stages are protected by the leaf capsule, which may provide an external barrier to pathogens until adulthood. Perhaps, like *B. mori*, there are even AMPs spun into the silk layer within the capsule (Pandiarajan, 2011). With that in mind, as well as the hypothesis that immune function varies through development, we predict that in *M. rotundata*, adult bees will have better immune function and survival than larval and pupal bees, and that AMP activity will be lowest during pupation.

2.2. Materials and methods

2.2.1. Animal care

Loose brood cells containing *M. rotundata* prepupae were purchased from JWM Leafcutter, Inc. (Nampa, ID, USA) in the spring of 2011 and 2012 and stored at 6°C for an

overwintering period of 8-9 months. To initiate metamorphosis, prepupal bees were placed in a humidified incubator at 29°C (Rheem Sherer Model CER 69M, Weaverville, NC). Insects were dissected from leaf capsules at specific time points in their development depending on the experiment, see Table 3 for descriptions and pictures. Dissected prepupae were stored in a 96-well plate, one bee per well, for the duration of pupal development. Adult bees were reared in small chambers constructed using plastic cups and lids, separated by males and females, and given *ad libitum* access to a 1:1 sugar water solution that was refreshed every three days (Bennett et al. 2013).

2.2.2. Survival assay

2.2.2.1. Treatments

To characterize immune responses throughout development, we used seven treatment groups: injection with one of four concentrations of *E. coli* (10^5, 10^4, 10^3, or 10^2 live cells per microliter), injection with a sterile phosphate buffered saline (PBS), sham-injection, or no injection. In all treatment groups, prepupal bees were extracted from their brood cells using sharp probes or forceps to break through the leaf capsule and soft forceps to carefully remove the prepupae from the cell. After bees were removed from their leaf capsules, they were stored in 96-well plates at 29°C until they reached the desired developmental stage and subjected to treatments. For experiments with adult bees, they were allowed to emerge normally from their brood cells, transferred to feeding chambers, and selected for treatments after no more than three days of captive feeding. Only bees that appeared in good condition, showed feeding behavior, and actively moved about their enclosures were chosen for experimentation. Injections of bacteria and PBS and sham injections were performed using a 30 gauge needle. All ages of *M. rotundata* were punctured on their dorsal side, in the intersegmental membrane between

abdominal third and fourth segments, counted from the posterior end. A 10 µl glass Hamilton syringe was held by a micromanipulator (Prior England, model 62864) and the bee placed on the movable stage of an Olympus SZX9 microscope. Bees and the syringe were positioned so that sliding the stage would allow the needle to puncture the cuticle in the same location in the same way each time. For sham-injections, bees were immediately removed from the needle to a well plate. Bees that receive injections of PBS or a bacterial suspension were injected with one µl and then removed to a well plate. Adult bees required chilling prior to injections (less than 5 minutes on ice) for ease of handling. Bees receiving no injection were handled in the exact same way, except for the injection.

2.2.2.2. Bacterial culture

DH5-α *Escherichia coli* were cultured with Luria broth in tubes and incubated at 37°C, shaking at 200 rpm, for at least eight hours (Lab-Line Environ-Shaker Model 3528-5, Melrose Park, IL). After eight hours, the optical density of the bacteria-LB solution was measured using a spectrophotometer at 600λ wavelength, to calculate the colony forming units (CFU). Bacterial cultures were resuspended at known concentrations. Our bacteria strain followed the equation (177790*OD) + 6141.5 = CFU/ml. Bacteria were then pelleted, washed and suspended in filter sterilized phosphate buffered saline (PBS) at the specified calculated volume to achieve 10^5 cells/µl, and diluted down to 10^4, 10^3, and 10^2 cells/µl. These *E. coli* concentrations were used for treatments with an immune challenge, to stimulate an immune response.

2.2.2.3. Survival assessments

To assess the overall efficacy of the immune system, we recorded survival of infection among the different treatment groups. The treatments were given at various stages of development; young prepupae at one day of incubation, older prepupae at six days of incubation, younger eye-pigmented pupa at 14 days of incubation, and older eye-pigmented pupa at 18 days of incubation. After treatment, bees were monitored for up to 30 days. We recorded developmental markers (Table 3), as well as indications of severe infection or mortality. Time of death was not easily measured during these developmental stages, so any coloration change (melanization, dehydration, mold spots) was recorded. If the discolored bees failed to develop to the next phase, death was confirmed.

Adult bee survival was similarly assessed, with the same treatment groups administered within five days of a bee maturing to adult. Days of survival after treatment were tracked for up to 30 days.

2.2.3. Hemolymph collection, AMP isolation, AMP activity assay

To determine AMP activity, we used four treatment groups. Bees were either untreated, sham-injected, PBS injected or injected with heat-killed *E. coli*. Treatment with heat-killed bacteria stimulates the immune system without an active infection. *E. coli* was cultured as described above and was then heat killed (at 65°C for 30 minutes) and kept refrigerated at 4°C, for no more than two weeks prior to injections. Bees were injected with 1 µl of 10^3 *E. coli*. After injection with bacteria, bees were incubated in at 29°C (Labnet 211DHS incubator, Edison, NJ) for 20 to 24 hours after treatment before hemolymph was collected. Hemolymph was gently squeezed out of the prepupae through a small puncture wound and collected using a 10 µl

pipette. This method yielded up to 12 µL of hemolymph (multiple collections using the 10 µl pipette for the larger volumes), on average 6.5 +/- 0.7 µl. Older pupating insects were fragile and could not be squeezed. They were instead centrifuged at a low speed to remove the hemolymph. Each pupa was placed in a 0.6 ml microcentrifuge tube with a hole (0.46mm outer diameter) in the bottom. A 30 gauge needle was used to wound the bee on its dorsal side near or on the head, and the bee was placed in the tube anterior side down. That tube was then placed in a larger microcentrifuge tube (1.5 ml) and spun in a Eppendorf Centrifuge 5417C for five minutes at 0.6-1.5 x g. This low speed avoided spinning out the fat body. Higher speeds were used for more sclerotized pupae. The adult bees were also spun in this manner to collect hemolymph, but at a much higher speed (5-7g), because they did not have as much fat as the pupae. Each successfully bled eye-pigmented pupa could yield 4.7 +/- 0.5 µL, and adults yielded 7.8 +/- 0.6 µl. Hemolymph was kept on ice until AMP isolation. The hemolymph was spun at 7000 x g for five minutes in microcentrifuge tubes. Hemolymph was then heated to 95°C for five minutes, and spun again at 13300 x g for 10 minutes (Kanost, 1990). The supernatant contained the heat-stable AMPs, usually 3 µl or less total volume.

Because the AMP activity of these bees is generally low, we used a bacterial killing assay to quantify their activity. Live DH5-α *E. coli* were diluted to 100 cells/µl in Hepes buffer (10 mM, pH = 7). Hepes buffer allows the *E. coli* to remain alive without growing, as it has no source of food for the bacteria. In a 96-well plate, 1 µl of *E. coli* and 1.5 µl isolated AMP sample were added to 27.5 µl Hepes for a total of 30 µl per well, and then samples are taken at an initial inoculation time point and at five hours of incubation, to measure the killing capacity of the AMPs. Hepes alone served as a negative control, and *E. coli* in Hepes served as a positive control. Isolated *M. sexta* AMP served as a positive control for AMP detection. For each

experimental replicate, there were a total of 24 treatment sample wells, with two biological reps per treatment per age group, in addition to the three control wells.

In duplicate, 2 µl of the AMP-bacteria mixture was removed from each well and added to 98 µl Hepes in a 0. 6 ml microcentrifuge tube and mixed thoroughly. From the 0.6 ml tube, 40 µl was plated on LB agar plates and incubated at 37°C (Labnet 211DHS, Edison, NJ) for 24 hours. To confirm our starting concentration of *E. coli*, we plated four ten-fold serial dilutions of *E. coli* alone.

