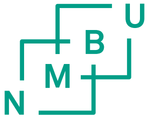

Norwegian University of Life Sciences
Faculty of Chemistry, Biotechnology and Food Science

Philosophiae Doctor (PhD)
Thesis 2019:52

Physiological features of aging in honey bees (*Apis mellifera*)

Fysiologiske egenskaper ved aldring i honningbier (*Apis mellifera*)

Eva Marit Hystad Byhrø

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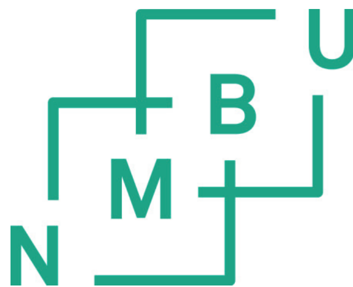
Ås (2019)



Thesis number 2019:52
ISSN 1894-6402
ISBN 978-82-575-1611-6

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Acknowledgments

The work in this thesis was financially supported by the Research Council of Norway and the Norwegian University of Life Sciences (NMBU). The work and experiments were carried out at the Faculty of Chemistry, Biotechnology, and Food Sciences (NMBU), Department of Medical Biochemistry (University of Oslo), School of Life Sciences (Arizona State University), and Faculty of Environmental Science and Natural Resource Management (NMBU).

I would like to express my gratitude to all those who gave me the opportunity to start and complete this thesis. I want to thank my great main supervisor, Prof. Gro V. Amdam, for accepting me as a PhD student. Together with Dr. Daniel Münch and Dr. Heli Salmela, you have been the best supervisor group I could have. I thank the three of you for your enthusiasm, encouragement, knowledge, wisdom, and for seeing opportunities where I find limitations. I am very grateful for your efforts and guidance through all these years. In addition to our common interest in honey bees, I must mention that we are very coordinated in having children. Altogether, seven children were born during my period as a PhD student.

I would also like to thank Lars Eide for guiding and supporting me through the first paper; Claus Kreibich for managing beehives, your support, and coffee breaks; and Erik Rasmussen and Jane Ludvigsen for always being helpful, positive, and such good colleagues. In addition, I would like to thank Ying Wang at ASU and Ana Vitlic for being part of the Arizona stay and as co-authors. I am also thankful to other people who were included in the group for being supportive and positive and exchanging knowledge.

I thank KBM and especially Wenche and Laila in administration for always being welcoming and answering all kinds of questions. Thanks to the Imaging Centre and research groups at KBM for keeping the doors open to use their machines; my work has been dependent on your hospitality. I thank the people at MINA for welcoming me when our lab moved, especially Kaja for coffee without cholesterol and that our paths crossed once more. I would also like to thank everyone else I met in labs or colleagues for supporting or helping me in one way or another.

I thank Denofa AS and my new colleagues in the quality department for being patient with me in my new role and in the final phase of my PhD.

I would like to thank my supportive family. I am endlessly thankful to my parents for everything. Together with my parents-in-law, I thank you for cheering me on, your time, and help. I thank my grandmother for being proud and supportive. I am thankful to my brothers, the rest of the family, and friends for being supportive and reminding me of who I am.

Joakim, you are my beloved husband on good and bad days. Thank you for your enthusiasm for science and your great patience. I could never manage this without you. To our sons, Felix Johan and Ulrik Olai, I thank you for reminding me what life is really about.

Skjeberg, April 2019
Eva Marit Hystad Byhrø

Abstract

Aging and a decline in physiological resilience occur in most living organisms, including honey bee workers. Aging research in different organisms—from yeast, worms, insects, and mice to humans—has investigated several causes. Aging is explained by different theories, often separated into programmed aging and stochastic-damage-caused aging. Programmed aging suggests that aging is controlled by a biological timetable, gene regulation, or a program plan. Stochastic-damage-caused theories explain aging by an accumulation of random damages in organs or cells. In addition, immunity and disease defense are reduced by increased age, together with learning ability and other cognitive functions. In aging studies, it can be advantageous to use model organisms to identify evolutionarily conserved mechanisms.

Model organisms offer the opportunity to perform controlled experiments, whole-lifespan studies, and experimental intervention with mortality as a direct readout. In this thesis, honey bee workers are used as the model organism. They have an interesting division of labor, where a young worker bee usually starts as nurse bee, which cleans and takes care of the brood within the hive. After a few weeks, she usually transfers to foraging activities to collect nectar, pollen, and water in the field. In honey bee workers, their physiological age and resilience are related to worker caste, where nurses are the physiologically youngest and foragers the physiologically oldest phenotype. A third caste of worker is the winter bees (*diutinus*), which are the most stress resilient phenotype and the longest-lived.

Through my thesis project, I have studied the three worker castes of the honey bee to in more depth. My research goal was to help build a better understanding of their phenotypic differences. One interesting finding in this thesis is that very old forager bees can be more resilient than younger forager bees. On the other hand, my work also demonstrates that nurses have better cellular immunity than foragers and winter bees, thereby supporting the idea that worker bees' aging is more dependent on their tasks than on chronological age, and that aging is most pronounced in foragers and less so in nurses and winter bees. The findings illustrate that aging patterns can be difficult to explain by a single theory or framework, since I can conclude that the most aged phenotype, the foragers, can be both resilient and fragile.

Abstrakt

Aldring og nedgang i fysiologisk motstandsdyktighet inntreffer i de aller fleste levende organismer, inkludert honningbienes arbeidere. Aldringsforskning har undersøkt flere årsaker i ulike organismer, fra gjærsopp, ormer, insekter, mus til mennesker. Aldring kan forklares av flere teorier hvor de ofte blir separert i programmert aldring og stokastisk-skade forårsaket aldring. Programmert aldring antyder at aldring styres av en biologisk klokke, genregulering eller en programplan. Stokastisk-skade forårsakede teorier forklarer aldring ved en akkumulering av tilfeldige skader i organer eller celler. Ved økende alder reduseres immunitet og motstandsdyktighet mot sykdommer, sammen med evnen til å lære og andre kognitive funksjoner. I aldringsstudier kan det være fordelaktig å bruke modellorganismer for å identifisere evolusjonært konserverte mekanismer.

Modellorganismer gir oss muligheten til å utføre kontrollerte eksperimenter, studere livsløps og utføre eksperimentelle inngrep med dødelighet som en direkte avlesning. I denne avhandlingen brukes honningbier som modellorganisme. De har en interessant arbeidsfordeling, hvor en ung arbeiderbie vanligvis begynner som en ammebie som rengjør og tar vare på larvene i bikuben. Etter noen uker går hun over til å være en trekkbie, dette innebærer å samle nektar, pollen og vann. En arbeiderbies fysiologiske alder og motstandsdyktighet har en sammenheng med arbeiderkasten den tilhører, hvor ammebiene er en fysiologisk yngre fenotype og trekkbiene er fysiologisk eldre. En tredje arbeidertype er vinterbier (*diutinus*), denne er den mest motstandsdyktige fenotypen mot stress og er den lengst-levende.

I denne avhandlingen har jeg studert de tre arbeiderkastene til honningbier i dybden. Mitt forskningsmål var å bygge en bedre forståelse av de fenotypiske forskjellene. Et interessant funn i denne avhandlingen er at svært gamle trekkbier kan være mer motstandsdyktige enn yngre trekkbier. På en annen side viser arbeidet at ammebier har bedre cellulær immunitet enn trekk og vinterbier og derfor støtter ideen om at arbeiderbienes aldring er mer avhengig av deres oppgave enn kronologisk alder, og at aldring er mest uttalt i trekkbier og mindre i amme og vinterbier. Funnene illustrerer at aldringsmønstre kan være vanskelig å forklare ved en enkelt teori eller rammeverk, ettersom at jeg kan konkludere med at den eldste fenotypen, trekkbiene, kan være både motstandsdyktige og sårbare.

List of papers

I. Hystad, E. M., Amdam, G. V., and Eide, L. (2014). "Mitochondrial DNA integrity changes with age but does not correlate with learning performance in honey bees." *Experimental Gerontology* 49: 12–18, doi.org/10.1016/j.exger.2013.10.013.

II. Hystad, E. M., Salmela, H., Amdam, G. V., and Münch, D. (2017). "Hemocyte-mediated phagocytosis differs between honey bee (*Apis mellifera*) worker castes." *PLoS ONE* 12(9): e0184108, doi.org/10.1371/journal.pone.0184108.

III. Byhrø, E. M. H., Salmela, H., Vitlic, A., Wang, Y., Munch, D., and Amdam, G. V. "Different activation of immune related genes in honey bee nurse and foragers (*Apis mellifera*)." *Manuscript is submitted and under revision.*

List of abbreviations

AMP	Antimicrobial peptides
IMD	Immune deficiency
Jak-stat	Janus kinase-signal transducer and activator of transcription
JNK	Jun N-terminal kinases
LPS	Lipopolysaccharide
PAMP	Pathogen-associated molecular patterns
PGN	Peptidoglycan
ROS	Reactive oxygen species
Vg	Vitellogenin (protein)
vg	Vitellogenin (gene)

1 Introduction

1.1 Physiological resilience and aging

Physiological resilience is defined as the organism's ability to respond to stressors and is often seen in relation to frailty and aging. Physiological resilience is expected to decrease and frailty to increase with age beyond adulthood. Both parameters can provide insight into predicting the health and physiological age of organisms (Blokh & Stambler 2017; Kirkland et al. 2016; Pollock et al. 2015; Schorr et al. 2018). With increasing age, accumulation of damages or dysfunctions in molecules, organelles, individual cells, or tissue organs may occur (reviewed by Gems & Partridge 2013; Kirkwood 2005; Lees et al. 2016). These changes, which can be seen systematically in the whole animal, lead to functional disorders such as a loss of cognitive function, reduced fitness, decrease in physiological functions, and reduced disease resistance and are related to increased frailty (reviewed by Lees et al. 2016; Lopez-Otin et al. 2013). The organism's resistance to such changes is known as physiological resilience.

Aging has fascinated many researchers, and many theories have tried to explain its causes. Aging theories can be separated into programmed aging and stochastic-damage-caused aging (Jin 2010). Programmed aging theory suggests that there is a biological timetable, gene regulation, or program plan that causes aging. The stochastic-damage-caused aging theories, such as the free radical theory of aging, suggest that accumulation of random damages in organs and cells causes aging (reviewed by Liochev 2013). An alternative theory related to the stochastic-damage-caused aging theories is the disposable soma theory of aging (Kirkwood 1977; Kirkwood 2005), which suggests that organisms have a limited amount of resources and that it is a tradeoff among cellular processes such as growth, reproduction, and DNA repair. It suggests that the organism prioritize maintenance of the reproductive tissue to inhibit damages in the germ line that will be inherited by the next generation. The somatic tissue survives only one generation; accumulated damages will therefore not be inherited due to limited maintenance (Kirkwood 1977; Kirkwood 2002).

1.1.1 Cognitive functions and aging

Aging affects cognitive functions such as learning and memory (reviewed by Anstey & Low 2004; Lees et al. 2016; Piotti et al. 2018; Teri et al. 1997). There are various forms of learning, for example, active learning and associative learning (reviewed by Waldrop et al. 2015; Wolff et al. 2015). Within associative learning, we find classical conditioning, also referred as Pavlovian learning (reviewed by Anselme 2015). Invertebrates can show associative learning in laboratory tests, where insect research has provided insights into mechanisms and genes in the brain involved in learning by classical olfactory conditioning (reviewed by Busto et al. 2010; Glanzman 2005). There are genetic and anatomical similarities between insects and mammals that can give us valuable insights into predicting aging and pathologies in humans by studying insects (reviewed by Davis 2004; Roman & Davis 2001). A decrease in memory and cognitive impairment may be a part of normal aging but can also be due to pathological reasons such as Alzheimer's disease in humans (reviewed by Antoniou & Wright 2017; Burke & Mackay 1997; Grady & Craik 2000).

1.1.2 Mitochondrial aging and genomic damage accumulation

In addition to being linked to age, a decline in cognitive functions is associated with an increase in oxidative stress (Haider et al. 2014) and reduced mitochondrial function (reviewed by Clay Montier et al. 2009; Falkenberg et al. 2007). Mitochondria are the respiratory organelle and main generator of ATP, which is used as chemical energy required in a cell. In production of ATP via the oxidative phosphorylation, reactive oxygen species (ROS) is also formed as a byproduct (Harman 1956). The mitochondrial genome is compact and circular double-stranded, which is organized into nucleotides associated with the inner mitochondrial membrane (Garrido et al. 2003). The mitochondrial genome encodes 24 RNAs and 13 subunits of the electron transport chain complexes that are essential for oxidative phosphorylation. The rest of the proteins a mitochondrion needs (between 1000 and 1500) are encoded by the nuclear genome (reviewed by Park & Larsson 2011). The number of mtDNA copies in a mitochondrion varies between two and ten (reviewed by Smeitink et al. 2001). In contrast to replication of the nuclear DNA, replication of the mitochondrial genome is independent of the cell cycle. The mtDNA copy number must be kept sufficient to preserve mitochondrial homeostasis. Mitochondrial biogenesis comprises the processes that vary mitochondrial mass, number, and size depending on the energy requirement of a cell

(reviewed by Jornayvaz & Shulman 2010). Variations in mtDNA copy number and mitochondrial mass are associated with disease and aging (reviewed by Clay Montier et al. 2009; Diaz & Moraes 2008; Lee & Wei 2005; Wallace 2010).

There are many studies that have observed increased levels of oxidative damage and ROS by increasing age and therefore support the free radical theory of aging, also known as the mitochondrial free radical theory of aging (reviewed by Barja 2004; Sanz 2016). Together with other free radicals produced endogenous cells, ROS and exogenous threats from outside the cell can lead to genomic instability (reviewed by Moskalev et al. 2013; Wallace 2010). Exogenous threats include x-rays, chemicals, and UV radiation, can, for example, lead to mutations or DNA breaks (reviewed by Hoeijmakers 2009; Larsen et al. 2005; Lord & Ashworth 2012). Genomic instability also includes changes in nucleobases and movement of transposable elements, deletions, and duplications in the genome (reviewed by Falkenberg et al. 2007; Wang et al. 2017). These threats can lead to accumulation of damages to cellular components, as well as point mutations, translocations, and gene disruption (Lopez-Otin et al. 2013). To eliminate damages, organisms have evolved mechanisms to repair nuclear and mitochondrial DNA (reviewed by Kazak et al. 2012; Lord & Ashworth 2012). Mitochondrial DNA is more vulnerable to damage than nuclear DNA and has a higher mutation rate, most likely due to the proximity to the production site of ROS, in addition to a lack of protective histones and a limited DNA repair system (reviewed by Barja 2004; Brown et al. 1979; Kregel & Zhang 2007; Richter et al. 1988). Aging and age-related neurodegenerative diseases can be linked to an accumulation of mtDNA damage and mutations (reviewed by Jeppesen et al. 2011; Mancuso et al. 2009).

1.1.3 Aging and changes in proteins

With increasing age, proteins of different types can be affected in various ways. Some proteins have antioxidant properties; like other antioxidants, they neutralize free radicals and help the organisms to eliminate detrimental internal damages. A decrease in antioxidant activity is common with aging and is therefore followed by increased oxidative stress (Mergener et al. 2009). In *Drosophila melanogaster,* the expression of antioxidants can extend lifespan in transgenic lines (Sun & Tower 1999; Sun et al. 2002). ROS can change proteins by carbonylation and glycoxidation, which can reduce their activity or alternate functions (Fernando et al. 2018; Gonos et al. 2018; Gruber et al.

2011). Additionally, impairment of proteostasis (protein homeostasis) can lead to an aggregation of proteins, accelerate aging, or lead to age-related diseases (Bagherniya et al. 2018; Labbadia & Morimoto 2015; Lamech & Haynes 2015). Proteostasis is the control of protein synthesis, folding, conformational maintenance, and degradation (Glick et al. 2010; Klaips et al. 2018; Meijer & Codogno 2004; Myung et al. 2001; Powers et al. 2009; Xie & Klionsky 2007). In humans, an aggregation of proteins is involved in diseases like Alzheimer's and Parkinson's (reviewed by Cohen et al. 2006; Cuanalo-Contreras et al. 2013; Powers et al. 2009).