The samples left in the 96-well plate were incubated (shaking) in a Lab-Line Environ-Shaker Model 3528-5 (Melrose Park, IL) at 37°C to allow time for the AMPs to kill the *E.coli*. After five hours, two more µl of the AMP/bacteria mixture was taken from each well and added to 98 µl in a microcentrifuge tube. Each 100 µl tube was then diluted out serially (10 µl into 90 µl PBS, mixed, then 10 µl taken again and added to 90 µl PBS, repeated out to 1:1000 dilution) as technical replicates, four for each sample. From each of these 100 µl dilution tubes, 40 µL was plated on labeled LB agar and incubated at 37°C for 24 hours. To stop growth, plates were placed in 6° for no more than 24 hours, and CFU were counted. For any plates with growth >100 colonies, plates were divided into quadrants with an indelible marker, to help track counting. Plates with more than 250-300 colonies were recorded as "lawns." For analysis, CFU were compared at the 100x dilution. Colony counts were converted to an AMP activity index by taking 1/colony count at that concentration.

2.2.4. Statistics

We used time-to-event statistical measurements, the Kaplan-Meier test and Cox regression to compare development times, as well as comparing overall survival rates, showing

time-to-event statistics. Adult average days of survival were tested with a univariate ANOVA, and a Cox regression was used for overall survival analysis. A univariate ANOVA was also used to analyze the AMP assay data at the 100x CFU concentration. Data are presented as means ± S.E.M., except for percentages.

2.3. Results

For overall development descriptions, novel untreated bees were used, and only bees that survived to adulthood were used to calculate average development times. Table 3 shows overall descriptions of the marked stages of pupal development. Development timing differed between the sexes (Figs. 1 and 2). Males developed significantly faster than females, by an average of 2.47 days faster at the first molt of pupation ($p = 0.017$), 2.32 days faster at the eye-pigmented stage ($p = 0.024$), 2.56 days faster at the time of sclerotization ($p = 0.015$) and reached adulthood 3.09 days earlier ($p = 0.003$) (see Fig. 1). Males took an average of 24.25 ± 0.6756 days to reach adulthood, while females took an average of 28.6 ± 1.4 days. Without a food source, males lived an average of 6.41 ± 0.964 days and females lived an average of 5.6 ± 1.53 days. Across development, the greatest difference and variation in development time was seen in the pupal stage for males and females.

Table 3. Characterization of normal development in *Megachile rotundata* pupae. Novel, untreated bees were dissected from leaf pods and tracked throughout metamorphosis. Daily visual inspections were performed and four stages were identified: prepupa, pupa, eye-pigmented pupa and sclerotized pupa. An adult female is also included. Descriptions of the characteristics used to identify each stage and corresponding images are listed.

developmental stage	**developmental markers**	**image**
Prepupa	Larval body form, yellow color, wiggling and responsive to touch soon after incubation.	
Pupa	Distinct head, thorax and abdominal segments, meconium is excreted, no color change.	
Eye-pigmented Pupa	Eyes clearly outlined and darkened, starting out cloudy and light pink, and transitioning to a darker reddish brown.	
Sclerotized Pupa	Darkening and hardening of body, darkening is gradual, and this phase is marked when all body segments are visibly darkened compared to larval coloring.	
Adult Bee	Characterized by escape behavior, fully formed wings, yellow markings become clear on the abdomen. Males are identified by their blunt abdomens with two yellow dots on the posterior end and green eyes. Females have black eyes, a pointed abdomen, and a stinger.	

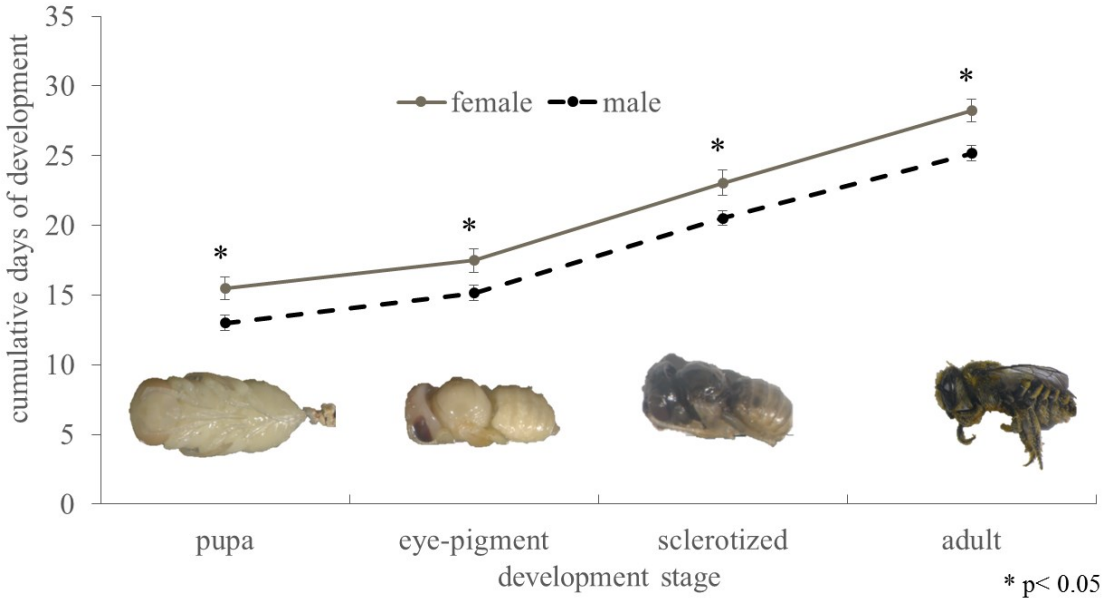

Figure 1. Development times of untreated *Megachile rotundata*. The development stages on the x-axis correspond to the descriptions in Table 3 and the average cumulative days to reach each stage is calculated for males (n = 20; black dashed line) and females (n = 17; gray solid line). Only bees that survived to adulthood were included in this analysis. Asterisks indicate significant differences between males and females at each stage, $p < 0.05$. Data shown are mean ± S.E.M.

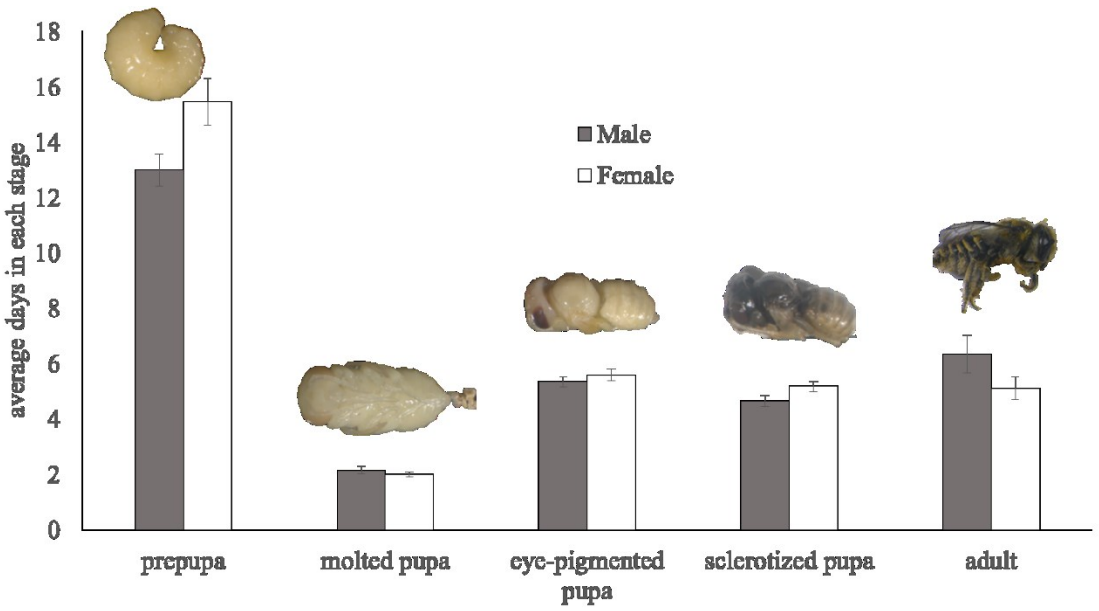

Figure 2. Development times of untreated bees, separated by sex and stage. The average number of days developing bees spent in each defined stage, shown for males (n = 20; gray bars) and females (n = 17; open bars). The largest difference in development timing is seen in the prepupal phase, with females taking more time to transition out of the prepupal body form than males. Data shown are mean ± S.E.M.

2.3.1. Pupal survival assays

To characterize the immune system throughout development, we recorded survival of bacterial infections from bees treated at different stages. Bees injected in the prepupal stages, 1 or 6 days after incubation, had poor survival, with 20% or fewer surviving to adulthood for all bacteria-injected groups (Fig. 3). Bees injected later in pupation were even less likely to reach adulthood, and they had limited ability to develop past the sclerotized stage (Figs. 3 and 4). The prepupal age group showed an effect of treatment on development time at each development stage, starting with the largest development delays reaching pupation, a delay that seems to carry through the rest of metamorphosis. This is shown for day 6 injections in Fig. 5 ($p = 0.023$ for eye-pigmented pupa, $p = 0.002$ for sclerotized pupa) and expanded in Figs. 6-9 ($p = 0.071$ for

molting to pupa, p = 0.008 for reaching eye pigmented pupa, p = 0.002 for sclerotization). For reaching adulthood, there was only a significant effect of bacteria treatment at the day 1 treatment age (p = 0.001). There was also a significant overall effect of treatment on how quickly the bees died (p < 0.001 for time to death). Development delays for the day 14 injected pupar are shown in Figure 10.

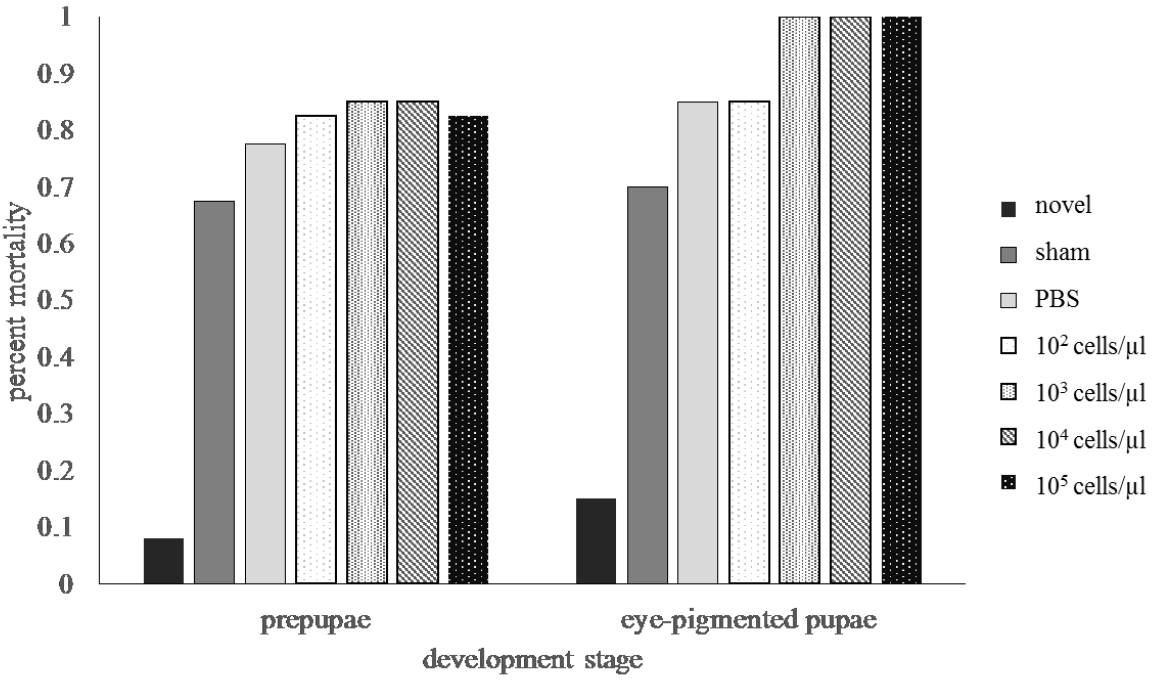

Figure 3. The effect of treatment on prepupal and pupal bee mortality. The percent of bees that succumbed to infection before reaching adulthood is shown. Prepupae bees here were injected on day 1 after initial incubation, and eye-pigmented pupae were injected on day 18 (n = 20 for each treatment group at each age).

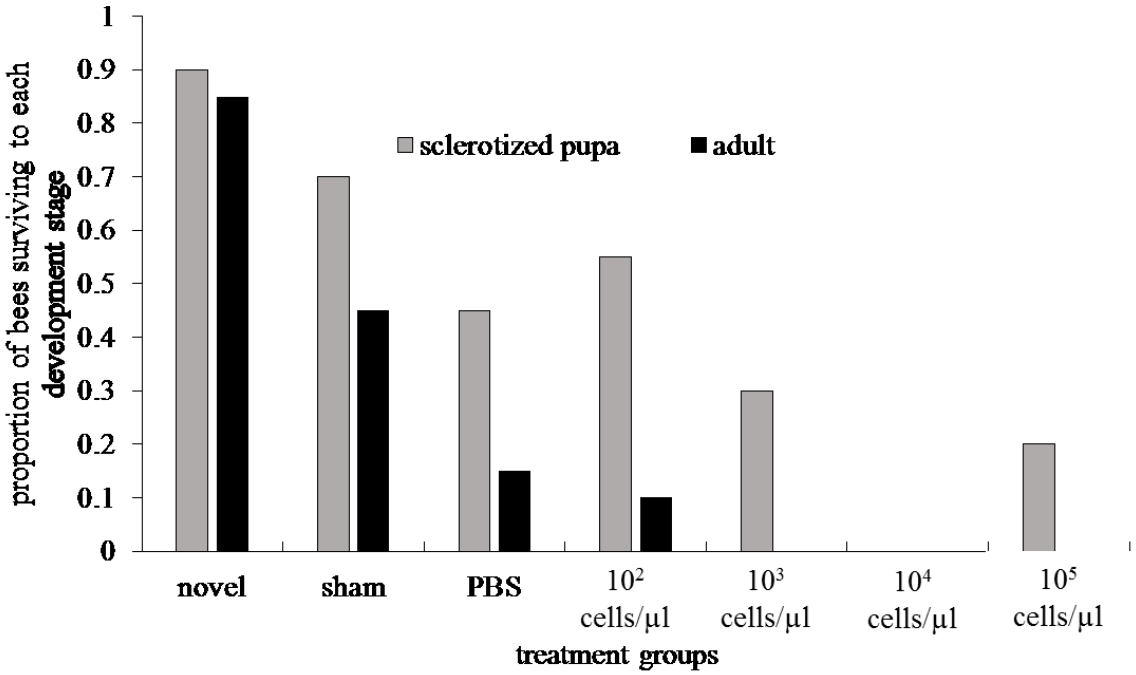

Figure 4. The effect of treatment on pupal bee survival and attainment of development milestones. The proportion of bees injected at the eye-pigmented stage (14 days after incubation) that reached sclerotization and/or adulthood is shown. (n = 20 for each treatment group).