1.1.4 Immunity and immunosenescence

Diseases can be caused by inner and outer factors. Previous sections mainly focus on inner factors that increase the individual's frailty, which can increase the risk of disease. In resistance against outer threats, the immune defense is important for eliminating pathogens. The first line of immune defense includes physical barriers such as the skin and mucous membranes in the respiratory system and the gastrointestinal tract. Physical barriers together with the immune system protect the organism against pathogens and harmful agents. With increasing age in adults, the efficiency of these physical barriers is reduced, which increases pathological exposure, making the individual less resistant against disease (reviewed by Castelo-Branco & Soveral 2014; DeVeale et al. 2004). The second line of immune defense is the immune system, which includes immune cells, tissue, and organs. However, there are differences between the immune system in invertebrates and vertebrates, mainly in that invertebrates such as insects rely on innate immunity with its predetermined receptor specificity, while vertebrates have an additional adaptive component with specific immunoglobulin triggered by the antigen from the invading pathogen (reviewed by Boehm & Swann 2014; Koonin & Krupovic 2015; Rinkevich 1999).

In insects, the key immune regulators are the fat body (a loose adipose tissue with multiple metabolic functions) and the circulating hemocytes (blood cells). The fat body is a production site of humoral effector molecules, also called antimicrobial peptides (AMP) (reviewed by Garschall & Flatt 2018; Zasloff 2002), while hemocytes are involved in phagocytosis, melanization, and encapsulation (reviewed by Lavine & Strand 2002; Marmaras & Lampropoulou 2009). However, to separate the immune system into humoral and cellular immunity can be artificial as these are interrelated and act

together in the defense against pathogens (reviewed by Hultmark 2003; Lemaitre & Hoffmann 2007).

Humoral immunity

The humoral immune component of insects acts mainly via AMPs, molecules that accumulate in hemolymph via the circulatory system of insects, defending the host against invading pathogens. Insects have four signaling pathways associated with immune response: Toll, immune deficiency (IMD), Janus kinase-signal transducer and activator of transcription (JAK-STAT), and Jun N-terminal kinases (JNK) (Evans et al. 2006). Innate immune responses are activated by structures in the pathogens' surface (pathogen-associated molecular patterns, often shortened as PAMPs) recognized by pattern-recognition receptors (reviewed by Hoffmann 2003; Hultmark 2003; Lemaitre & Hoffmann 2007; Marmaras & Lampropoulou 2009). When pattern-recognition receptors bind to PAMPs on the pathogen, the immune system activates signal cascades to produce AMPs or activate cellular responses (reviewed by Garschall & Flatt 2018; Hoffmann 2003; Min & Tatar 2018; Zasloff 2002).

Cellular immunity

There are different systems of classification of hemocytes in insects. Some hemocytes share morphology and features between species. Others are not comparable to each other or not identified in all insects. Additionally, studies have used different conditions to classify the hemocytes, which can make comparisons between studies more difficult. In Lepidoptera (Butt & Shields 1996; Stoepler et al. 2013), Hemiptera (Joshi & Lambdin 1996), and *Anopheles* (Hillyer & Christensen 2002), five main types of hemocytes can be distinguished based on morphological features. In general, plasmatocytes are the encapsulating cell type (Negri et al. 2014), and some report that it is a phagocytic (engulfing pathogens or particles) cell type (reviewed by Lavine & Strand 2002; Ribeiro & Brehelin 2006). However, granulocytes are considered the main phagocytic cell type (reviewed by Lavine & Strand 2002; Ribeiro & Brehelin 2006). Oenocytoids contain phenoloxidase, an enzyme involved in melanization (reviewed by Hillyer & Christensen 2002; Lavine & Strand 2002). It has been suggested that spherule cells transport cuticular elements, and prohemocytes are considered as hemocyte stem cells (reviewed by Lavine & Strand 2002). In contrast, the model insect *D. melanogaster* has three immune cell types identified, with a different way of naming the cell populations as

compared to other insects. Based on cellular features, their cell types are plasmatocytes (phagocytic cells), crystal cells (involved in melanization), and lamellocytes (encapsulating cell type) (Kounatidis & Ligoxygakis 2012; Ribeiro & Brehelin 2006). This, of course, complicates the comparability of immune studies between *D. melanogaster* and other insects.

Circulating hemocytes in adult insects are formed in the hematopoietic glands at larval and pupal stages (Akai & Sato 1971; Gardiner & Strand 2000; Jung et al. 2005). There has been a traditional thought that new hemocytes are not formed in adult insects. However, a few studies have shown that adult insects recruit new hemocytes by mitosis (Amaral et al. 2010; King & Hillyer 2013).

Immunosenescence

The immune system's decline in immune function and competence during aging is called immunosenescence, which occurs in both innate and adaptive immunity. Disorders in the immune system can cause reduced immunity, leaving the organism more prone to diseases. Interestingly, the decline in immune functions also leads to a poorer response to vaccination in elderly humans (Roukens et al. 2011; Sasaki et al. 2011). The immune system is reduced by having both fewer immune cells and less effective immune reactions (reviewed by Kurz & Tan 2004; Mancuso et al. 2018; Sadighi Akha 2018). For example, in humans, the phagocytic capability in macrophages and neutrophils are reduced (reviewed by Shaw et al. 2013; Ventura et al. 2017). The macrophages also produce less ROS, and, following, their ability to kill an invader decreases (reviewed by Müller & Pawelec 2014; Shaw et al. 2013). Nevertheless, some immune functions remain unaffected by age or even show an increase in activity. For example, the number of natural killer cells increases by age in humans. However, their functions per cell seem to decrease, and the higher cell number is explained as a compensatory mechanism to maintain levels of functionality (Hazeldine et al. 2012). In *D. melanogaster*, aging is associated with an upregulation of some immunity-related genes but not all (Felix et al. 2012; Zerofsky et al. 2005).

1.1.5 Model organisms in aging research

In aging research, there are many advantages with usage of model organisms as they are controllable and can help one to avoid many of the ethical challenges met with usage of humans. A good model organism must have a relatively short lifespan so that one can follow the individual from birth to death. Many cellular pathways are conserved between animal phyla and share similar properties, which is a criterion of a good research model (reviewed by Lee et al. 2015; Lees et al. 2016). Advantages of insects as models are that they show aging traits and their lifespan makes them effective models. In comparison to humans (who live for years), the lifespans of insects are measured in weeks. Insects are relatively easy to control and manipulate, and they are cheap to grow and hold. Additionally, the model organism can be interesting to study. Advantages of using the fruit fly *D. melanogaster* and worm *C. elegans* are that they have genomic databases and genetic tools (reviewed by Lees et al. 2016). Silkworm moths and honey bees (*Apis mellifera*) are relatively large-sized insect, so one can analyze single individuals (reviewed by Lee et al. 2015). Social insects, such as honey bees and ants, differ according to their social caste and not only genotype, whereby their phenotypical age can be reversed (reviewed by Lee et al. 2015). The plasticity of aging makes honey bees an ideal organism for longevity studies and is the subject of this thesis.

1.2 Honey bees as a research model for physiological resilience and aging

Honey bees show many aging traits like other animals, including humans, and are a widely used model organism for aging studies. They are so-called eusocial insects with flexible aging patterns and have different traits in long-lived and short-lived individuals. This can challenge and extend the understanding of health, resilience, and aging (Amdam et al. 2005; Herb et al. 2012; Huang & Robinson 1996). Considering the disposable theory of soma (Kirkwood 1977), it can be presumed that worker bees are the soma and the queen is the reproductive. Thus, honey bee society invests more in maintenance of the queen rather than each individual worker bee (Corona et al. 2007; Haddad et al. 2007). In the context of programmed aging, honey bees can be considered as a system, with the transition from nurses to foragers as part of the program plan for aging.

1.2.1 Honey bee biology

The honey bee is kept as a domestic animal for its honey and wax production and is an economically important pollinator. Pollination of fruits and other flowering plants by honey bees and wild pollinators give higher yields to and therefore increase the economic value of many plant crops. Honey bees are generalists, meaning that they forage for and pollinate many different crops (Seeley 1995). In recent years, they have come into focus due to colony losses, known as colony collapse disorder (CCD). This phenomenon was described for the first time in spring 2007. CCD caused winter losses of 24 to 36 percent in 2006 and 2007 (vanEngelsdorp et al. 2007 ; vanEngelsdorp et al. 2009). It is characterized by the disappearance of worker bees, where only few dead bees are found in the hive or the apiary. Several factors have been investigated for involvement, including pathogens and diseases, monocultures (leading to poor nutrition), and pesticides (Alaux et al. 2010; Di Prisco et al. 2013; Nazzi & Pennacchio 2018). However, the findings cannot point out one single cause for CCD, which is most likely due to a number of co-occurring events (reviewed by vanEngelsdorp et al. 2017).

Honey bees live in colonies, each consisting of ten to fifty thousand worker bees headed by a reproductive queen (Winston 1987). Queen and worker phenotypes, including lifespan potential, are determined by larval diet. While larvae that develop into queen bees are continuously fed with royal jelly, worker bee larvae are initially fed royal jelly but later they are primarily fed pollen and honey (Snodgrass 1910). The diet has an influence on epigenetic mechanisms and other pathways that determine whether the larvae develop into a queen or worker bee (Kucharski et al. 2008; Lyko et al. 2010; Spannhoff et al. 2011).

Queens typically live two to five years, whereas worker bees live twenty to forty days in summer and up to six to eight months in winter (Amdam 2011; Maurizio & Hodges 1950; Rueppell et al. 2007; Visscher & Dukas 1997). After worker bees emerge from their cells, they usually carry out tasks within the colony as nurse bees, whereby they perform larval care, cleaning, and comb building (fig. 1). After two to three weeks, they start foraging for nectar, pollen, propolis, or water in the field (Seeley 1995). As a forager bee, she has an expected lifespan between seven and eighteen days (Neukirch 1982; Visscher & Dukas 1997). In temperate climates, in absence of a brood, worker bees develop into a third worker type during late summer and autumn, the so-called winter bees (*diutinus*). Winter bees are long-lived bees that support the colony and queen by

temperature regulation during wintertime (reviewed by Amdam 2011). Typically, they show low juvenile hormone titers and high accumulation of vitellogenin (Vg) (Fluri et al. 1977; Smedal et al. 2009). In spring, they conduct nursing and foraging tasks for the colony until they die (reviewed by Amdam 2011). Physiologically, foragers are the aged phenotype, independent of chronological age. Their older physiological age is shown in that they have fewer intact immune cells (Amdam et al. 2005), are more vulnerable to starvation (Speth et al. 2015), have more oxidative damage (Seehuus et al. 2006b), and have lower stores of lipids and protein than nurses (Remolina et al. 2007; Toth & Robinson 2005). Remarkably, honey bees have the ability to postpone and accelerate aging depending on their phenotype (reviewed by Amdam 2011; Elekonich & Roberts 2005; Münch & Amdam 2010).

Figure 1: A) The colony is headed by a single reproductive queen. B–F) The 10,000 to 50,000 faculty sterile worker bees usually start as nurses (B) before they progress to cleaning the nest (C), building activity (D), or guarding the hive (E). Usually they end their lives by foraging nectar, pollen, water, and propolis in the field (F). (Figure adopted from (Rangberg et al. 2012)

This aging plasticity is demonstrated by the ability to revert tasks and physiological aging, whereby a forager can revert into a nurse bee and perform nursing tasks within the colony (Amdam et al. 2005; Herb et al. 2012; Huang & Robinson 1996). In addition, flight restriction has been shown to increase lifespan and maintain cognitive functions in foragers (Rueppell et al. 2007; Tolfsen et al. 2011).

Interestingly, honey bee colonies can be manipulated by establishing a colony with newly emerged bees of the same chronological age, a so-called single cohort colony (Huang & Robinson 1992; Toth & Robinson 2005). In these colonies, some worker bees turn into precocious forages that show an earlier onset of foraging, including receiving aging features (fig. 2). Other worker bees develop into typical nurse bees. In such

colonies, it is important to remove the brood to hinder new recruitment of more bees into the colony, which can lead to a worker-type switch (Münch et al. 2013).

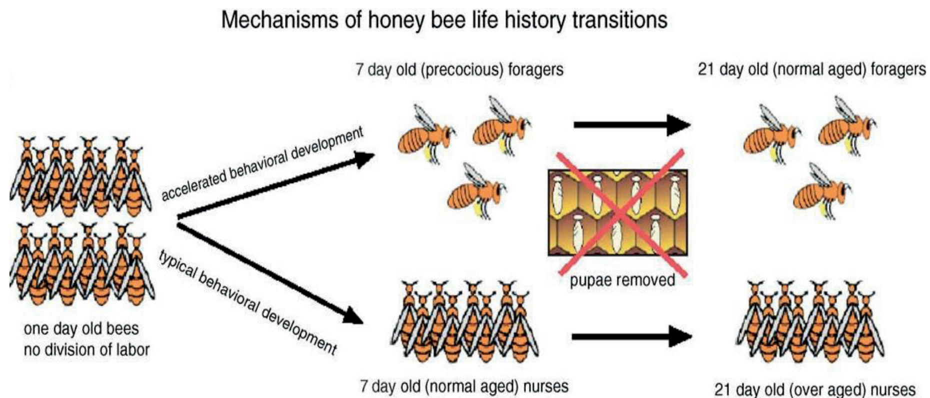

Figure 2: Development of the worker bees in a single cohort colony. The colonies are established with newly emerged bees only, and all worker bees therefore have the same chronological age. To fulfill all tasks required within a hive, some worker bees develop into precocious foragers; others follow the typical behavioral development into nurses. The pupae are removed to hinder a switch of worker bees. After twenty-one days, the foragers have a normal chronological age for a forager bee, and the nurse bees are overaged compared to a colony with natural switch. (Figure adapted from (Elekonich & Roberts 2005)

1.2.2 Cognitive aging in honey bees

Cognitive functions and learning ability are very important traits for a forager bee to succeed in her foraging trips (reviewed by Menzel et al. 2006). These abilities must mature as precocious foragers have increased mortality risks in their foraging flights (Perry et al. 2015). A precocious forager orientates poorer than a mature forager orientates and performs poorer when the environment changes (Ushitani et al. 2016). The forager uses landmarks and the sun to orient during its flights from and back to the hive again (von Frisch & Lindauer 1956; Winston 1987). The honey bee is a powerful model for cognitive functions as it can learn to associate different odors with sugar rewards. However, old foragers and infected foragers show a decline in learning capability (Behrends et al. 2007; Gage et al. 2018). A poorer learning performance is one of the aging symptoms in forager bees (after fifteen days of flight) (Behrends et al. 2007; Munch et al. 2010; Scheiner & Amdam 2009).

1.2.3 Mitochondrial aging, ROS, and instability in aging honey bees

During flights, forager bees have a high need of energy, and a high energy production leads to higher ROS production (Beenakkers et al. 1984). There are findings that support the free radical theory of aging in honey bees, for example, accumulation of proteins with oxidative carbonylation (Seehuus et al. 2006b) and accumulation of lipofusin (oxidation product of unsaturated fatty acids) in hypopharyngeal glands (Munch et al. 2013) due to aging.

Mitochondrial DNA are less intact in forager bees than in nurses and queen bees (Aamodt 2009), and mtDNA content decreases with age (Weinrich et al. 2017). Interestingly, a study by Cervoni et al. has shown that the respiration rate by mitochondria is lower in foragers than in nurses, although they have similar numbers of mitochondria (Cervoni et al. 2017). This is controversial compared to earlier findings in that energy needs can be expected to be higher in flying foragers, followed by an increased ROS production. Similarities are detected in *D. melanogaster* as the respiration rate decreases (Brandt et al. 2017; Weinrich et al. 2017) and the ROS production increases with age (Weinrich et al. 2017). The decrease of respiration in *D. melanogaster* is most likely due to inactivate mitochondria in the aged flies (Brandt et al. 2017). In *C. elegans* and mice, there are findings that increased ROS does not affect their lifespan or that oxidative damage accelerates aging (Doonan et al. 2008; Gruber et al. 2011; Van Remmen et al. 2003; Zhang et al. 2009). Similar findings are also seen in honey bees as oxidative damage is not always accumulated with age (Cervoni et al. 2017; Tolfsen et al. 2011; Williams et al. 2008). There can even be higher oxidative stress damage in nurses compared to foragers, measured by protein carbonylation and lipid peroxidation (Cervoni et al. 2017).

However, multiple data is contrary with the free radical theory of aging. In the same study where they detected a decrease in respiration rate in *D. melanogaster* with age, they could not find the same in aging mice (Brandt et al. 2017), indicating that the causes of aging can be different between species.

1.2.4 Aging and changes in protein in honey bees

As mentioned in the section "Mitochondrial aging, ROS, and instability in aging honey bees," ROS can lead to carbonylation of proteins. Carbonylated proteins are accumulated with age in foragers (Seehuus et al. 2006b), and an increase in carbonylated proteins is also found in aging *C. elegans* (Gruber et al. 2011).

Vg is an interesting honey bee protein in the context of aging. It is a phospholipoglyco protein primarily known for its function in egg-yolk formation. Its expression is higher overall in queens than worker bees, but Vg is also expressed in non-egg-laying worker bees. This multifunctional protein is involved in the worker bees' aging and immunity. It is found to function as an antioxidant in adult worker bees, where elevated Vg levels are associated with prolonged lifespan (Amdam et al. 2004b; Corona et al. 2007; Salmela et al. 2015; Seehuus et al. 2006b). Vg is expressed higher in young worker bees compared to old workers (Rueppell et al. 2017). The regulation of Vg is negatively affected by juvenile hormone. Titers of juvenile hormone increase in the transition from nurse to forager bee, and this increase is associated with a decrease in Vg (Fluri et al. 1977; Huang & Robinson 1992; Huang & Robinson 1996). This relationship has been shown by *vg* gene knockdown (RNAi), where juvenile hormone titers in worker bees increase in *vg* knockdown bees (Guidugli et al. 2005). Worker bees with their *vg* knocked down initiate foraging earlier in life than control bees (Ihle et al. 2010; Nelson et al. 2007). In winter bees, juvenile hormone titers are low (Huang & Robinson 1995), and Vg levels are relatively high, which is associated with their long lifespan (Amdam et al. 2003; Amdam et al. 2004a; Smedal et al. 2009).
The honey bee Vg is also found to bind to dead bacteria (Salmela et al. 2015), and is recently suggested to be a part of an immune-priming pathway by that bacterial fragments are transported to the hypopharyngeal glands (glands that are used to feed queen and larva with royal jelly) in worker bees that express Vg (Harwood et al. 2019). In addition, Vg has been shown to have antimicrobial and antioxidant properties in *Apis cerana*, the Asiatic honey bee (Park et al. 2018) Interestingly, Vg's functions in immune defense are reported in fish. For example, Vg is an immunologically active, acute phase protein that is rapidly upregulated after injection of bacterial components (Tong et al. 2010). Additionally, Vg functions as an opsonin, which enhances macrophage phagocytosis and causes lysis of bacteria (Li et al. 2009; Liu et al. 2009; Tong et al. 2010).

Proteostasis and aging in honey bees are not a well-studied network. Proteostasis is the control of protein synthesis, folding, conformational maintenance, and degradation. Heat shock proteins and unfolded protein response (UPR), identified in honey bees, are parts of the proteostasis network in response to stressors (Johnston et al. 2016; McKinstry et al. 2017). Honey bees express heat shock proteins that protect against

increasing temperatures and impact protein folding (McKinstry et al. 2017). However, these studies did not investigate aging in the proteostasis network.

1.2.5 Immunosenescence in the honey bee

As mentioned, the decline of the immune system during aging is known as immunosenescence. The honey bee, with its flexible aging patterns, offers the opportunity to study aging of the immune system (Amdam et al. 2004b; Amdam et al. 2005).

There is a limited number of efforts in the literature to classify honey bee hemocytes. Part of the challenge in this classification is the low level of morphological differences between the cell types (de Graaf et al. 2002; Marringa et al. 2014; Price & Ratcliffe 1974). However, one can use the descriptions from other insects or count total number of hemocytes. Hemocyte counts in adult honey bees are effects of labor division and parasite infection, which support an active regulation, similar to Vg abundance (Amdam et al. 2004a; Marringa et al. 2014; Wilson-Rich et al. 2008). The number of hemocytes in honey bees is found to decrease with age (Schmid et al. 2008) and in transition from nurse to forager bees (Amdam et al. 2005; Fluri et al. 1977). Additionally, larger portions of the hemocytes are pycnotic and therefore nonfunctional (Amdam et al. 2005) and lacking nodulation reaction in foragers (Bedick et al. 2001).

One fascinating characteristic of immunosenescence in honey bees is its connection to the behavioral role and dependence on social caste, seen in the fact that reversion of foragers to nursing tasks restores the numbers of previously lost functional hemocytes (Amdam et al. 2005; Muench 2010). In *D. melanogaster*, the percentage of phagocytic cells is lower in older flies, and the number of hemocytes decreases in aging females (Mackenzie et al. 2011). A decrease of hemocytes with age is also observed in mosquitos (Castillo et al. 2006). Other cellular immune functions, like phenol oxidase activity, increase in overaged nurses compared to younger ones (Schmid et al. 2008).

Interestingly, despite demonstrating all four signaling pathways associated with immune response—Toll, IMD, JAK-STAT, as well as JNK—the known number of immune-related genes involved in these pathways in honey bee is only one-third of what is described in e.g., *Drosophila* and *Anopheles*. The lower number of immune-related genes could be due to an incomplete annotation of the honey bee genome or a functional

difference with genes or responses that do not exist or are unidentified in other species (Evans et al. 2006). A study that compares summer and winter bees found that the latter have relatively lower expression of immune-related genes (Steinmann et al. 2015). Additionally, some studies have found that honey bee foragers have a greater expression of immune-related genes (Vannette et al. 2015). Also, *D. melanogaster* show higher expression of antimicrobial effector genes in aging flies (Eleftherianos & Castillo 2012). In addition, there are several immunity studies of adult honey bees of unknown ages investigating gene responses after different infections such as *Varroa destructor* (Doublet et al. 2017), *Nosema ceranae* (Chaimanee et al. 2013; Doublet et al. 2017; Huang et al. 2016), and *Escherichia coli* (Gaetschenberger et al. 2013).

Nutrition has additional effects on immunity, antioxidant levels, and DNA methylation in worker bees. For example, the substances curcumin and caffeine seem to have a positive effect on longevity and immunity in honey bees (Strachecka et al. 2014; Strachecka et al. 2015). Another study examined five- and ten-day-old caged bees fed with polyfloral and monofloral diets. It found that hemocyte concentration, but not phenol oxidase activity, increased with a polyfloral diet (Alaux et al. 2010).

In addition to individual immunity, honey bees have social immunity, which involves hygienic behavior and grooming to resist diseases and survive (reviewed by Cremer et al. 2018; Evans & Spivak 2010). Pathogen-treated bees tend to spend more time on grooming and foraging than control bees (Natsopoulou et al. 2016). Somatic maintenance is reduced in foragers and following reduced stress resilience and lower survival (Seehuus et al. 2006b; Speth et al. 2015). The start of foraging can be an energy saving strategy for the colony, where it prioritizes the energy and resources on maintenance of the society rather than individuals.

2 Aim of the study

My research goal of this thesis is to help build a better understanding of the phenotypic differences between worker castes of the honey bee. The main aim is to improve understanding of mitochondrial parameters and cellular and humoral immunity in honey bee workers and to link results to the different aging rates and the physiological resilience observed in worker bee castes. The first part of this thesis, presented in paper I, uses young and old forager bees and investigates their learning performances and mitochondrial parameters. As previously known, it is an important trait for forager bees to learn and be able to orientate in the field. The objective is to assess the learning capability and foraging experience and to relate this to the mitochondrial DNA intactness, mitochondrial damage, and gene expression. The second part, presented in paper II and III, continues focusing on different honey bee workers and their distinct age phenotypes. Paper II, therefore, uses the three worker types—nurse, forager, and winter bees—to investigate their differences in immunity by phagocytosis and Vg levels in hemocytes and to find out whether the young phenotype, old phenotype, or winter bees have the best immune parameters. Paper III questions whether the phenotype—nurse or forager—of similar chronologically aged bees could influence the response to immune challenges, monitoring their survival after such a challenge and their gene expression of immune-related genes and *vg*.

3 Methods

3.1 Methods overview

The main topic of this PhD thesis is aging and physiological resilience, and the methods used in the three papers can be separated into in vivo (whole organism) and in vitro (cellular or molecular) experiments. In the former, bees were emerged in an incubator in a laboratory, and the adult honey bees were handled by marking and collecting them from their hive to ensure investigation of the specific phenotype with the right age for the in vitro experiments. This experimental design is essential for the best comparisons in studies using honey bees as a model. In the in vitro experiments, PCR, confocal microscopy, and flow cytometer were used to investigate different parameters.

3.2 Learning performance and mitochondrial aging

3.2.1 Design of experiment

In paper I, only outdoor hives were used. Honey bee foragers were marked on their returning flight back to the outdoor hives. Marking was avoided during their orientation flights, as these are training flights for younger bees that have not yet become foragers. The foragers were recognized by their marks and collected when they had >5 and >15 days of flight experience. A forager that has >5 days of foraging experience is considered a young mature forager bee, and after >15 days of foraging, she is considered an old forager bee. In this in vivo experiment, foraging experience is critical in the comparison of forager bees. After collection, the foragers were brought into the laboratory for learning tests, followed by brain isolation for molecular analysis.

3.2.2 Learning tests for honey bees

Honey bees can learn to associate different odors to a sugar reward. Prior to the learning test, their gustatory responses to different sugar concentrations were measured to know their motivation toward sugar as a reward. In the learning test, the odor is presented with a following sugar reward. If she sticks out her tongue the next trial when the odor is presented, she has learned to associate the odor with a sugar reward. She is given the sugar reward combined the odor anyway, however, she is then given a lower learning score (fig. 3). This session was repeated six times, and she received a learning score based on how many times she stuck out her tongue. If the forager stuck out her tongue five times, she was scored as a good learner. Poor learners needed more learning

trials to learn to associate the odor with a sugar reward; indeed, some foragers do not even learn at all. However, poor and good learners are usually found in every colony (Scheiner et al. 1999; Scheiner et al. 2003). Before mitochondrial analyses, the foragers were separated into four groups: young foragers with poor or good learning capacity and old foragers with poor or good learning capacity.

Figure 3: Showing how a bee learns to associate an odor with a sugar reward. A) The bee is presented an odor from a syringe; she does not respond by sticking out her tongue. B) The odor is paired with a sugar reward. C) The bee has learned to associate the odor with a sugar reward and sticks out her tongue before the sugar is presented.

3.2.3 Polymerase chain reaction

For this thesis, polymerase chain reaction (PCR) was used for both DNA and RNA analyses. DNA and RNA isolation require different methods, and therefore different kits were used (for details, see papers I and III).

In paper I, the DNA integrity method is dependent on good DNA quality without damage introduced by handling and DNA isolation. To estimate damage, the PCR method amplifies a 10 kb fragment and relates it to the same amount of amplification of a smaller fragment. Amplification of the 10 kb fragment is inhibited by DNA damage in the sequence, and the relation to the smaller fragment gives an indication of the mtDNA intactness. In this protocol, low extension temperatures were used to succeed in amplification of a large 10 kb product as the mitochondrial genomes in honey bees are very A-T rich (Crozier & Crozier 1993). It has previously been reported that A-T–rich genomes are sensitive to high extension temperatures; therefore, lower temperatures for extension are suggested as routine for such genomes (Su et al. 1996). The amplified 10 kb fragment is quite large and gives a good estimate of mtDNA damage but not a good estimate rate. An additional mtDNA damage assay was therefore established and included, where mtDNA damage rate in a restriction enzyme site by Taq1 could be estimated. Taq1's restriction enzyme site consists of the four nucleobases with the sequence 5' T-C-G-A. A mitochondrial copy number varies between cell types but can also give an indication of pathology or health in similar cell types, and this was also

analyzed. After establishing the assays, a positive control for mtDNA damage was included; however, the mitochondrial copy number was not included in this. Paraquat, known to induce oxidative damage, was injected into the bees (Seehuus et al. 2006b; Tawara et al. 1996) and killed them in a dose-dependent manner. Furthermore, as expected, the paraquat exposure reduced the integrity of brain mtDNA and increased damage rate in the Taq1 restriction site assay.

3.3 Immunity and aging

3.3.1 Design of experiments

In paper II, the experiments were performed during wintertime to allow simultaneous testing of summer and winter bees. Winter bee colonies were transferred into a flight room to induce summer activities. To induce foraging activity, feeders with pollen and 30 percent sucrose were provided. Newly emerged bees were marked and introduced to the colonies; ten to twenty days later, they were collected as mature nurse bees. Foragers were marked during foraging flights and had an unknown chronological age as they were former winter bees. On the collection day, they had been foraging for at least three days. Previous studies considered foragers to be mature when they have at least five days of flight and foraging experience. However, it was difficult to collect enough foragers with this length of foraging experience in the flight room. This limits the direct comparability with studies using regular free-flight, outdoor setups. Yet using an artificial flight room to simulate summer conditions has a significant advantage, that is allowing to directly compare summer- like worker types and regular winter bees collected from outdoor hives. Our approach hence overcomes disadvantages of longitudinal collection schemes, that compare summer and winter phenotypes from population at very different time points (seasons).

In paper III, single cohort colonies were used, meaning that all worker bees had a similar age. On day twenty-one to twenty-two, bees were collected and injected with bacterial cell wall components to activate their immune systems before monitoring their survival and gene response.

3.3.2 Characterization of hemocytes

The naked eye has limitations in resolving power, and microscopy is used to magnify the specimen for study (fig. 4).

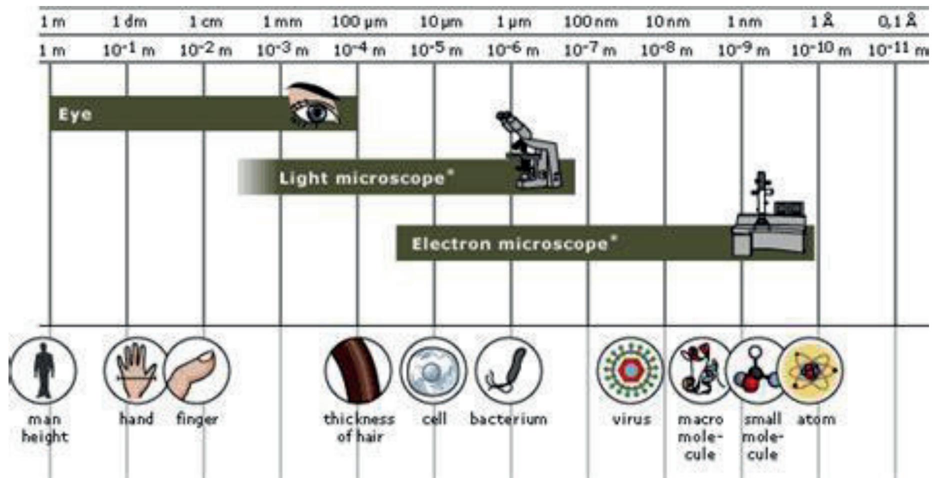

Figure 4: An overview of different resolving power in the naked human eye, light microscopy, and electron microscopy (figure adapted from www.nobelprize.org)

Usage of light microscopy is a relatively easy method as it utilizes visible light and lenses to magnify the object. Confocal laser scanning microscopy allows detecting fluorescence signals in a three-dimensional space at – for light microscopy - very high resolution. The detected fluorescence can originate from a fluorophore-labeled marker (e.g., an antibody) or from auto fluorescence. The comparability high resolution and three-dimensional image volume recording with confocal microscopes is based on blocking out-of-focus light which produces in-focus images from selected depths in a biological sample (Cullander 1998; Nwaneshiudu et al. 2012). In contrast, electron microscopy uses electrons instead of light to detect structures and therefore has the potential for greater resolution (since the wavelength of an electron is smaller than a photon). In this work, confocal microscopy was used to detect hemocytes in the hemolymph of honey bees. The laser beam excites the fluorophore, and light is emitted at a higher wavelength that can be detected by the detectors and is specific for each fluorophore (Nwaneshiudu et al. 2012).

Like a microscope, the flow cytometer can detect cells based on optical features. In contrast to the microscope, the flow cytometer is a high throughput quantification method that rapidly analyzes and distinguish large cells numbers based on certain characteristics, such as size and specific staining. Results are often given as plots and not images (Picot et al. 2012). Briefly, in flow cytometers each cell or particle passing

through one or more beams of focused light (Snow 2004). Specific light scattering and fluorescence emission provides information about the individual properties of an object. For details, see paper II.

4 Summary of Papers

4.1 Cognitive functions and mitochondrial aging

4.1.1 Paper I: Mitochondrial DNA integrity changes with age but does not correlate with learning performance in honey bees

The aim of this published paper is to investigate mitochondrial parameters in young and old forager bees with known learning performance. We measured their learning performance and tested their brain tissue for mitochondrial parameters with different PCR methods. We compared honey bees that had >5 days and >15 days of foraging experience and found no significant difference in copy number of mitochondrial DNA between the groups. When we compared the mtDNA integrity in our groups, we identified a significant difference between the young and old forager bees. However, to our surprise, the old foragers had a higher mtDNA integrity (amplification of a large mtDNA fragment) than the young foragers. These results could be related to the resilience in old foragers as >15 days of foraging is relatively old for a forager bee. As mentioned, the average foraging span is eight to seventeen days of foraging (Neukirch 1982; Visscher & Dukas 1997). Our finding could indicate that only the most resilient foragers survive >15 days of foraging. However, we were not able to detect significant differences between young and old foragers in the second PCR method, which detects damage and mutation rate in the site of a selected restriction enzyme. Our results indicate that accumulated oxidative damage and ROS are not the determinant of age in honey bees; based on them, we cannot support the notion that oxidative damage is the major cause of aging. However, it should be noted that we did not investigate levels of ROS by itself, only indirectly as integrity and damage rate. Interestingly, we could not find a difference in learning capacity and mitochondrial DNA damage in forager bees. Learning is a very important ability in forager bees. However, our results indicate that the mitochondrial parameters we investigated are not relevant in learning capacity in honey bee foragers.