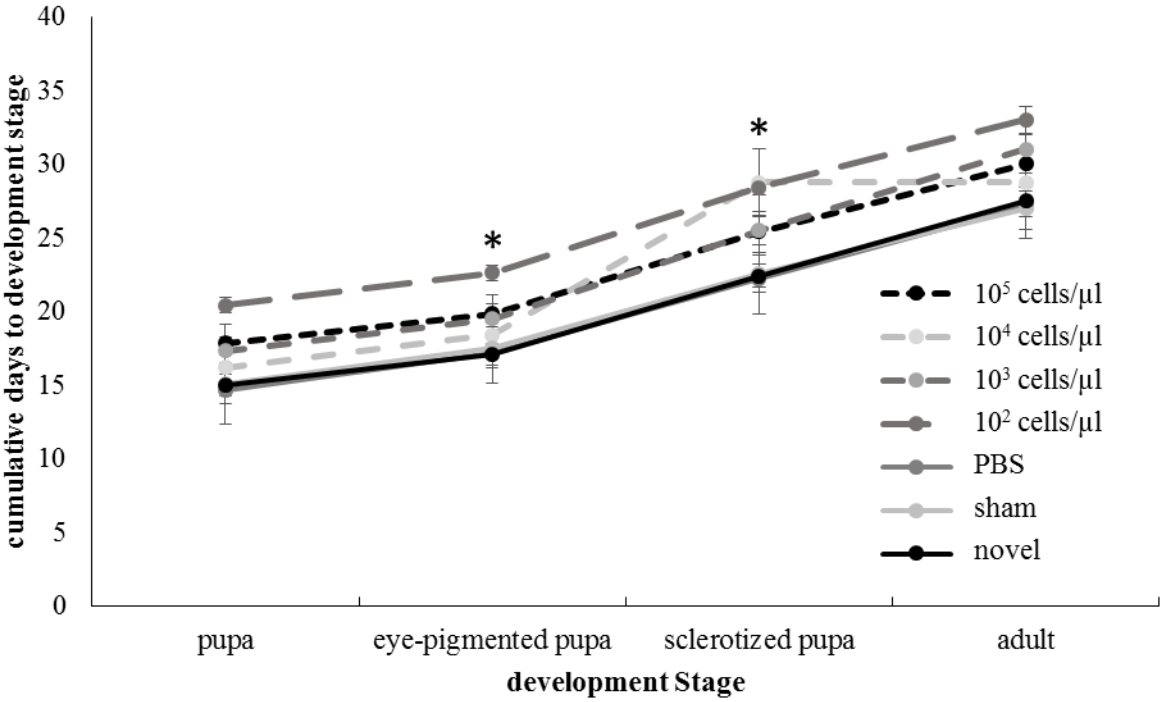

Figure 5. The effect of treatment on overall development timing across milestones. Cumulative development time for prepupae injected on day 6 after incubation at 29°C. . The few bees that survived the treatments to adults (10^2 and 10^4: n =4, 10^3 and 10^5: n = 3) showed developmental delays compared to novel (n = 20), sham (n = 8), and PBS (n = 6) control groups. Asterisks indicate an overall effect of treatment on development at that stage

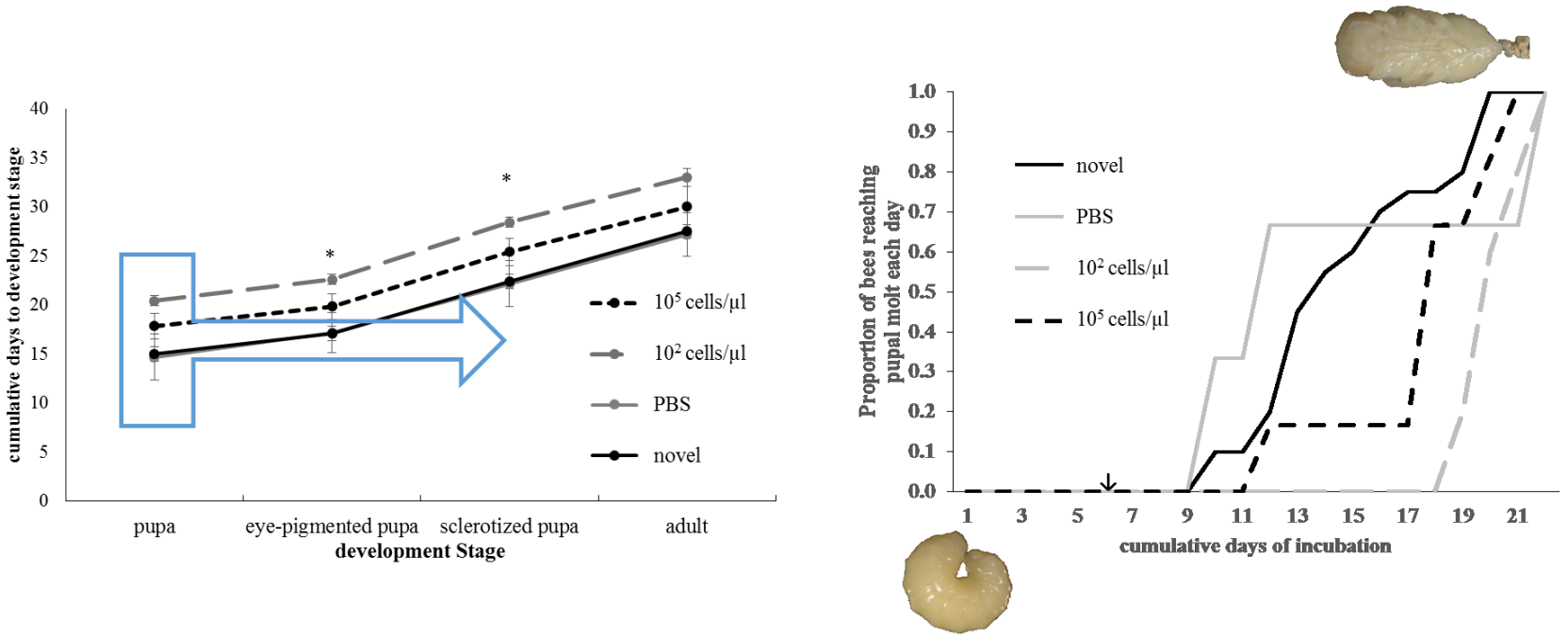

Figure 6. The effect of treatment on how quickly prepupal bees develop to the pupal molt stage. Proportion of bees that survive to pupation remaining in prepupal stage each day of incubation is shown in the right side graph (novel n = 20, PBS n = 6, 10^2 n = 5, 10^5 n = 6), which is shown in context of overall development timing on the left side graph. Treatments here were administered on day 6 of incubation at 29°C.