In addition, we investigated gene expression of three genes encoded by the mitochondrial genome. However, we could not identify a difference in expression between our groups of the ND1 gene, CytB gene, or ribosomal L-RNA in the honey bee's brain. A limitation of the study is that we did not analyze all genes expressed by the

mitochondrion, so whether other mitochondrial genes have an influence remains unknown.

4.2 Immunity and aging

4.2.1 Paper II: Hemocyte-mediated phagocytosis differs between honey bee (*Apis mellifera*) worker castes

In this published article, we focused our studies on honey bee immunity and vitellogenin in nurses, foragers, and winter bees. To study honey bee immunity, we established a microscopy assay to observe hemocytes. We focused on phagocytic hemocytes as phagocytosis is an important immune process not sufficiently studied in honey bees. We adapted two established methods from *D. melanogaster* and *Anopheles gambiae* to quantify phagocytosis. We labeled the cell's nucleus by DAPI and the f-actin, which is a part of the cellular cytoskeleton, by fluorescently labeled phalloidin. For measuring the phagocytosis, we injected fluorescent-labeled beads or CM-Dil stain into the worker bee's abdomen. The fluorescently labeled beads or CM-Dil were then taken up by free-floating phagocytic hemocytes, which were thereafter extracted from the worker bee. By usage of confocal microscopy, both staining methods were found to have equal uptake by the hemocytes. We chose to continue with latex beads only as they were easier to handle in the flow cytometer. Furthermore, we tested whether injection of *E.coli* could change phagocytic rate, but our results did not demonstrate this.

We found that nurses have higher rates of phagocytic hemocytes than forager and winter bees. These findings match previous ones in other insects as immunosenescence has been observed as a decline in hemocyte numbers and phagocytosis in, for example, fruit flies and crickets (Horn et al. 2014; Mackenzie et al. 2011; Park et al. 2011). There has also previously been shown an increase of pycnotic, nonfunctional hemocytes in honey bees (Amdam et al. 2005).

Earlier studies have connected the protein Vg to immunity in honey bee workers as it is a zinc carrier (Amdam et al. 2004b). Zinc is a trace mineral critical for proper functioning of white blood cells in mammals (Mocchegiani et al. 2000). We wanted, therefore, to investigate Vg's relationship to hemocytes, specifically whether it is located inside hemocytes. For this, we used an antibody for Vg that was made in another study

(Seehuus et al. 2007). A secondary antibody that was fluorescently labeled visualized the Vg antibody. We confirmed that Vg is also located within hemocytes and therefore measured Vg-positive hemocytes in the same task groups as the rest of the study. We found that winter bees had statically significant higher levels of Vg-positive hemocytes than nurses and foragers. No statistical difference was found between nurses and foragers. In our experiment, foragers and winter bees were of similar chronological age, and this indicates that foragers reduce their Vg levels when they start foraging. This clearly demonstrates that phenotype is more dependent on chronological age in honey bees. Furthermore, we correlated phagocytic rate and Vg levels to mitosis. In nurse bees, we found a significant negative correlation between phagocytic rate and mitosis, but we could not find this in foragers and winter bees. This means that nurses with high levels of phagocytosis recruit fewer hemocytes than nurses with low phagocytic rates. In the relationship between Vg and mitosis, we found a significant negative correlation in winter bees and no relation in nurses or foragers. This indicates that in winter bees with lower levels of Vg, there is a need for recruitment of new hemocytes. From other studies, Vg is associated with health and longevity; our results are, therefore, in accordance with this.

An advantage of this study was that we simultaneously compared three different worker castes: nurses, foragers, and winter bees. We found significant differences between nurses and foragers in phagocytic activity, which are in line with the assumption that foragers are less resilient than nurses are. However, there was a limitation. In an ideal situation, we would have preferred to co-analyze phagocytic levels and Vg levels simultaneously in the same samples. This was not possible in our experiment as there was an overlap between our fluorophore emission and the laser excitations in the flow cytometer, and distinguishing Vg levels and phagocytosis was not possible. In principle, it is possible to analyze with three different lasers at the same time, but this requires different fluorophores than we had available.

4.2.2 Paper III: Different activation of immune-related genes in honey bee nurses and foragers (*Apis mellifera*)

In this submitted manuscript, we test whether immune challenges could affect survival and gene expression in similarly aged nurses and forager bees. We found that nurses survive significantly longer than foragers of the same chronological age after immune

stimulation. This was independent of immune challenges, and control injection did not show statistical significance from LPS and PGN injections. Furthermore, we found that nurse bees upregulate the AMP defensin-2 after LPS and PGN treatment, compared to control injected bees. Expression of the AMP hymenoptaecin and Vg does not show significant differences. Foragers upregulate defensin-2 after PGN treatment but not after LPS treatment. Additionally, they upregulate hymenoptaecin after both LPS and PGN treatment compared to control. Gene expression of Vg was not significantly affected in either nurses or foragers.

The dissimilar gene expression patterns after immune challenges of nurses and foragers suggests a socially controlled or age-related different immune pathway activation with different expressed antimicrobial peptides. In *D. melanogaster*, age-dependent transcriptional changes are detected, mainly an increase in expression of immune-related genes (reviewed by Garschall & Flatt 2018; Min & Tatar 2018). Defensin-like peptides, belonging to the Toll pathway, are effective against gram-positive bacteria and less active toward gram negative (reviewed by Hillyer 2016). The IMD pathway, with hymenoptaecin, is usually effective against gram negative (reviewed by Hillyer 2016). Foragers are potentially more exposed to other pathogens than nurses are during their flights, which might lead to other gene expression patterns. In addition, we investigated Vg gene expression due to its relationship with immunity. However, gene expression of Vg does not differ after immune injections.
A limitation of the paper is that the project was intended differently, and we therefore did not include a non-injected control. This would have been very valuable, especially in our gene expression analysis, since we would have had the potential to reveal wounding and inflammatory effects related to immune reactions.

5 Concluding Remarks

The work presented in this thesis improved our understanding of the phenotypic differences between the worker castes. My aim was to investigate mitochondrial and immunity factors that influence honey bee resilience and aging.

This thesis work found that the mtDNA is more intact in aged forager bees as compared to young forager bees. This is opposite of the expectations of theories of stochastic-damage-caused aging, where one expects DNA damage to accumulate with increasing age. In recent years, the different aging theories have been under discussion. Especially the free radical theory of aging has been disputed, which might have been one of the most popular theories of aging (Gems & Partridge 2013). However, multiple studies have reported data that support this theory, and therefore we cannot dismiss the theories of stochastic-damage-caused aging. On the other hand, several studies indicate results that are in contrast with this theory as they have not identified increased damage accumulation with age (reviewed by Lopez-Otin et al. 2013; Lucas & Keller 2014; Møller et al. 2010). However, biology rarely has only one cause, and oxidative damage can be a primary cause of aging, or the aging spectrum might be wider with oxidative damage as a minor or major determent of aging (reviewed by Gems & Partridge 2013).

Additionally, from our results and previous ones, we can conclude that foragers are less resilient to survival and have poorer immune defenses than nurse bees, which is in line with the consideration that honey bees have programmed aging. However, our finding that foragers have more intact mtDNA could indicate that the most resilient foragers, with the best antioxidant defense, DNA repair, or a combination of different physiological functions, survive the longest. Individuals within a species having different genetic backgrounds, meeting different challenges, accidents, and environmental factors, and having different nutrition statuses, can increase variations in aging trajectories. These factors can affect the resistance against diseases and damages at different levels (reviewed by Ponton et al. 2013). Our honey bee workers have similar, if not identical, genetic backgrounds, and they have met many of the same exogenous challenges in our controlled experiments (reviewed by Amdam 2011); thus, their differences must therefore mainly be explained by other causes.

This work reveals that foragers and nurse bees have different gene expression patterns after an immune challenge. Whether it is related to their age or phenotype—where

progression into a forager bee changes the gene expression of immune-related genes—or the fact that they meet different immune challenges remains unknown. Our findings on the survival rates of nurses and foragers are comparable with results in other studies. We demonstrate that forager bees are less resilient than nurse bees as the latter survive significantly longer than the former. In addition, the longer survival of nurse bees is independent of immune or control challenges.

Our results confirm that the type of worker bee is more determinative of the resilience and lifespan than chronological age (Behrends et al. 2007; Seehuus et al. 2006a; Speth et al. 2015). Parts of our findings support the notion that foragers are the least resilient age phenotype and that nurses are the healthy and fit phenotype (as the nurses survive significantly longer and have more hemocytes that are phagocytic). Other results show that foragers express immune-related genes differently, which suggests other pathways for immune defense (as nurses and foragers express immunity genes at different levels). Moreover, other findings indicate that foragers that get relatively old are quite resilient, and these results do not support the free radical theory of aging. Overall, this thesis work highlights that physiological resilience and aging can be complex topics.

6 Future Perspectives

Vg has been found to have multiple functions in worker bees. Our results identified Vg inside hemocytes, but we did not investigate its immune functions or its purpose there. The function of Vg in hemocytes is a topic relevant to immunity as the protein has immune-supportive functions, or is involved in immune defense and immune-priming. There is interesting data from Vg in fish, where it functions as an oposin and promotes phagocytosis (Li et al. 2008; Li et al. 2009; Zhang et al. 2011). Future studies can build on findings in this study and other studies to investigate levels of Vg and its relationship to immune parameters.

One personal observation that has not been experimentally tested is that, when extracting their blood, foragers tend to have more melanized blood than nurses. One speculation is that foragers spend their energy on propheonol oxidase activity, and nurses have a rather better cellular defense by phagocytosis to clear pathogens. The antimicrobial peptide expression might be supportive of these defenses (in paper III).

There are descriptions of hemocytic mitosis in adult honey bees, but there are few reports in adult insects in general (Amaral et al. 2010; King & Hillyer 2013). Our result in detecting mitosis in adult honey bees is novel. The textbook thought is that circulating hemocytes in insects are formed at the larval stage. Yet it must be further studied to conclude whether hemocytes are considerably recruited by mitosis in adults or if it is more as a curiosity. It is recommended, however, that future studies keep in mind that mitosis occurs in adult honey bees. Additionally, their cell division could be related to the recent novel field that addresses adaptive immunity in honey bees.

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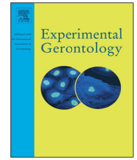
Paper I

Experimental Gerontology 49 (2014) 12–18

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero



Mitochondrial DNA integrity changes with age but does not correlate with learning performance in honey bees

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ARTICLE INFO

Article history:
Received 4 February 2013
Received in revised form 22 October 2013
Accepted 28 October 2013
Available online 6 November 2013

Section Editor: T.E. Johnson

Keywords:
mtDNA
rRNA
DNA damage
Learning
Honey bee
Aging

ABSTRACT

The honey bee is a well-established model organism to study aging, learning and memory. Here, we used young and old forager honey bees to investigate whether age-related learning capacity correlates with mitochondrial function. The bees were selected for age and learning performance and mitochondrial function was evaluated by measuring mtDNA integrity, mtDNA copy number and mitochondrial gene expression. Quite unexpectedly, mtDNA from young bees showed more damage than mtDNA from older bees, but neither mtDNA integrity, nor mtDNA copy number nor mitochondrial gene expression correlated with learning performance. Although not statistically significant ($p = 0.07$) the level of *L-rRNA* increased with age in good learners whereas it decreased in poor learners. Our results show that learning performance in honey bee does not correlate with absolute mitochondrial parameters like mtDNA damage, copy number or expression of mitochondrial genes, but may be associated with the ability to regulate mitochondrial activity.

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1. Introduction

Age is a major cause of mitochondrial impairment and cognitive decline. Many models are employed to demonstrate how oxidative stress and mitochondrial dysfunction result in learning deficits and cognitive dysfunction. Mitochondrial function has been targeted, either chemically or genetically to demonstrate an effect on cognitive function (Aksenov et al., 2013; Hayashi et al., 2008; McManus et al., 2011). Despite the correlation between mitochondrial impairment and learning deficit that is associated with age, it is still uncertain whether variations in learning performance in a population of a given age correlate with individual differences in mitochondrial capacity. The topic is difficult to address because the variability in mitochondrial function between healthy individuals is not expected to be large. Additionally, diversities in genetic background, environmental conditions and possible pre-training effects may occlude the impact of heterogeneous mitochondrial function. Honey bees represent a well-established model for learning and memory (Giurfa and Sandoz, 2012; Menzel et al., 2006), and can be used to study age-related changes in brain functions (Munch and Amdam, 2010). Honey bees are eusocial insects with colonies consisting of ten to forty thousand individuals with similar genetic background. Within the colony the vast majority are sterile female workers headed by a single reproductive queen. Queens usually outlive the relatively short lived workers by 2–3 years (Seeley, 1995; Winston, 1987). The worker bees show flexible aging that emerges by task-switching, making them a powerful model for aging research (Amdam et al., 2005). A worker bee starts as a 'nurse', a social task group that is active inside the nest, performing larval care, cleaning and building work. After 2–3 weeks the worker bee abandons nursing task, and starts collecting nectar, pollen, propolis or water in the field as a forager (Seeley, 1995). The lifespan as a forager spans from 7 to 18 days (Visscher, 1997). Interestingly, the aging phenotype in bees has a strong task-dependent feature; nurses age slowly while foragers age fast, and the flexibility in aging is demonstrated by the ability to postpone, accelerate or even reverse aging, dependent on the nursing task (Amdam et al., 2005; Behrends et al., 2007; Huang and Robinson, 1996; Seeley, 1995). Forager bees use landmarks to orient their flights, remember previously visited flowers and learn which floral odors are associated with food regards (Frisch and Lindauer, 1956; Winston, 1987). Learning and memory are therefore important abilities during foraging (Srinivasan, 2010). Brain function is a trait that senesces during foraging, measured as associative (Pavlovian) learning ability in the laboratory (Behrends et al., 2007). High energy processes like learning, memory and sensory input require efficient neuronal mitochondria (Han et al., 2011; Lovell and Markesbery, 2007; Niven et al., 2007).

Aging in honey bees, as in vertebrates, occurs in parallel with the accumulation of oxidative damage in macromolecules like proteins, lipids and DNA (Barja, 2004; Seehuus et al., 2006a). Accumulation of oxidative

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0531-5565/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.exger.2013.10.013

damage in mitochondrial DNA (mtDNA) increases with age in rodents (Hudson et al., 1998) and correlates with lifespan in mammals (Barja, 2004). Alterations in mtDNA that are known to cause neurological dysfunction include mtDNA deletions as in Kearns-Sayre syndrome (Oldfors et al., 1990), reduced copy number (Bartesaghi et al., 2010), mtDNA damage (Lauritzen et al., 2010) and replicative errors caused by mutations in mtDNA polymerase γ (Santoro et al., 2006). The non-coding region of mtDNA is particularly sensitive to damage, as this can alter replication and transcription of mtDNA (Coskun et al., 2004; Lee and Wei, 2005). The mtDNA non-coding region, involved in mtDNA transcription and replication, is known as the A-T rich region in insects (Wei et al., 2010).

In this study, we have employed the honey bee model to investigate whether age-dependent learning performance in a population correlates with mitochondrial characteristics. Parameters like mtDNA integrity and copy number, and mitochondrial gene expression levels were quantified to address this question. Foraging bees of two different ages were selected according to learning performance prior to brain mtDNA and mtRNA characterization.

2. Materials and methods

2.1. Bee related methods

2.1.1. Animal handling and identification

The bees were collected and analyzed in June 2011 at the Norwegian University of Life Sciences, Aas, Norway. Two commercial colonies of around 40,000 *Apis mellifera carnica* workers were used. Foragers of unknown chronological age were paint-marked on their thorax when they returned back from foraging flights to indicate length of foraging experience. Care was taken to not mark bees during periods of orientation flights, when pre-forager worker bees also fly out and construct a map of the hive's surroundings. The evening before the learning experiment we collected foragers who had been marked either 4–6 or 14–16 days earlier. Bees with 4–6 days of foraging experience (= 5 DF) and 14–16 days of foraging experience (= 15 DF) were kept evenly distributed between four wooden boxes. The bees were kept overnight with *ad libitum* access to 30% sucrose and H_2O in an incubator (Hera cell 150—Thermo scientific) set to 30 °C. Four hours before the bees entered the behavioral test, sucrose feeders were removed and bees were starved for this period. Two hours before the tests the bees were chilled on ice and mounted in holders. After mounting they were force-fed with 2 μL 30% sucrose, to ensure that all the bees had comparable satiation state.