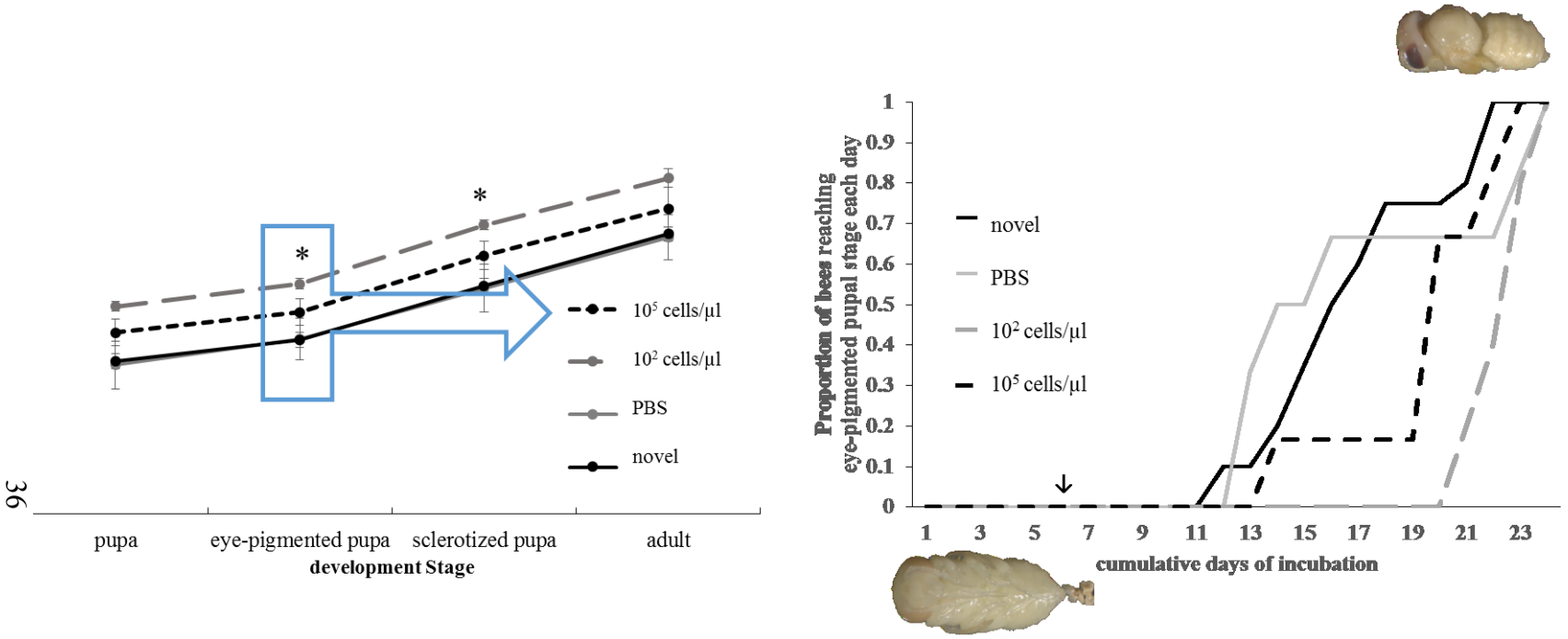

Figure 7. The effect of treatment on how quickly prepupal bees develop to the eye-pigmented pupal stage. Percent of surviving bees that reached eye-pigmentation stage each day of incubation is shown in the right side graph (novel n = 20, PBS n = 6, 10^2 n = 5, 10^5 n = 6), which is shown in context of overall development timing on the left side graph. Treatments here were administered on day 6 of incubation at 29°C.

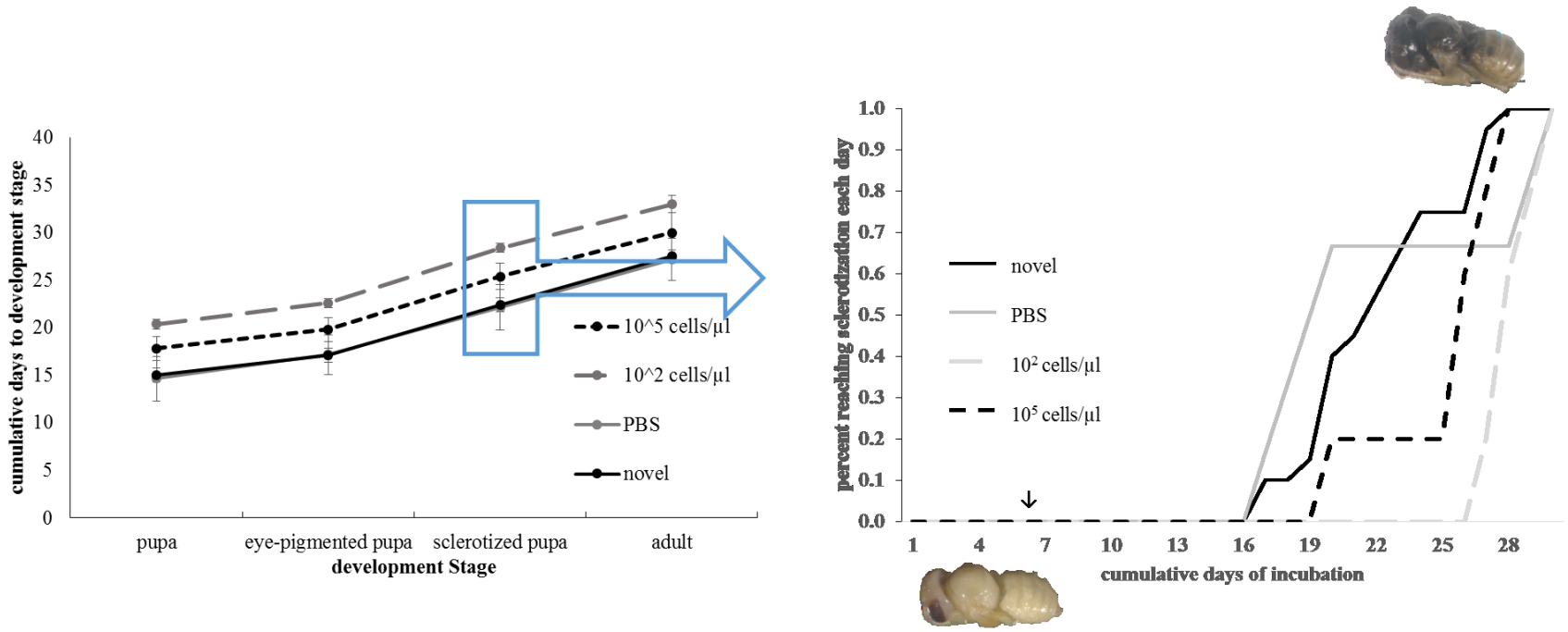

Figure 8. The effect of treatment on how quickly pupal bees develop to the sclerotized pupal stage. Percent of surviving bees that reached sclerotization stage each day of incubation is shown in the right side graph (novel n = 17, PBS n = 3, 10^2 n = 3, 10^5 n = 3), which is shown in context of overall development timing on the left side graph. Treatments were administered on day 6 of incubation at 29°C.

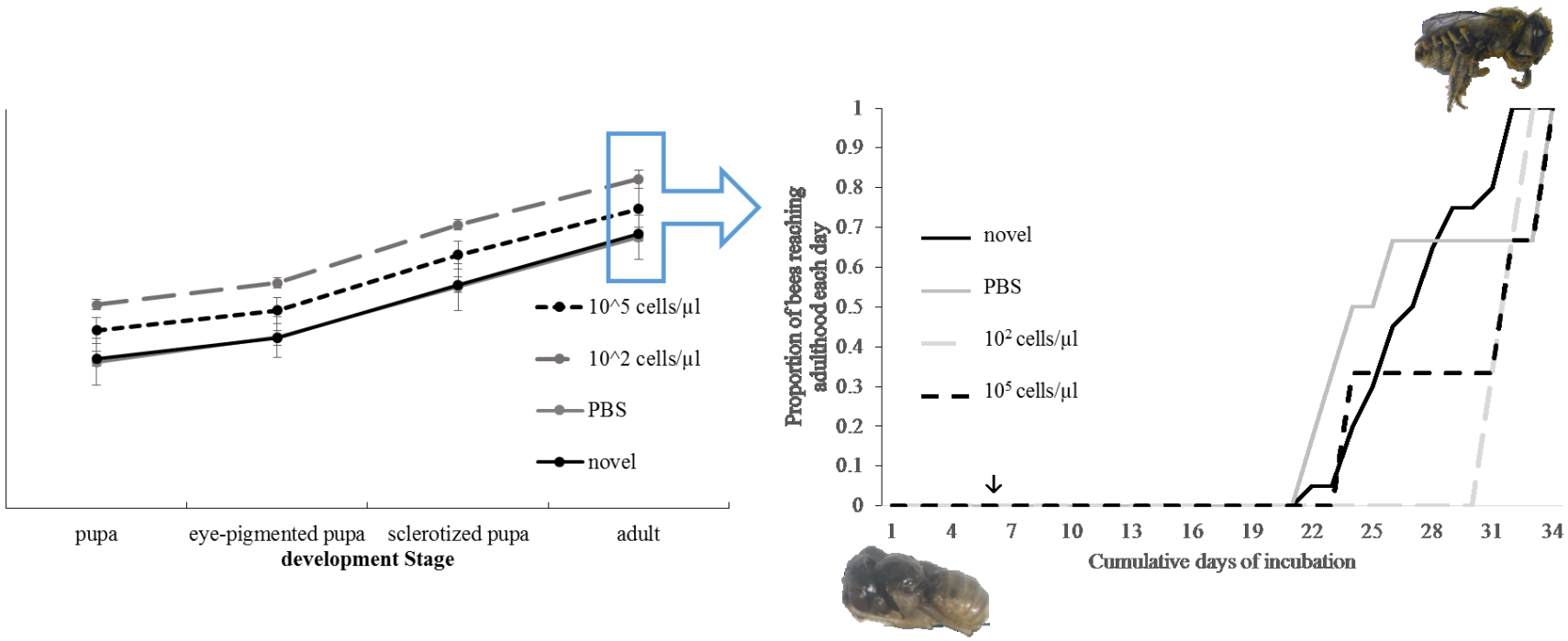

Figure 9. The effect of treatment on how quickly pupal bees develop to adulthood. Percent of surviving bees that reached adulthood each day of incubation is shown in the right side graph (novel n = 20, PBS n = 6, 10^2 n = 3, 10^5 n = 3), which is shown in context of overall development timing on the left side graph. Treatments were administered on day 6 of incubation at 29°C.