2.1.2. Quantification of olfactory learning performance

Individual performance was assessed with a classical olfactory learning assay (Bitterman et al., 1983; Giurfa and Sandoz, 2012), in which bees learn to associate an odor (conditioned stimulus, CS) with a sucrose reward (unconditioned stimulus, US).

2.1.3. Evaluation of gustatory responsiveness

To control for an individual's motivation, we first tested all bees for their responsiveness to the sucrose reward. The gustatory response score (GRS) was measured as described previously (Behrends et al., 2007). Briefly, the bee's antennae were presented with seven different sucrose solutions in an ascending order (0%, 0.1%, 0.3%, 1.0%, 3.0%, 10% and 30% sucrose). A responsive bee would extend its proboscis (tongue) when the antennae were touched with a droplet of sucrose solution. The sum of proboscis extension response (PER) to the seven sucrose solutions is given as the gustatory response score (GRS). Consequently, bees with a poor response to the sucrose reward received a low GRS (0–3), while good responders received a high GRS (4–7). Bees that fail to respond to sucrose (PER = 0) could not be rewarded and were therefore excluded.

2.1.3.1. Quantification of associative learning ability. Learning performance was measured during 6 learning trials by monitoring when individuals responded to the conditioned stimulus (CS), the rewarded odor. Two different odors, carnation (Natur & Helse, Oslo, Norway) and cineole oil (Sigma-Aldrich, Germany) were used as the CS and an alternative unrewarded stimulus (neutral odor). To avoid effects specific to one of the two odors, the use of odors as either CS or the neutral odor was balanced between the experimental replicates. The neutral alternative odor was tested before the first and after the sixth CS/reward pairing. This was to test whether bees could discriminate between the CS and the unrewarded odor. For odor application, 2 μL of each odor was applied to 1 × 1 cm paper, and placed into different 10 mL syringes (BD Plastipak 10 mL and BD Microlance, Madrid, Spain). Bees were trained to the CS in six consecutive learning trials, in which the odor was paired with the reward (30% sucrose). The inter-trial intervals were 15–20 min long to avoid confounding effects of habituation. In all trials, bees were placed in a ventilated hood for around 10 s before and 5 s after odor stimulation, for acclimatization. Specifically, a single learning trial lasted for 5 s. During these 5 s, the bees were first presented with the CS alone (airstream with approximately 9 mL of the syringe volume), before combining the remaining odor volume with feeding 1 μL of the 30% sucrose solution for the last 2 s. The bees that responded to the CS alone in the first learning trial before presenting the reward were assigned as 'spontaneous' responders, but were excluded from the *in vitro* analyses. A final last CS presentation was rewarded with 3 μL 30% sucrose instead of 1 μL, to avoid starvation and induced mortality before dissection.

2.1.4. Selection criteria and tissue dissection for mitochondrial analysis

For further analyses individuals were classified into two categories: 1) the poor learners (learning score 0–3) and, 2) good learners (learning score 5). Before dissecting out brains, bees were transferred to ice, to anesthetize the subjects. Then brains were separated from the head capsule and adjacent tissue, retinal tissue was scraped off, and brains were snap frozen in liquid nitrogen and stored at −80 °C.

2.1.5. Paraquat exposure

Paraquat dichloride x-hydrate Pastanat® (Sigma-Aldrich, Germany) at different concentrations in a total of 0.5 μL was injected into the brain of a separate subset of worker bees of unknown age that were randomly picked from one colony. Mortality was recorded 6 h after injection. The heads were removed from the surviving bees, snap frozen and stored at −80 °C. Paraquat is known to induce mitochondrial oxidative stress (Tawara et al., 1996), and was used to control whether mtDNA damage could be used as a sensor of oxidative stress.

2.2. Molecular biology methods

2.2.1. DNA and RNA extraction

Total DNA, both nuclear and mitochondrial DNA was isolated with the DNeasy® Blood & Tissue kit (Qiagen) following the manufacturer's protocol with an extra temperature step at 70 °C for 10 min to improve yield. Total RNA from brain tissue was isolated using the RNeasy®Mini kit (Qiagen) according to the manufacturer's protocol, with an additional DNA digestion step. The concentration of nucleic acid was determined using NanoDrop (NanoDrop 1000 spectrophotometer, Thermo Fischer Scientific).

2.2.2. mtDNA copy number

Quantitative real time PCR was used to determine mtDNA copy number by measuring the difference in CT values from the nuclear actin gene and mitochondrial *L-rRNA* gene. The values were related to 5 DF good learners, and the relative mtDNA copy number calculated by Pfaffl correction (Pfaffl, 2001). The qPCR mixture (20 μL) consisted of Power SYBR® green PCR master mix (Applied Biosystems), 0.5 mM of each forward and reverse primer, 0.1 ng DNA and DEPC water. The PCR program was run on Step One Plus PCR System using the following

program: one cycle of 95 °C for 10 min, and 40 cycles of 95 °C (30 s)–54 °C (45 s)–65 °C (45 s). The *L-rRNA* primer sets were used as mtDNA marker: 5′-TTA AAT TCT ATA GGG TCT TAT CGT CCC ATA-3′ (sense) and 5′-TAG CTG CAG TAT GTT GAC TGT ACA AAG G-3′ (antisense), and the actin primer sets were used as nuclear DNA marker: 5′-TGC CAA CAC TGT CCT TTC TG-3′ (sense) and 5′-AGA ATT GAC CCA CCA ATC CA-3′ (antisense). Melt curve analyses were performed to ensure amplification of single products. Standard curves were made from serial dilutions of DNA to determine qPCR efficiency.

2.2.3. mtDNA damage detection

mtDNA integrity was evaluated by two different methods. 1) The mtDNA damage-mediated inhibition of PCR amplification, and 2) the mtDNA damage-mediated inhibition of restriction cleavage. The first method scores for the ability of mtDNA to serve template in amplification of a large PCR product (10 kb). The PCR mixture (45 μL) contained 5 or 10 ng of total DNA, with 100 mg/mL BSA, 200 μM of each dNTP, 440 nM sense and antisense primer, 1.3 mM $Mg(OAc)_2$ and 1 unit of rTth polymerase. Primers used to amplify the region between nt 3287 and 13,873 (10 kb) were 5′-ACC ACC TCT AGA TCA TTC ACA TTT AG-3′(sense) and 5′-TAG CTG CAG TAT GTT GAC TGT ACA AAG G-3′ (antisense). The PCR mixture was separated by agarose gel electrophoresis including SYBR safe (Invitrogen, Carlsbad, CA, USA). Product was quantified by densitometric analyses. The second method scores for the ability to inhibit TaqI cleavage at position 10388 in *A. mellifera* mtDNA. The method was established previously (Wang et al., 2011) and adapted to honey bee. The degree of TaqI inhibition was evaluated by the ability to amplify the mtDNA region (nt 10,055 to 10,530) containing the TaqI restriction site with or without TaqI pretreatment, in a combined qPCR analysis. The digestion/qPCR mixture (20 μL) contained 1 ng of total DNA (+/− TaqI (1 U)), 10 μL Power SYBR® green PCR master mix (Applied Biosystems, USA), 0.5 mM primers; 5′-AAT GAT AAA CCT AAA ACT AAC TCA CAT ACA G-3′ (sense) and 5′-ATT AGT AAT ATG GAA GGT CTA TTA AAG ATG-3′ (antisense). The mixture was incubated at 65 °C (15 min) for TaqI digestion, followed by inactivation for 10 min at 95 °C. The subsequent qPCR continued for 40 cycles including the following steps: 95 °C (30 s)–53 °C (45 s)–65 °C (45 s) mtDNA damage was estimated by the difference in CT values from digested and nondigested DNA using the pfaffl relation and presented relative to the 5 DF good learner group.

2.2.4. Gene expression analysis

cDNA strand synthesis was performed with the high capacity cDNA reverse transcription kit (Applied Biosystems, USA). The reaction (20 μL) contained 250 ng RNA and buffer, dNTP mix, random primers and MultiScribe Reverse Transcriptase according to manufacturer's recommendation. The cDNA synthesis and the subsequent Reverse Transcriptase inactivation were performed by stepwise incubation at 25 °C for 25 min, 37 °C for 120 min and 85 °C for 5 min. The cDNA was stored at −20 °C until analyses.

Transcript levels were assessed by qPCR analyses of cDNA, related to the house keeping gene actin. The qPCR, with the same composition as in Section 2.2.3 was initiated for 10 min at 95 °C for 10 min, followed by 40 cycles of 95 °C (30 s)–54 °C (45 s)–65 °C (45 s). Variations in qPCR efficiency were corrected for by the Pfaffl method (Pfaffl, 2001) to calculate relative expression level, using average CT values from all 5 DF good learners as control for *ND1*, *CYTB* and *L-rRNA*; actin was used as reference.

The following genes were analyzed for expression: actin and *L-rRNA* (see sequences in Section 2.2.3); *ND1* (5′-CCA AAT CAA GGA TAC AAA ATT CAT ATA-3′ (sense); 5′-GTT TGG TAT ATT TCA ACC TTT TAG TGA TGC -3′ (antisense)) and *CYTB* (5′-ATT TCC ATA TCA TTT AGG AGA TCC AGA CAA-3′ (sense); 5′-ATA TGC AAA TAG GAA ATA TCA TTC AGG TTT-3′ (antisense)).

2.3. Statistical analysis

Factorial ANOVA was used for statistical analysis of how mitochondrial parameters correlate with learning and age unless otherwise specified. Multifactorial ANOVA was used to rule out significant plate to plate variations in qPCR analyses. For the data set with paraquat treated bees ($N = 5$ in each group), Kruskal–Wallis test was used to compare all groups. When a significant result was detected, we employed Mann–Whitney *U* test in post hoc comparison between the two groups. All tests used the program Statistica 10 (StatSoft®, Tulsa, OK, USA).

3. Results

3.1. Worker bee learning performance

Worker bees at 5 days of foraging (5 DF) and 15 days of foraging (15 DF) were selected as described in Materials and methods and examined in the Pavlovian learning test. A total of 288 bees completed learning tests and the score distributions are shown in Fig. 1. Based on these scores, the two age groups were further separated into poor (score 0–3) and good (score 5) learners. Learning skills as foragers are reported to be age dependent (Behrends et al., 2007), and although our data set contained more good learners among the young foragers, we do not have the statistical confidence to state this. It should also be noted that the chronological age-dependent learning skill is not ubiquitously agreed on, as winter bees do not show learning senescence (Behrends and Scheiner, 2010; Munch et al., 2013).

3.2. Lethal oxidative stress induces mtDNA damage in honey bee

PCR-based methods for assessing mtDNA damage and mtDNA copy number have been established for different eukaryotes including *Drosophila* (Hunter et al., 2010; Santos et al., 2006), but not for *A. mellifera*. Primers for *A. m. carnica* mtDNA were designed based on the mitochondrial DNA sequence of *A. m. ligustica* (ACCESSION L06178) (see Materials and methods). Paraquat has previously been shown to kill honey bee in an oxidative stress-dependent manner (Seehuus et al., 2006b). We tested whether paraquat-mediated killing of honey bees involved accumulation of mtDNA damage in the honey bee brain. This assay was included as a positive control in order to confirm the responsiveness of the assay to mtDNA damage. As seen in Fig. 2A, paraquat killed bees in a dose-dependent manner, confirming earlier reports. The lowest concentration of paraquat killed approximately 25% of the bees. The brains from the survivors were isolated and the DNA extracted and analyzed for mtDNA damage. Paraquat exposure significantly reduced the integrity of brain mtDNA in the survivors inferred from

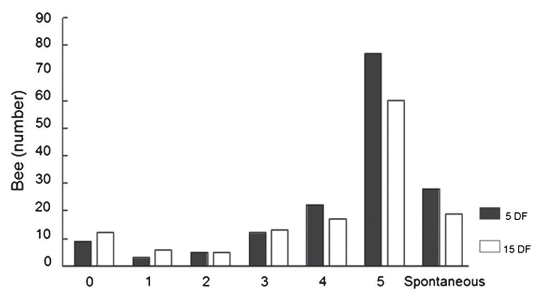

Fig. 1. Presentation of learning score distribution for bees with 5 days of foraging experience (5 DF) and 15 DF. The learning scores of 288 bees were evaluated. Score 0 was given to bees that did not demonstrate learning, and 5 was given to the individuals that showed learning response after the first trial, and is considered as the best score. The bees that responded spontaneously before any stimulus was presented are categorized as "spontaneous". See Materials and methods for score criteria.

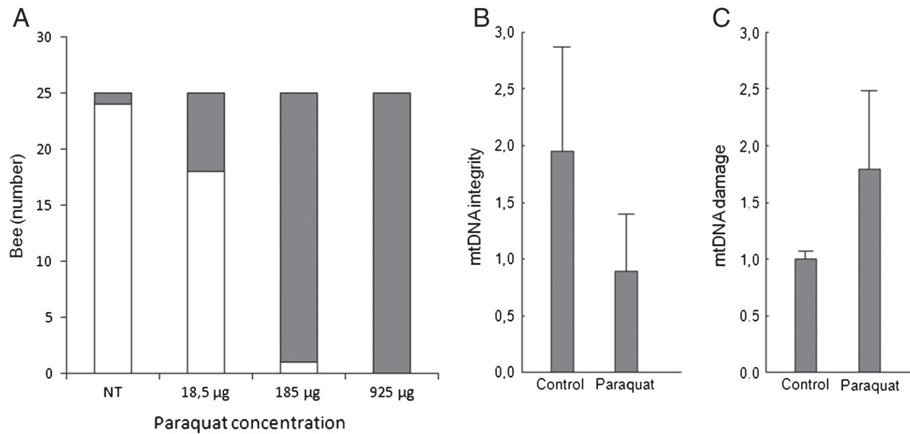

Fig. 2. Paraquat-induced killing of bees involves mtDNA damage. (A) Paraquat kills bees in a dose-dependent manner. The fraction of survivors (white) and dead (gray) were determined 6 h after injection. (B) The integrity of mtDNA in the surviving bees receiving 18.5 μg paraquat (75% survival) was estimated by the ability to amplify a large PCR product ($Z = 1.671145$, $p = 0.094694$, $N = 10$). (C) mtDNA damage was separately estimated by the ability to inhibit TaqI restriction cleavage ($Z = -1.67115$, $p = 0.094694$, $N = 10$). The values are mean with SE from 10 individual treated bees. The Mann–Whitney *U* test showed a significant effect of paraquat on mtDNA integrity on both detection methods ($p < 0.1$ for one-sided test).

one-sided Mann–Whitney *U* test (Fig. 2B and C). Damage to mtDNA accumulates in Poisson dependent manner and the average number of lesions can be calculated from the result in Fig. 2B (Santos et al., 2006). The amount of paraquat sufficient to kill 25% of the bees produced in average one lesion per mtDNA molecule in the survivor brains.

3.3. mtDNA content and integrity in good and poor learners of different age

Mitochondrial content in the different performance and age groups was evaluated by the mtDNA copy numbers. Regardless of comparisons between the age groups (Fig. 3A), the learning categories (Fig. 3B), and the interaction between age and learning (Fig. 3C), mitochondrial content did not vary significantly between any of the groups.

We then tested the integrity of mtDNA by two separate methods. Unexpectedly, we found that mtDNA from young foragers (5 DF) contained significantly more damages than mtDNA from older foragers (15 DF) (Fig. 4). However, there was no significant difference in mtDNA integrity between poor and good learners at either age, or between different groups defined by age and learning skills. In combination, the analyses demonstrate that learning performance in honey bees is not related to the quantity or quality of mtDNA.

3.4. Similar expression of mitochondrial L-rRNA and mitochondrial structural genes in good and poor learners, as in young and old bees

Expression of mtDNA encoded genes is regulated by promoters in the A-T rich region of insect mtDNA. The genes encoding the complex I subunit NADH reductase 1 and complex III subunit Cytochrome B (*ND1* and *CYTB*, respectively) are located on separate strands of the mtDNA and are therefore expressed from different promoters. The apparent increase in *ND1* expression with age, which was similar in poor and good learners, was not statistically significant ($p = 0.075$: Supplementary Fig. 1). When expression of *ND1* in the four groups was analyzed for possible interaction between age and learning, no significant correlation was evident (Fig. 5A). Expression of *CYTB* showed similar pattern in that there were no significant differences with age or learning performance, or significant interaction with age and learning skills (Supplementary Fig. 1 and Fig. 5B).