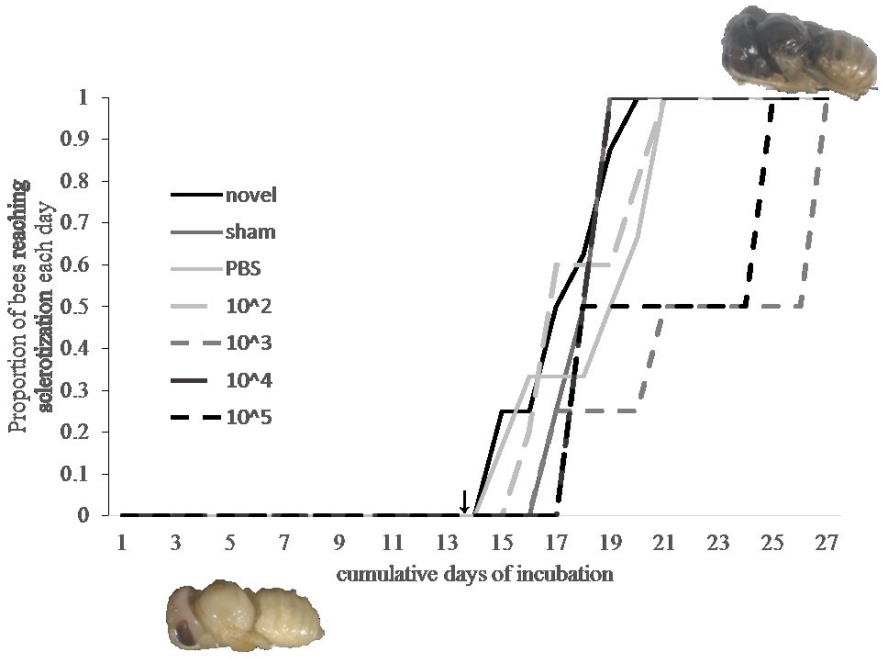

Figure 10. The effect of later treatment on how quickly pupal bees develop to the sclerotized pupal stage. Percent of surviving bees that reached sclerotization stage each day of incubation is shown (novel n = 16, sham n = 5, PBS n = 7, 10^2 n = 6, 10^3 n = 4, 10^4 n = 2, 10^5 n = 2). Small arrow indicates treatments were administered on day 14 of incubation at 29°C. This group had mortality too high to generate the development delay graph that accompanies the other graphs.

2.3.2. Adult survival assays

Adult bees showed much greater ability than younger insects to survive an immune challenge. Females lived longer than males overall ($p < 0.04$, $F_{1, 6} = 6.835$), and average survival time dropped significantly with increasing *E. coli* concentrations (Fig. 11). There was no significant effect of treatment ($p = 0.242$, $F_{6, 1} = 1.821$) if sex were taken into account, but there was an overall effect of sex on survival time ($p = 0.037$, $F_1, 6 = 6.835$). In few cases, bees treated with the highest concentration injections survived up to 30 days (Figs. 11 and 12).

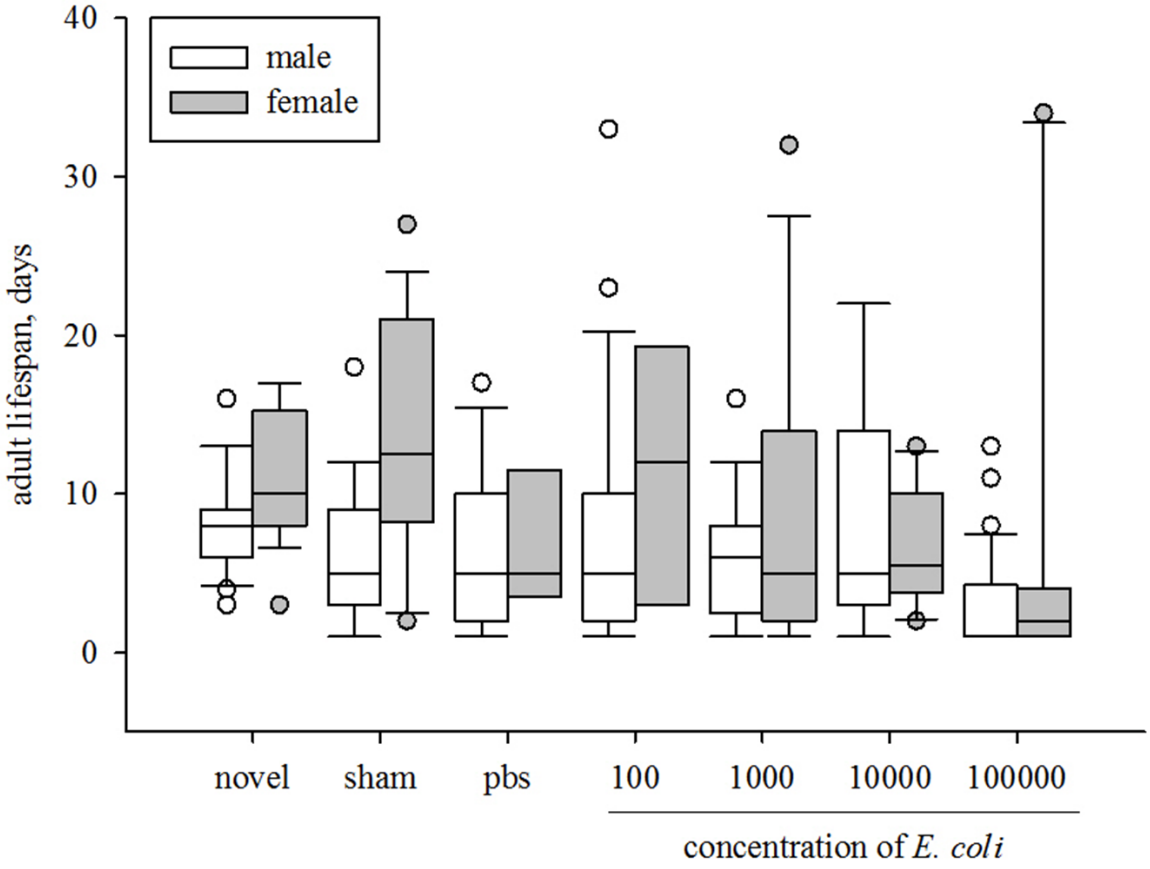

Figure 11. The effect of treatment on average days of adult bee survival, separated by sex. Males and females differed significantly in the novel ($p = 0.008$) and sham ($p = 0.001$) groups