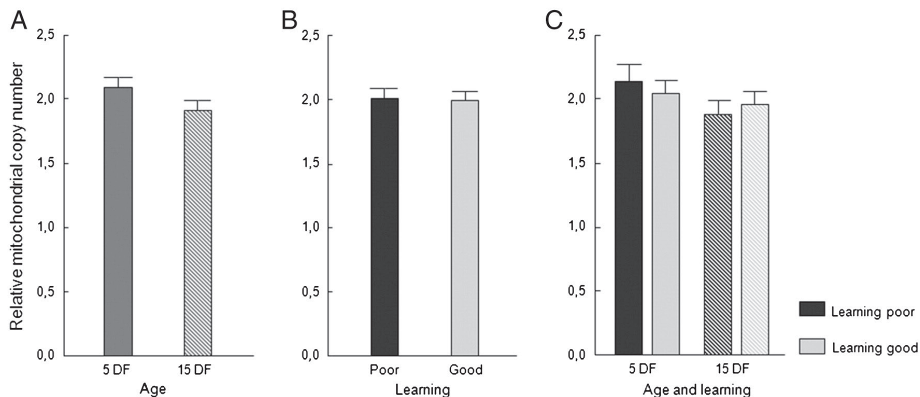

Fig. 3. Similar mtDNA copy number in honey bees of different age and learning performances. mtDNA copy number was determined by qPCR analyses, and presented relative to 5 DF good learners. (A) 5 DF versus 15 DF (ANOVA: $F_{1, 39} = 2.6203$, $p = 0.11356$, $N = 46$), (B) good learners versus poor learners (ANOVA: $F_{1, 39} = 0.01428$, $p = 0.90551$, $N = 46$), and (C) comparison of groups with respect to age and learning (good learners are in white; poor learners are in gray (ANOVA: $F_{1, 39} = 0.63748$, $p = 0.42946$, $N = 46$)). Values are mean with SE.

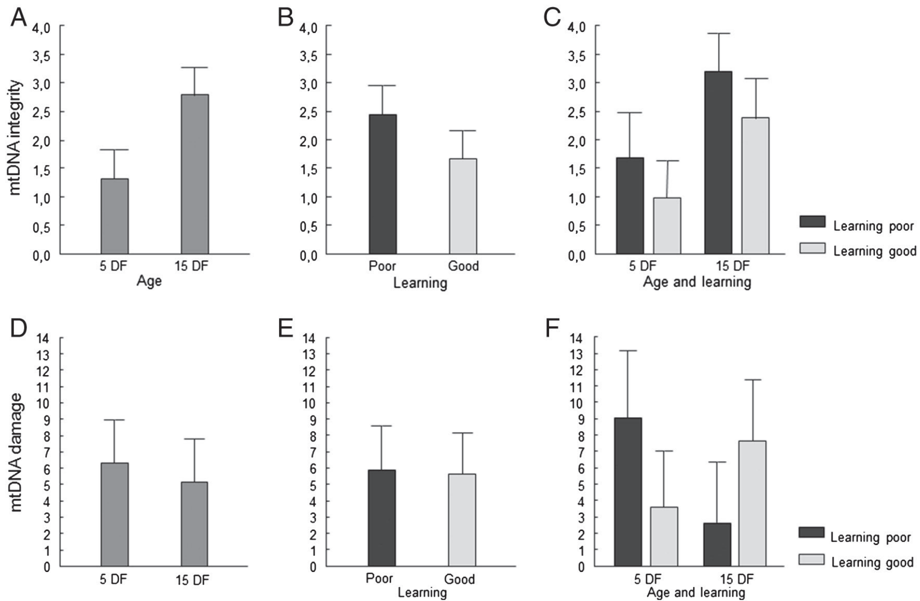

Fig. 4. Lower integrity of mtDNA in young honey bees is independent of learning skills. Integrity of mtDNA was assessed by the ability to amplify a large product by PCR (A–C) or by the ability to inhibit TaqI restriction cleavage (D–F). (A, D) Comparison of the 5 DF to the 15 DF (A: ANOVA: $F_{1,38} = 4.2493$, $p = 0.04616$, $N = 46$; D: ANOVA: $F_{1,41} = 0.09849$, $p = 0.75524$, $N = 41$). (B, E) Comparison of mtDNA integrity in poor and good learners (B: ANOVA: $F_{1,38} = 1.1427$, $p = 0.29183$, $N = 46$; E: ANOVA: $F_{1,41} = 0.00341$, $p = 0.95371$, $N = 41$). (C, F) Comparison of age and learning capacity (good learners in white; poor learners in gray). C: ANOVA: $F_{1,38} = 0.00649$, $p = 0.93619$, $N = 46$); F: ANOVA: $F_{1,41} = 1.9393$, $p = 0.17125$, $N = 41$. Values are mean with SE.

Like vertebrates, insect mtDNA contains two ribosomal RNAs (the small (S) and large (L) rRNA) that are located in immediate downstream positions of the promoter region, separated by the $tRNA_{VAL}$ gene. Compared to the *CYTB* transcript, the level of *L-rRNA* is present in 300-fold excess. The *ND1* level is approximately 50% of that of *CYTB*. As was the case for the structural genes *ND1* and *CYTB*, the levels of *L-rRNA* in 5 DF and 15 DF were generally similar, and the poor learners had comparable *L-rRNA* levels to good learners (Supplementary Fig. 2). Interestingly, we discovered a borderline significant ($p = 0.068$) correlation between age and learning performance whereby poor learners were characterized by decreased *L-rRNA* expression with age whereas good learners displayed increased *L-rRNA* expression with age (Fig. 5C). These data indicate that mitochondrial parameters *per se* do not distinguish poor learners from good learners, but rather determined their ability to regulate mitochondrial activity.

4. Discussion

In this study, we have tested how learning performance correlates with mitochondrial parameters during aging in honey bees. From our

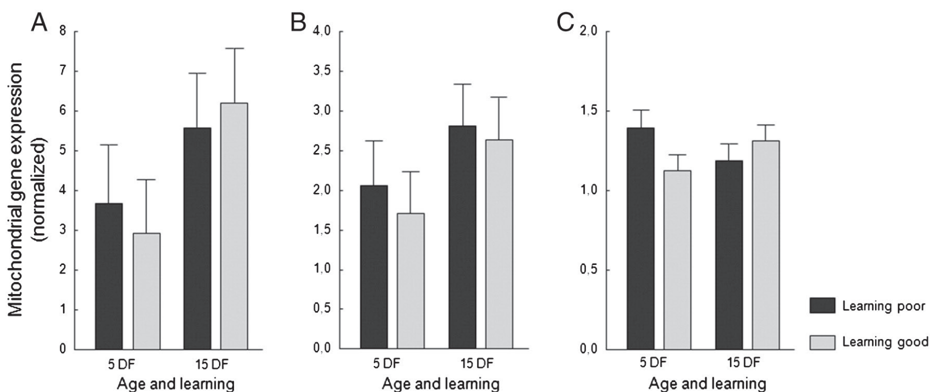

Fig. 5. Age- and learning-associated changes in mitochondrial gene expression. (A) ND1 transcript levels were analyzed by real-time qPCR, using β-actin as reference and analyzed for interaction between age and learning (ANOVA: $F_{1,24} = 0.24553$, $p = 0.62474$, $N = 31$). (B) Similar interaction analyses for *CYTB* expression (ANOVA: $F_{1,24} = 0.02754$, $p = 0.86958$, $N = 31$). (C) Ribosomal L-RNA displays borderline significant interaction between age and learning (ANOVA: $F_{1,24} = 3.6479$, $p = 0.06816$, $N = 31$). White bars represent good learners; gray bars represent poor learners. The levels were normalized to 5 DF. Values are mean with SE.

results, we conclude that individual differences in learning performance do not correlate with mitochondrial function, assessed by mtDNA damage, copy number or mitochondrial gene expression. Mitochondrial *L-rRNA* was similar in the two age groups as well as between good and poor learners. However, there was a trend (close to statistical significance) showing that *L-rRNA* was differently regulated with age in good and poor learners. In our experimental system, we did not find any evidence of reduced mitochondrial function with age. The findings are partly in contradiction to the situation in another insect model, *Drosophila melanogaster*, as aging in this insect correlates with decreased expression of mitochondrial genes (Schwarze et al., 1998). There might be several reasons for this discrepancy and we believe that the social task-dependent aging typical for honey bees could be a mediating factor.

Honey bee aging is an inevitable side effect of foraging. Aging and declining learning capacity depend on the number of days of foraging rather than the chronological age (Wolschin et al., 2009). Foraging activity has an influence on the learning score which is generally lower in older foragers than nurses of the same chronological age (Behrends et al., 2007; Scheiner and Amdam, 2009). The explanation is possibly due to differing weather conditions as it has been shown that rain-induced flight restriction prevents a decline in associative learning in honey bees (Tolfsen et al., 2011). In the same report, flight restriction was also found to affect age-dependent oxidation. Our results nevertheless demonstrate that mtDNA from old foragers contained less damage than mtDNA from young foragers.

Accumulation of mtDNA damage has been shown to correlate with lifespan expectancy in general (Barja, 2004) and mtDNA damage is reported to increase with age (Hudson et al., 1998), and was assumed to be the net result of increased oxidative stress. However, recent discoveries question the general impact of increased oxidative stress as a general cause of aging. Systematic deletions of ROS scavenging enzymes generally did not affect life span (Perez et al., 2009). These findings are in line with ours, as young foragers have more mtDNA damage than old foragers. It is a possibility that elevated mtDNA damage in the young foragers is merely a secondary effect of a high metabolic rate as honey bees during flight have one of the highest metabolic rates measured (Suarez et al., 2000). It is therefore a possibility that the high level of mtDNA damage in young foragers contributes to metabolic dysfunction with age. If such dysfunction reduces the bees' ability to survive and forage, then we might observe less mtDNA damage in older foragers as an artifact of this selective mortality.

An alternative hypothesis for the increased mtDNA damage in young foragers might be that the bees have adapted to the atmosphere inside the hive, which has been reported to be 100 times enriched in CO_2, with reduced O_2 (Ohashi et al., 2009). Bees are additionally able to actively ventilate the hive (Southwick and Moritz, 1987), resulting in alternating O_2 and CO_2 cycles. It is possible that low O_2, cycling O_2/CO_2 or a combination of both, might reduce the toxic effect of oxygen. Changing work task to foragers then exposes the bee to a higher oxygen content, potentially inducing more mtDNA damage. As a follow-up to this hypothesis, it would be interesting to know the impact of hive atmosphere on the different lifespan potentials of nurse bees and foragers (Tolfsen et al., 2011). The temperature changes associated with foraging could potentially additionally increase the probability of mtDNA damage in the non-acclimatized young forager bees' temperature (Jones et al., 2004).

Fifteen days of foraging represents a relatively high foraging age (Amdam, 2011) and suggests that longevity-promoting phenotypes are selected for in the survivors reaching this age. Accumulation of mtDNA damage is counteracted by mtDNA repair and/or antioxidant buffering against mitochondrial oxidative stress. These are two factors that are important for longevity in mice (Russo et al., 2004; Schriner et al., 2005) and might be particularly elevated in the surviving 15 DF bees.

Two fundamentally different mtDNA damage detection methods were used to quantify mtDNA damage in this study. The ability to amplify a long PCR fragment depends on the level of blocking lesion, such as base adducts, oxidized sugar residue and single strand nicks that inhibit the PCR polymerase. This method is frequently used to quantify mtDNA damage and has previously been used to analyze mtDNA damage in insects (Hunter et al., 2010). The second assay was established by this research group previously (Wang et al., 2010) and scores for the ability of a lesion to inhibit Taq1 restriction cleavage. Despite limitations in both methods, they give comparable responses and suggest that artificial errors have minor influence on the output.

The relative expression levels of *CYTB* and *ND1* are comparable. The *ND1* gene is encoded by the same strand as *L-rRNA* and the latter is 600 times more abundant. A similar pattern has been described previously in mosquito, where an alternative transcription termination directly after the *L-rRNA* gene, but before the *ND1* gene, was suggested (Neira-Oviedo et al., 2011). This is similar to transcription in mammals, where two heavy strand promoters are identified and one of them is terminated directly after the *16S-rRNA* gene (Falkenberg et al., 2007).

We believe the borderline significant effect of age and learning task on *L-rRNA* is interesting as it may reflect resilience in mitochondrial biology that is beneficial for cognitive function. Although statistical significance would be stronger with more samples, we believe that the differences between the groups could be more pronounced if the region involved in learning (calyx) in honey bees was studied specifically (Wolschin et al., 2009). Provided that learning performance reflects an inherent ability of individual bees to regulate mitochondrial biogenesis, it would be interesting to test whether the good learners also score better on other types of tests where mitochondrial adaptions are required.

5. Conclusion

In general, we did not find correlation between learning performance and age, and mtDNA parameters. We observed slightly improved mtDNA integrity in the older individuals, which was independent of cognitive function. We believe that the borderline significant correlation between *L-rRNA* and learning with age is interesting and may indicate that learning capacity in honey bees correlates with the ability to regulate mitochondrial biogenesis rather than the absolute mitochondrial capacity. Thus, mitochondrial resilience should not be neglected when addressing mitochondrial function.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.exger.2013.10.013.

Conflict of interest

The authors have no conflicts of interest.

Acknowledgements

We thank Claus Kreibich for managing the bee colonies and to Daniel Münch for discussions and comments. We are indebted to one of the reviewers of the first version of this manuscript, who came up with the idea that hive oxygen pressure might have an impact on mtDNA damage in the young foragers. We thank Alexander Rowe for critical reading of the manuscript. L.E. received support from the University of Oslo and the Norwegian Research Council (NevroNOR). G.V.A. was supported by the Research Council of Norway (180504, 191699, and 213976).

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Paper II

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RESEARCH ARTICLE

Hemocyte-mediated phagocytosis differs between honey bee (*Apis mellifera*) worker castes

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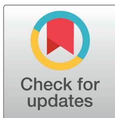

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Citation: Hystad EM, Salmela H, Amdam GV, Münch D (2017) Hemocyte-mediated phagocytosis differs between honey bee (*Apis mellifera*) worker castes. PLoS ONE 12(9): e0184108. https://doi.org/10.1371/journal.pone.0184108

Editor: Bok-Luel Lee, Pusan National University, REPUBLIC OF KOREA

Received: June 13, 2017

Accepted: August 17, 2017

Published: September 6, 2017

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The Research Council of Norway (213976 to EMH, GVA and DM) and Academy of Finland (265971 to HS) supported this study. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Honey bees as other insects rely on the innate immune system for protection against diseases. The innate immune system includes the circulating hemocytes (immune cells) that clear pathogens from hemolymph (blood) by phagocytosis, nodulation or encapsulation. Honey bee hemocyte numbers have been linked to hemolymph levels of vitellogenin. Vitellogenin is a multifunctional protein with immune-supportive functions identified in a range of species, including the honey bee. Hemocyte numbers can increase via mitosis, and this recruitment process can be important for immune system function and maintenance. Here, we tested if hemocyte mediated phagocytosis differs among the physiologically different honey bee worker castes (nurses, foragers and winter bees), and study possible interactions with vitellogenin and hemocyte recruitment. To this end, we adapted phagocytosis assays, which—together with confocal microscopy and flow cytometry—allow qualitative and quantitative assessment of hemocyte performance. We found that nurses are more efficient in phagocytic uptake than both foragers and winter bees. We detected vitellogenin within the hemocytes, and found that winter bees have the highest numbers of vitellogenin-positive hemocytes. Connections between phagocytosis, hemocyte-vitellogenin and mitosis were worker caste dependent. Our results demonstrate that the phagocytic performance of immune cells differs significantly between honey bee worker castes, and support increased immune competence in nurses as compared to forager bees. Our data, moreover, provides support for roles of vitellogenin in hemocyte activity.

Introduction

The honey bee (*Apis mellifera*) is an important pollinator. The alarming decline of managed colonies has spurred much research into disease epidemics and mechanisms to counteract pathogens [1–3]. Highly social honey bees show a flexible division of labor among female workers that are all functionally sterile and assist the queen. After emergence, young workers

typically start as nest bees that nurse the brood and other colony members, including the queen. After 2–3 weeks, workers can progress to outside tasks, and collect nectar, pollen and water (foragers) (see [4] and references therein). The physiological changes associated with worker caste progression from nurses to foragers include changes in their nutrient storage [5], hormonal regulation [6, 7], oxidative stress resilience [8], mortality and aging dynamics [5, 9–11] as well as in immune pathways [6, 9, 12]. Brood-less, over-wintering colonies are maintained by another worker caste—winter (*diutinus*) bees, which actively engage in thermoregulation and have the longest worker-lifespan (reviewed by [13]).