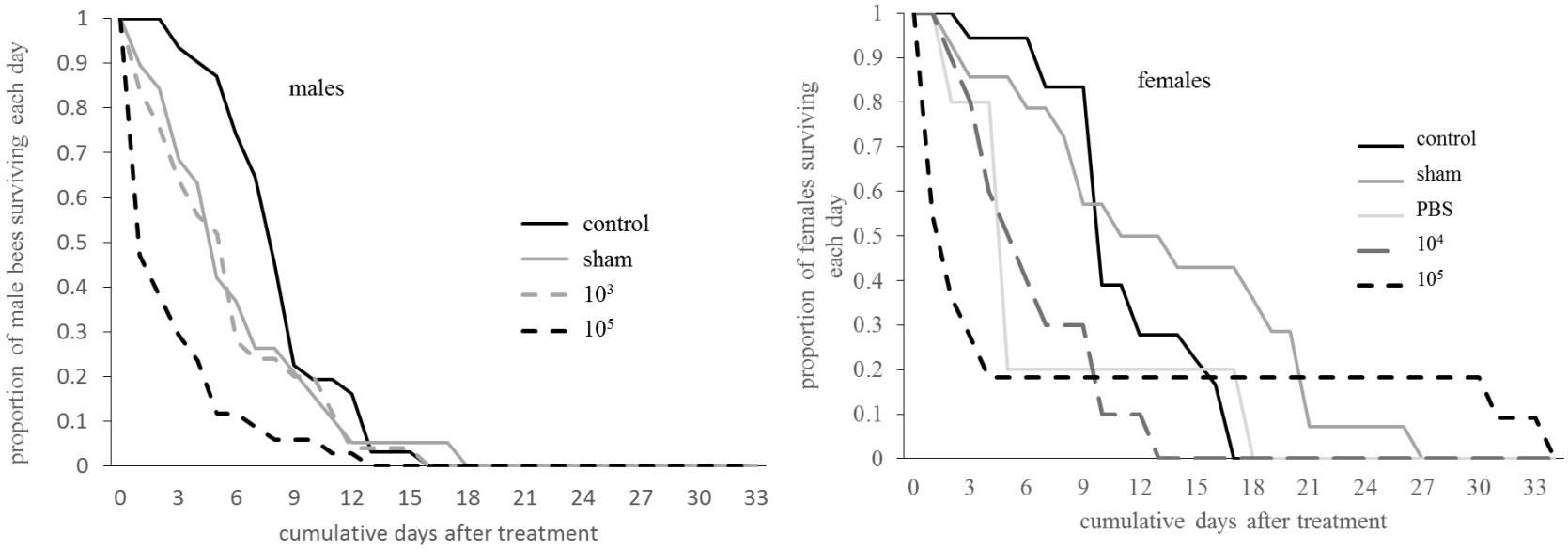

Figure 12. The effect of adult stage treatment on adult bee survival time, separated by sex. A steeper curve indicates a higher mortality rate. All adult bees were treated within 3 days of emergence. For the treatment groups, novel n = 49 (31 male, 18 female), sham n = 33 (19 male, 14 female), PBS n = 32 (27 male, 5 female), 10^2 n = 39 (33 male, 6 female), 10^3 n = 37 (25 male, 12 female), 10^4 n = 39 (29 male, 10 female), 10^5 n = 45 (34 male, 11 female). The left side graph shows males, the right side shows females.

2.3.3. Antimicrobial peptide assays

AMP activity was affected by age (Fig. 13), ($p < 0.001$, $F_{2, 122} = 16.480$). Within the adult age group, there was a significant effect of treatment ($p < 0.001$, $F_{3, 36} = 10.558$). For adults and prepupae, AMP activity was highest when bees were primed with heat-killed bacteria. Injection of PBS also caused elevation in AMP activity for these groups, and for the pupae AMPs were elevated slightly with a sham injection. AMP activity was undetectable in pupal bees, with no effect of treatment ($p = 0.728$, $F_3 = 0.436$). There was also no significant effect of treatment ($p = 0.118$, $F_{3, 39} = 2.084$) in prepupal bees. Although they showed a similar pattern to adult bees in AMP activity, with higher AMP activity among those primed with heat-killed *E.coli* compared to novel and sham groups.

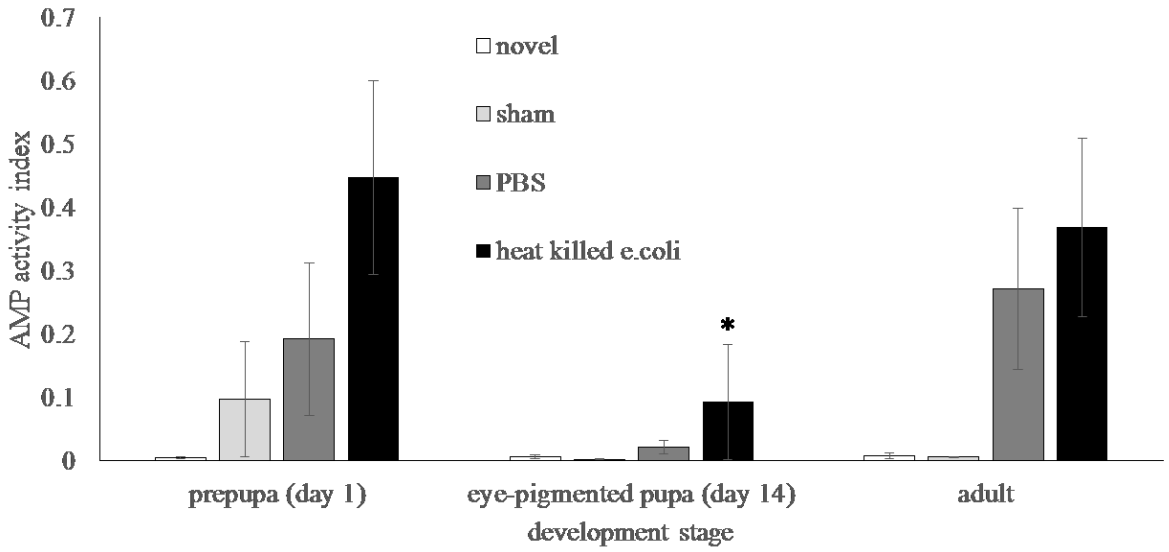

Figure 13. Antimicrobial peptide activity across development stages. The effect of treatment on AMP activity, shown as an index calculated by one divided by the average colony count is shown. A higher index value indicates greater AMP activity. n = 10 for each treatment group of adults, n = 10 for prepupae treated with heat killed *E. coli* and eye-pigmented pupae treated with PBS and sham injections. n = 11 for all other groups. Data shown are mean +/- SE.

2.4. Discussion

We expect immune function to be variable across development in insects and across taxa, due to the variable demands on metabolism and interaction with life cycles, environments, and evolutionary factors. The overall picture of the immune function of *M. rotundata* across pupal development is that it is most vulnerable, immunologically, in the midst of the greatest physiological and morphological changes seen in metamorphosis. We see this trend in both in the pupal bee's inability to survive a live inoculation of a supposedly non-infectious *E. coli* strain (Fig. 3), and in the humoral immune indicator of AMP activity being largely absent in pupal bees (Fig. 13). These juvenile bees are very sensitive to any immune challenge. Pupal and prepupal insects in many species have shown immunological vulnerability, and studies of insects

approaching pupation can also give an idea of what is to come in metamorphosis (Table 3). This is a first description of *M. rotundata's* immune function throughout pupal metamorphosis and will allow for greater understanding of the bee's defenses against infection and when it is most vulnerable. The techniques described here to measure immune function can be used as markers of bee quality and to compare various commercial rearing methods, which is exciting for farmers and academics alike. This is also a novel approach to measuring AMPs in insects with lower activity (compared to model animals like *M. sexta*), which will hopefully transfer to other non-model insects in need of immunological study.

2.4.1. Survival and development of *M. rotundata*

Our hypothesis that for *M. rotundata*, adult bees would have a more robust immune function compared to the relatively sheltered prepupal and pupal bees was supported. The bees treated before and during pupation struggled to overcome infection, with the pupal groups proving to be the most vulnerable. Pupal and prepupal bees that did survive the initial infection exhibited delays in development, though it is worth considering for the apparent development delays during pupation, the number of surviving bees is so low that the development differences we are showing may be attributed to differences between males and females, as we cannot differentiate the sexes until (or unless) they reach adulthood. Pupal and prepupal bees are so vulnerable that even our PBS injections and sham controls caused major mortality, suggesting they are sensitive to any break in the cuticle. It is reasonable to conclude that at pupal life stages, *M. rotundata* relies heavily on the protection of the leaf capsule. These results are consistent with recent studies of honey bees, showing vulnerability to infection and no AMP activity during pupation of worker and drone bees (Gaetschenberger et al., 2013).