Bee colonies encounter a multitude of infectious pathogens, including bacteria (e.g. *Paenibacillus larvae* and *Melissococcus plutonius*), fungi (e.g. *Ascospaera apis*) and viruses (e.g. deformed wing virus) [1–3]. To fight pathogens, honey bees and other invertebrates depend on an innate immune system, and do not feature the acquired, antibody-mediated immune response of vertebrates. Several recent studies, however, show that immune priming occurs in insects (reviewed by [14]), including trans-generational immune priming ([15], reviewed by [16]). The innate immune system is comprised of cellular and non-cellular (humoral) response pathways. Briefly, the three lines of cellular immune responses are phagocytosis, encapsulation and nodulation [17, 18]. Relatively few studies in honey bees have assessed the cellular responses directly, e.g. by measuring hemocyte (blood cells) counts [9, 19], or by analyzing nodulation [20, 21] and encapsulation [22]. In contrast, research into honey bee immunity typically has focused on gene expression of antimicrobial peptides (humoral response) [23–25] and phenoloxidase activity, which is involved in both humoral and cellular responses [19, 26].

Phagocytosis is a rapid, cellular cleaning process of the hemolymph, where individual hemocytes engulf large particles, such as bacteria and dead cells [17, 27, 28]. Hemocyte-mediated phagocytosis has been confirmed in honey bees [29], but functional aspects have not been studied in detail, including how this process may vary between the worker castes.

Contrasting immune responses among worker castes revealed that forager bees have a higher number of pycnotic (suggestive of cell death) hemocytes compared to nurses, which coincides with lower vitellogenin (Vg) levels in foragers [9]. Vg is a protein involved in honey bee worker stress tolerance, behavior and immunity. Vg is considered as a donor of zinc that is necessary for immune system function [6]. Lack of zinc in honey bees induces pycnosis, which may explain the loss of functional hemocytes in foragers [6]. Vg has been found in many cell types and in different honeybee tissues. Vg often adheres to membrane structures [30], where it can protect cellular function via antioxidative [30, 31] and anti-inflammatory [30] actions. In several fish species, Vg also enhances the phagocytic activity of immune cells [32–34]. However, to our best knowledge any direct interaction between Vg and hemocytes has not been described in insects.

Circulating hemocytes in adult insects are formed in the hematopoietic glands at larval and pupal stages [35–37]. In adult insects, new hemocytes are typically recruited by mitosis [38, 39]. Similar to vitellogenin abundance, hemocyte counts in adult honey bees are effects of labor division and parasite infection, which supports an active regulation [6, 40, 41]. Yet, links between hemocyte recruitment and Vg are not yet identified in insects.

The aim of this study was, firstly, to establish an efficient approach for studying phagocytosis in honey bee workers. Secondly, we aimed at using such quantitative phagocytosis assay to explore phagocytic differences between three worker castes (nurses, foragers and winter bees), for which previous studies had already identified differences in immunocompetence [6, 9, 12, 41]. We use a bead-based and a dye-based assay measuring phagocytosis and incorporation. We test if co-inoculation with *Escherichia coli* can affect phagocytic rate in the worker bees, as it does in *Anopheles gambiae* and *Aedes aegypti* [39, 42, 43]. Furthermore, we test if Vg is

directly associated with the hemocytes. Finally, we explore correlations between phagocytic activity or Vg levels, and the recruitment of new hemocytes by mitosis in the worker castes.

Materials and methods

Subjects

All tests were performed with honey bees (*Apis mellifera carnica* Pollmann) collected from apiaries of the Norwegian University of Life Sciences (Aas, Norway). To allow simultaneous testing of phagocytic rate and vitellogenin levels in summer and winter worker castes, we transferred winter bee colonies into a flight room ($N_{conolny}$ = 2). This triggers the transition to summer behaviors, including nursing and foraging [10]. Indoor colonies experienced a day: night cycle set to 12h. Apart from artificial light sources, temperatures also changed at 12h intervals with 25˚C and 17˚C as maximum and minimum, respectively.

To establish tests that assess phagocytic activity, we exclusively used mature nurse bees with a chronological age between 10–20 days. In order to obtain nurse bees of known age, newly emerged bees (age = 1 day) were paint marked (Uni Posca, Tiverton, United Kingdom) on their thorax for later identification, and were returned to two replicate host hives. When they were between 10–20 days, mature nurses were identified based on their paint mark and by performing nursing activity, i.e. having their heads in cells of the brood comb. Nurse bees where collected from two replicate hives on two different days (N = 20 each sampling day).

To induce foraging activity, feeders with pollen and 30% sucrose were provided. At the time of collection, winter bees collected from outside locations (N_{colony} = 2) and flight room foragers (former winter bees) had a chronological age of at least 5 months. Foragers were identified by paint marks they had received when visiting feeders. A minimum foraging duration of 3 days was ensured, by re-collecting foragers at least 3 days after they had initially being marked in the flight room. Mature nurse bees in these tests were born in the flight room, and again had a chronological age of 10–20 days (see before for identification).

Injection of phagocytic markers

For quantification of phagocytosis in honey bees we adapted two methods that have been established for other insects. One such approach includes the injection and hemocyte uptake of fluorescently labeled latex beads [27, 44–47]. Another is based on injecting the fluorescent dye CM-Dil, and has been successfully used in *Anopheles gambiae*, where the dye is incorporated in more than 70% of circulating phagocytic hemocytes [39, 43]. We prepared injection solutions with latex beads (0.5μm fluorescent carboxylate-modified polysterene latex beads, Sigma- Aldrich, Saint Louis, Missouri, USA) by dilution in Grace's insect medium (1:5, Sigma-Aldrich, Saint Louis, Missouri, USA). The alternative CM-Dil solutions (Vybrant® CM-Dil Cell-labeling solution, Molecular probes, Eugene, Oregon, USA) contained 0.75mM CM-Dil in Grace's insect medium. According King and Hillyer 2012 [43] CM-Dil was prepared fresh and has been injected within 15 min after preparation.

After collection, mature nurse bees were chilled at 4˚C and fixated with insect pins to a wax plate. Then, honeybees were injected with 1.5μL of either latex beads or CM-Dil solution. Following this, individuals injected with latex beads were kept in an incubator set at 32˚C for 2h before hemolymph extraction. Incubation time was chosen based on published protocols and pilot test runs, to ensure that incubation periods are sufficient to induce robust phagocytosis. Bees injected with CM-Dil were also kept at 32˚C. Yet, in line with available CM-Dil based protocols we have chosen a shorter incubation time of only 20min before hemolymph extraction [43].

After puncturing the abdomen between the fourth and fifth tergite with a sterile needle (31 G, Sigma Aldrich Saint Louis, Missouri, USA), approximately 3μL hemolymph per animal were extracted with 1μL Drummond micropipettes (Drummond Scientific Co/ Sigma-Aldrich, Schnelldorf, Germany). Collected hemolymph was suspended into 50μL collection buffer consisting of 70% Grace's insect medium, 20% anticoagulant solution (98mM NaOH, 186mM NaCl, 1.7mM EDTA and 41mM citric acid, buffer pH 4.5) and 10% Fetal Bovine Serum [47].

Imaging for qualitative assessment of phagocytosis

High-resolution microscopic imaging was used to confirm phagocytosis and incorporation of latex beads and CM-Dil (compare Fig 1). For both treatments, the collected and re-suspended hemolymph was pipetted on poly-L-lysine coated slides. The slides were kept in an incubator at 32˚C for 40min in order to let the hemocytes adhere to the slide. Subsequently, slides were fixed for 20min with 4% paraformaldehyde (PFA, Sigma-Aldrich, Saint Louis, Missouri, USA) dissolved in phosphate buffered saline (PBS, Sigma-Aldrich, Saint Louis, Missouri, USA). Cells were permeabilized for 5min in 0.2% Triton-X 100 in PBS and washed 3 times for 5min in PBS. Finally, samples were co-stained for 45min with the nuclear marker (4',6-diamidino-2-phenylindole, DAPI; 1:1,000 from 0.5mg/mL stock; Sigma-Aldrich, Saint Louis Missouri, USA), and a marker for the cellular matrix protein F-actin (Alexa Flour 488 Phalloidin; Molecular Probes, Eugene, Oregon, USA). After staining, slides were washed 3 times for 5 min before they were mounted in 30% glycerol/PBS, and were sealed with nail polish.

Confocal micrographs were acquired on a Leica TCS SP5 laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany). High-resolution images were taken with a 40x immersion oil objective (numerical aperture 1.25). The simultaneous acquisition mode was used to minimize spatial shifts between color channels; the z-step size was kept at 0.5μm. Signal detection bandwidth differed slightly between latex beads (excitation = 561nm, detection = 580-630nm) or CM-Dil stain (excitation = 561nm, detection = 570-617nm). Settings for the remaining two channels were kept constant, independent of treatment, namely 405nm/430-480nm for excitation/detection of DAPI and 488nm/580-650nm for excitation/detection of Alexa Flour 488 Phalloidin. Image processing with the ImageJ software package [48] included maximum intensity projection of image stacks, and Gaussian filtering (kernel size = 1) to remove high spatial frequency noise.

Morphological information from imaging allowed us to classify hemocytes by using established classification systems. Based on detailed descriptions for several insects [18, 38, 49–52], we identified granulocytes, plasmatocytes and prohemocytes in our samples from honey bees.

Activation of the immune system with *E. coli*

We tested if *E. coli* inoculation prior to marker injection can affect phagocytosis. To this end, we contrasted three treatment groups that all were injected with latex beads: one group with prior injection of 1μL of *E.coli*/Grace's insect medium solution (strain ATTC 25404 K-12), a first control with prior injection of 1μL Grace's insect medium only and a second control without prior injection. *E.coli* was grown overnight in Lysogeny broth (Sigma-Aldrich, Saint Louis, Missouri, USA) at 37˚C on a shaker. Cell counts were diluted to approximately 800.000 per μL. Cells were centrifuged at 5200 rpm for 5min, washed twice and re-suspended to the same concentration in Grace's insect medium. Prior to latex bead injection, honeybees were incubated for 2h. After latex bead injection, the honeybees were incubated for additional 2h as before, in total 4h.

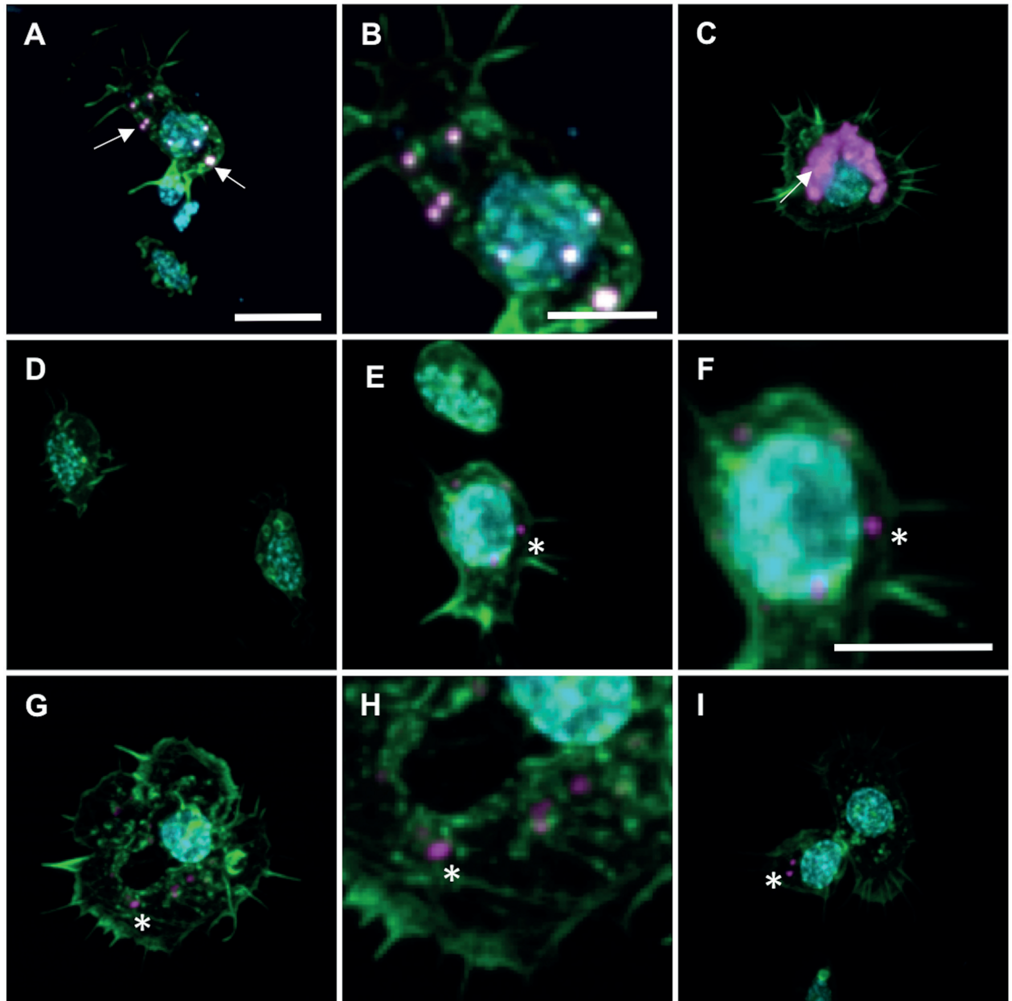

Fig 1. Circulating hemocytes from honey bee's hemolymph take up the phagocytic markers; latex beads and CM-Dil. A-C Images showing hemocytes with latex beads (magenta, arrow) detected in the cytosol (labeled with phalloidin in green, nuclei labelled with DAPI in cyan). **B** is a zoomed image of the same hemocyte (plasmatocyte) detected in A. **C** Shows a hemocyte (granulocyte) that has internalized multiple latex beads. Such hemocytes were occasionally found. **D** An example of hemocytes without detectable phagocytosis of latex beads. **E-I** Hemocytes with detectable CM-Dil (arrow) in the cytosol (green, nuclei labeled with DAPI in cyan). Both F and H are zoomed images of E and G, respectively. **E** and **F** Shows a plasmatocyte. **G** Shows a granulocyte. **I** The upper cell to the right is an example of a hemocytes without detectable incorporation of CM-Dil, whereas the cell to the left show incorporation of CM-Dil (asterisks). Scale bar for A, C, D, E, G, I in A = 10μm, scale bar for B, F, H in B = 5μm, scale bar in F = 5μm.

https://doi.org/10.1371/journal.pone.0184108.g001

Imaging for quantifying phagocytosis

For quantitative analysis of microscopic images samples were handled essentially as before (previous section). Minor protocol differences include that for staining with DAPI only, no

permeabilization with Triton-X was needed. A lower magnification objective (20x oil, numerical aperture 0.7) was used to acquire images with a larger overall dimension, and hence a greater number of hemocytes. To reduce technical bias in signal acquisition, laser power and sensor sensitivity settings were kept constant for all images. Five image replicates were taken for representing each individual; all cells in each image were counted, >50 cells were counted for each individual.

Contrasting nurses, foragers and winter bees by high throughput quantification of phagocytosis

In order to measure phagocytosis with flow cytometry, individual foragers, nurse and winter bees were collected simultaneously and injected with latex beads (see before). After hemolymph extraction, the sample preparation was essentially the same, apart from the following modifications. Hemolymph was collected directly into tubes with 100μL collection buffer on ice. 100μL of 4% PFA and a 1:1000 dilution of DAPI in PBS was added to the hemolymph. Samples were analyzed using a MACSQuant® analyzer, >3000 cells were counted for each individual (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany). DAPI was detected using the V1 channel (excitation = 405nm, detection = 450nm), fluorescent latex beads were detected in the R1 channel (excitation = 635nm, detection = 655-730nm). Flow cytometry data were analyzed with the MASCQuantify™ software package (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany). Negative controls for phagocytosis and hemocyte markers were included, by omitting latex bead and DAPI staining, respectively. Non-hemocyte particles were excluded by gating the cell population in forward scatter (FSC) vs side scatter (SSC). Gating adjustments to remove additional background noise, i.e. particles without DAPI or without latex beads, were made by using the negative control samples. The resulting data allowed to calculate the phagocytic rate by contrasting the counts for DAPI only objects (hemocytes), and objects that were both positive for DAPI and for the phagocytosis marker (hemocytes with latex beads).

Using flow cytometry, we could not distinguish the hemocyte types based on size and granularity dot plots, a typical approach used for blood testing. However, our results are in line with a previous study (de Graaf et al., 2002), that explains the failure to detect type dependent differences with the relatively small size of honey bee hemocytes.