Previous studies examining the development of *M. rotundata* used x-ray protocols to track the timing of their development within the leaf capsules every three days (Kemp and Bosch, 2000), which does not show specific timing for pupation, eye-pigmentation, and sclerotization. Our study tracked the bees visually daily and is more detailed in describing development markers. The sexual differences in development timing and survival we observed are also consistent with these previous studies (Kemp and Bosch, 2000), with males developing faster by about three days on average, and adult females surviving an average of three days longer (without treatment). Our methods differ from other studies of *M. rotundata* through development, in that they included extracting bees from their leaf capsules. This accounts for the differences seen in development time to adults for our bees compared to the studies of Kemp and Bosch (2000), which measured actual emergence time from the leaf capsules and found that males and females emerged 33 and 35.8 days after incubation, respectively. Trostle and Torchio (1994) observed that pharate adults remain in the cell capsules with proboscis and wings fully extended for two to three days before the wings harden and the proboscis is retracted just prior to emergence, something we could not see in the absence of the leaf capsule.

Adult survival assays in this study included feeding with 50/50 sugar water, which increased longevity compared previous studies that used longevity without feeding as a measure of vigor (Kemp, 2000). Injections of *E. coli* as an immune challenge were not as immediately deadly to adults compared to pupae, with some adult females living a maximum of 34 days after injection of the highest concentration *E. coli* treatment. In other adult insects, *Aedes aegypti* mosquito exhibits an age associated mortality increase in response to an *E. coli* immune challenge. Hillyer et. al. (2005) attributed this mortality to a decrease in hemocyte count by 24%

in day zero versus day five post adult emergence, which suggests that adult mosquitos may not be able to produce more hemocytes.

Other holometabolous insects have a mixed result when measuring immune responses across development. Pupae of red turpentine beetles, *Dendroctonus valens,* show the highest encapsulation capacity compared to larva and adults, and in contrast, the lowest PO activity (Shi and Sun, 2010). For honey bees, encapsulation doesn't vary across development, but hemocytes are more abundant in pupae compared to adults and larvae, while adults showed the highest PO activity (Wilson-Rich et al., 2008). As insects have such a broad and diverse physiology when it comes to immune responses, making specific predictions is often challenging.

2.4.2. AMP activity

AMP activity is weaker in prepupae than in adult bees, and seemingly absent from eye-pigmented pupae. We used a modified AMP assay as no specific protocol was available for *M. rotundata*. It is worth noting that the AMPs, though clearly present in these bees, are far weaker than what has previously been observed from lab-reared *Manduca sexta* we used as controls. The initial experimental design was a zone of inhibition assay, and though some AMP activity was seen from *M. rotundata* using the assay described by Moret (2000), there was not enough contrast to consistently see differences in treatment groups. The assay also required too much hemolymph or isolated AMP to execute for what we were able to collect from *M. rotundata*. Haine et al. (2008) solved this problem by using a different strain of bacteria, *Arthrobacter globiformis*, which was less resistant to weak AMPs when studying similarly weak AMPs of honey bees. However, we were unable to obtain this bacterium and were compelled to seek other protocols. We tried another experimental design to see if there would be an effect of

AMPs to slow the *E. coli* growth over time by comparing optical densities at multiple time points of the bacteria's growth phase. Isolated AMPs were put into an LB culture *E. coli* in a 96-well plate, and as the bacteria grew over 6 hours, optical density was measured to see any slowed growth rate, but again there was not enough contrast between *M. rotundata* groups. In each of these experimental designs, the *M. sexta* AMPs were extremely effective at eliminating microbes, and in survival assays *M. sexta* injected with the same DH5-α *E. coli* had almost no mortality for the larval caterpillars (Booth, 2013). The protocol that finally worked was successful because the bacteria were able to survive, but not able to grow in the HEPES buffer, and the serial dilution of the *E. coli* allowed for observable differences between *M. rotundata* groups for their capacity to kill the bacteria over 5 hours, when plated out on agar.

Both AMP activity and survival of treatment immune challenges in *M. rotundata* is highest in adult bees, which makes sense in the context of their life cycle. They may be relatively protected in their leaf capsule for their entire larval and pupal phases, so they would not need as robust an immune response until after they emerge as adults. The silk moth *B. mori* has been shown to express AMP activity during pupation in the hard casing of its pupal cocoon (Pandiarajan et al., 2011), and the pupation of *Carabus lefebvrei* involves volatile chemical secretions into their underground chamber as antimicrobial and antifungal defense while they develop (Giglio, 2009). We predict there is AMP activity (or another form of immune defense) present in the leaf capsule itself for *M. rotundata* as it is constructed by the mother. During pupation in the leaf capsule, the bees may have to defend from fungus like chalkbrood (*A. aggregate*) (McManus and Youssef, 1984), as well as parasitoid wasps of the *Melittobia* genera (Woodward, 1994).

AMP activity is just one measure of immune function in *M. rotundata*, but the pattern holds that when we see the least AMP activity is when we see the lowest survival of an immune challenge (Figs 3 and 4). AMPs are produced as a result of immune system activation, by the presence of bacteria or fungal infection (Hoffmann et al., 1995), but during pupal development of *M. rotundata*, those infection signals did not elicit a response (Fig 13). During the critical time of pupation, no AMP activity was detected, suggesting that the pupation process may be too energetically costly for the bees to also mount an immune response. This is similarly the case with the ground beetle *Carabus lefeburie*, which has the lowest phenoloxidase (PO) activity in its pupal phase (Giglio and Giulianini, 2013). *M. sexta* caterpillars also have shown a decrease in cellular defense responses as they transition to wandering stages before pupation, with significantly lower PO, AMP, and nodulation activity, and fewer hemocytes compared to the early 5th instar larvae (Eleftherianos et al., 2008). In the wax moth *G. mellonella*, PO activity peaks at the larva to pupa molt and at the transition from pupa to adult, but the lowest PO levels also occurred during pupation (Benesova et al., 2009).

2.4.3. Conclusion

The results of this study are intriguing due to *M. rotundata's* importance as a pollinator and study organism and encourages further examination of this bee's immune function and ecoimmunology. In the context of commercial rearing, categorizing the immunological development of *M. rotundata,* and in particular, describing any developmental vulnerabilities they suffer during pupation informs both farmers and researchers. Future studies investigating improvements in rearing techniques can use these result and methods as markers of quality. Any

improvement in survivability of commercial bees will make *M. rotundata* more effective and efficient as a key pollinator species.

Further questions outside the context of commercial rearing remain. For example, we recorded little to no AMP activity during pupation: which other immune defenses must take over until the adult bees emerge and produce effective AMPs once more? In the context of insect immunology and development, examination of the evolutionarily conserved innate immune system may play a role in understanding immunity for vertebrates, and could even ultimately be applied to human medicine. The more we know about this bee's physiology, life cycle, and ecological interactions, both in the wild and commercially, the better equipped we will be to deal with increasing challenges of climate change, growing demand for pollinated foods, and the need for insects to play a larger role in the lives of humans.

2.4.4. Future directions

Additional study of *M. rotundata's* physiology will allow for biological markers that can be used to optimize rearing practices for this commercially important pollinator. To have a full picture of the bee's development of immunity, the larval phase of *M. rotundata* should be examined to find its ability to survive an immune challenge and develop to adulthood, as well as measuring AMP activity. Further immunity studies across development stages of *M. rotundata* could be used as indicators of immune function. These methods could also be used to test the effect of toxins such as pesticides and herbicides the bees are likely to encounter in pollinating situations for commercial farms, as well as in the wild.

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