Imaging of Vg positive hemocytes

Hemolymph extraction and imaging was essentially done as described above in the section of qualitative assessment of phagocytosis. For Vg specific immunochemistry, (compare S1 Fig), slides with hemocytes were first blocked for 30min with 5% BSA/PBS, followed by overnight incubation with the rabbit-anti-Vg-antibody 1:500 (except for negative controls) at 4˚C. Samples were again washed once in PBS for 5min and subsequently incubated with the secondary antibody (1:200 for 1h, Alexa-647 conjugated goat-anti-rabbit antibody in PBS-1% BSA). Finally, samples were co-stained for 45min with 1:1000 DAPI and Alexa Flour 488 Phalloidin. After staining, slides were washed 2 times for 5min before they were mounted and imaged as described before.

Quantification of Vg positive hemocytes in nurses, foragers and winter bees

For quantification of Vg positive hemocytes, we contrasted nurses, foragers and winter bees. Hemolymph was collected directly into tubes with 100μL collection buffer on ice. 100μL of 4%

PFA in PBS was added to the hemolymph and incubated at room temperature for 20min, followed by centrifugation at 2000g for 5min at room temperature. Cells were re-suspended in 0.2% Triton-X in PBS (5min) for permeabilization. This was followed by one washing with PBS, and blocking with PBS-5% bovine serum albumin (BSA). Samples were then incubated overnight at 4˚C with a specific rabbit anti-Vg-antibody (1:500 in PBS-1% BSA, Salmela et al., 2015), again followed by 2 washes with PBS. Samples were then incubated for 20min with Alexa-647 secondary antibody. After three washes, samples were re-suspended in PBS together with a 1:1000 dilution of DAPI. Extensive washing of hemocytes typically results in a reduction of hemocyte numbers in the samples. Yet, at least 1000 events per each sample were recorded in the flow cytometric measurement. In the flow cytometer, DAPI was detected as before, Vg was detected in R2 (excitation = 635nm, detection = 750nm). Single color controls included a DAPI-only control, and a negative control for Vg, with only secondary antibody incubation, additionally, background noise was removed.

Correlation analysis for phagocytic rate and Vg levels related to mitosis

Data on phagocytic rate and Vg levels were obtained from experiments described in the sections "Contrasting nurses, foragers and winter bees by high throughput quantification of phagocytosis" and "Quantification of Vg positive hemocytes in nurses, foragers and winter bees", respectively. Mitosis levels were calculated based on the different DAPI-intensity distributions. Briefly, a more intense DAPI staining due to their greater amount of DNA and a second peak in the DAPI-signal histogram [53, 54], identifies mitotic cells.

Statistical analyses

The T-test was used to test different phagocytic rate between CM-Dil and latex bead injections. Phagocytic rates in tests with *E.coli* pre-injection and two controls were contrasted using one-way ANOVA. Phagocytic rates and Vg levels in different worker types were analyzed by a one-way ANOVA, and post hoc Fisher Least Significant Difference (LSD) test. Possible links between phagocytosis and mitosis, as well as vitellogenin and mitosis were tested with Pearson's correlation analysis. All analyses were performed with the Statistica 13.2 software package (Round Rock, TX, USA).

Results

Establishing phagocytic markers in honey bees

To facilitate our studies, we established protocols for incorporation of two phagocytic markers in honey bees; fluorescent latex beads and CM-Dil stain. Microscopy confirmed that both markers could be incorporated by hemocytes (Fig 1). Using available morphological classification systems [18, 38, 49–52], we found that plasmatocytes and granulocytes were taking up both fluorescent markers (e.g., compare Fig 1A and 1C). Typically, the phagocytic cells took up only few latex beads (Fig 1A and 1B). Likewise, only a small fraction of the cells' cytosol was found to be positive for the CM-Dil stain (Fig 1E–1I). In contrast, observations of the uptake of larger bead numbers by a single hemocyte was rare (Fig 1C). Similar for both labeling techniques, we found that about 3% of hemocytes were positive for the two markers (Fig 2A, T-test: $N_{latex\ beads} = 11$, $N_{CM\text{-}Dil} = 15$, $F = 2.003$, $P = 0.27$). This supports that latex beads and CM-Dil are both suitable as phagocytic markers. The data are presented in detail in S1 Table. Lastly, we tested if immune activation by *E.coli* 2h prior to latex bead injection affects the level of detectable phagocytosis. We found that phagocytosis levels were not different in individuals with prior *E. coli* injection and in the two controls, i.e. with prior Grace's medium injection

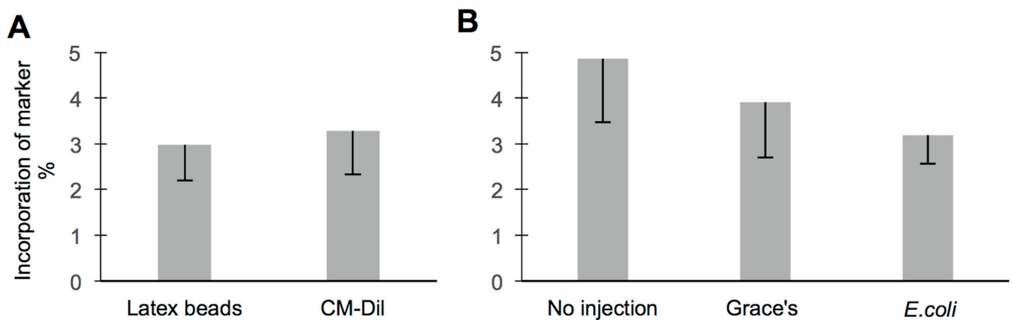

Fig 2. Measurements of phagocytic events do not differ between phagocytic markers and are not affected by a bacterial challenge. A Quantitative analyses of the latex bead and CM-Dil uptake with high-resolution microscopy yield similar results (T-test: $P>0.05$). The relative ratio of marker positive hemocytes in mature nurse bees is shown as mean ± SE. **B** Activation with *E.coli* or Grace's insect media as control does not change the relative ratio of the phagocytic cells in mature nurse bees compared to the individuals with no activation, shown as mean ± SE.

https://doi.org/10.1371/journal.pone.0184108.g002

and no prior injection (Breakdown and one-way ANOVA: $N_{no\ injection} = 9$, $N_{grace's\ medium} = 12$, $N_{e.coli} = 12$, F = 0.57, *P* = 0.57, Fig 2B). Hence, we concluded that relative phagocytosis levels in honeybees could be quantified using latex bead injection without a prior immune activation by *E. coli* inoculation. Data are presented in S2 Table.

Comparing phagocytic activity in different worker castes

We next established a flow cytometry assay for high-throughput analysis of phagocytosis in the honey bee. Honey bees were injected with latex beads—the phagocytic marker. The nuclear marker DAPI was used as a reference to count hemocyte numbers. No clear clustering of hemocyte population could be distinguished (Fig 3A for a representative example). The DAPI-labeled objects were further separated (gated) into phagocytic (co-labeled with DAPI and latex beads) or non-phagocytic (labeled only with DAPI) hemocytes (Fig 3C, compare Material and Methods section). Fig 3B shows the histogram for a DAPI labeled sample and a non-DAPI control.

We contrasted phagocytic activity in three different worker castes: mature nurse bees (n) and foragers (f) as well as winter bees (w). A flight-room was used to induce typical summer worker-type behavior, allowing the simultaneous collection of typical summer worker castes in the flight-room (nurses, foragers) and the winter worker caste at outside locations (winter bees). The average percentage of phagocytic hemocytes detected with flow cytometry was 20.1% in nurses, as compared to only 9.0% in foragers, and 8.3% in winter bees (see Discussion). We detected a significant difference in the phagocytic activity among worker castes (One-way ANOVA; $N_n = 15$, $N_f = 13$, $N_w = 18$, F = 6.8, $P<0.01$, Fig 3D). Post-hoc analyses revealed a significantly higher phagocytic activity in nurses as compared to foragers and winter bees (Fisher LSD test; $P_{(n\ vs\ f)} < 0.01$, $P_{(n\ vs\ w)} < 0.01$) but no significant difference between foragers and winter bees ($P_{(f\ vs\ w)} = 0.85$).

Comparing levels of Vg in hemocytes in different worker castes

With high-resolution imaging, we established the presence of Vg within plasmatocytes and granulocytes (S1 Fig). We used flow cytometry to determine the number of Vg-positive cells in the hemolymph of nurse, forager and winter bees (Fig 4). The percentage of Vg- positive cells

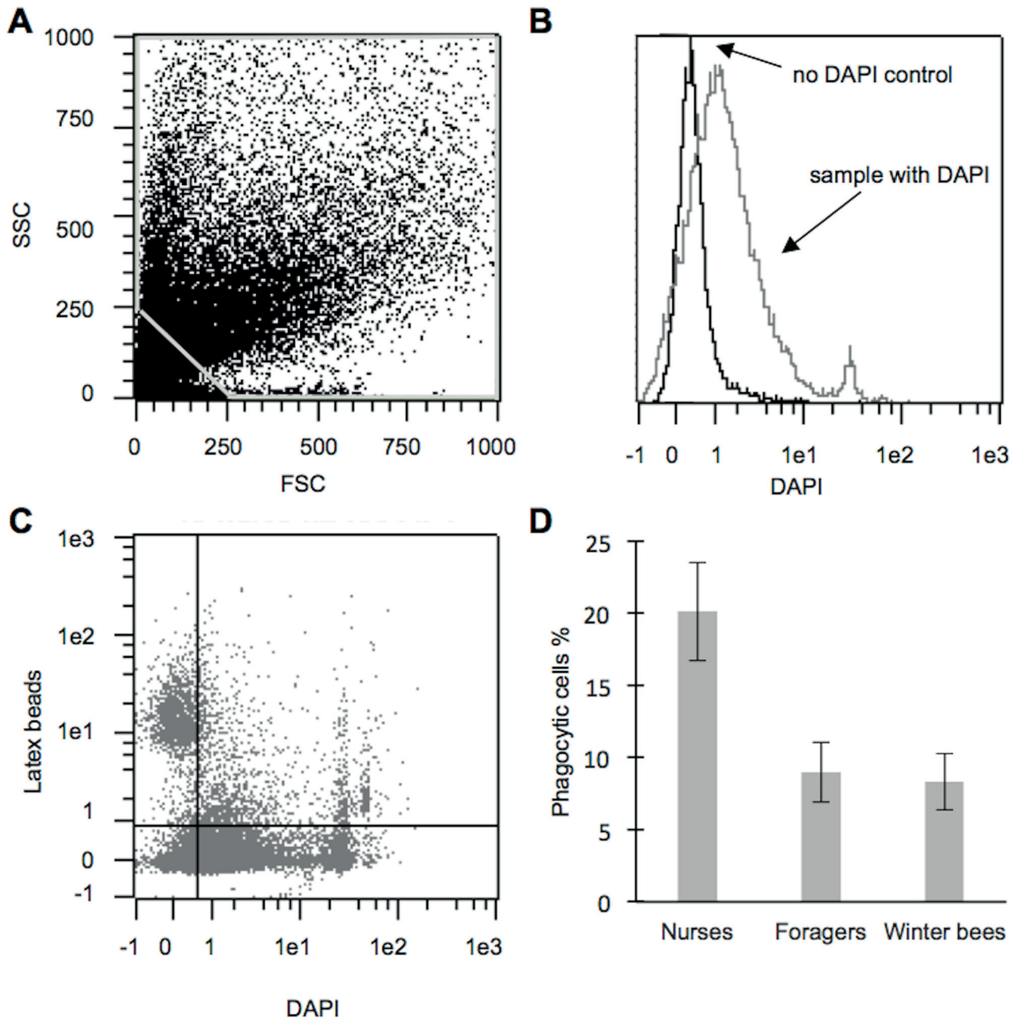

Fig 3. The phagocytic activity is higher in nurses than in foragers or winter bees. A Dot plot separated with FSC (size) and SSC (granularity) exemplifying the gating used to remove small particles and background noise. No clusters in hemocyte populations are detected. **B** A histogram of one hemocyte sample incubated with DAPI (grey), and a hemocyte control sample incubated without DAPI (black). The histogram illustrates how to exclude background and false positives from the DAPI-specific fluorescence. **C** Dot plot of latex beads *versus* DAPI. Each dot represents a single particle from gating in A. DAPI positive cells with nuclei are shown to the right in the plot. To the left DAPI negative particles are shown, which were excluded from further analysis (separated by the vertical line). Hemocytes in the upper right region are counted as phagocytic, and hemocytes in the lower right region as non-phagocytic (separated by the horizontal line). **D** Mature nurse bees have a statistically significantly higher percentage of phagocytic activity than foragers or winter bees (One-way ANOVA: $P<0.01$). Data are presented as means ± SEM.

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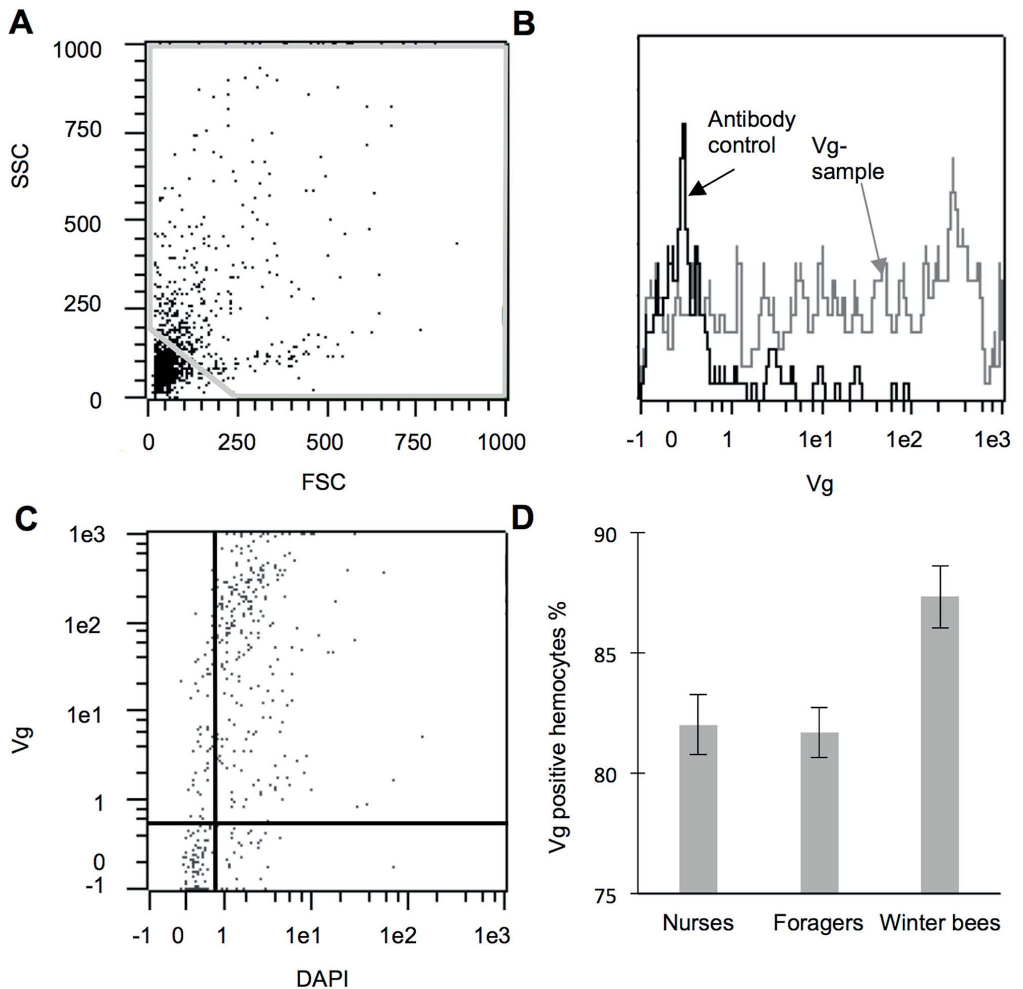

Fig 4. Quantification of Vg positive cells from hemolymph in nurses, foragers and winter bees. A A dot plot separated with FSC and SSC shows the gating for removing small particles and the background noise. **B** Immunostaining of hemocytes with and without the Vg primary antibody. The histogram shows hemocytes incubated with the secondary antibody only (control: black) and hemocytes incubated with both the Vg primary and secondary antibodies (grey). **C** Dot plot from a Vg positive sample, where the gating approach (the horizontal and vertical lines) are adapted from the controls of DAPI and Vg antibody. The two regions to the right are counted as hemocytes with the upper right region as Vg positive hemocytes. **D** The mean percentage of the Vg positive hemocytes is higher in winter bees compared to nurses and forager bees (One-way ANOVA: *P*<0.01). The data are presented as means ± SEM.

https://doi.org/10.1371/journal.pone.0184108.g004

was calculated based on detected Vg and DAPI signals (compare previous section; Fig 4C). As illustrated in the histogram (Fig 4B), Vg-positive cells can be separated from Vg- negative objects by using a Vg-negative control as a reference. We found an overall effect of worker caste on the rate of Vg-positive hemocytes (One-way ANOVA; $N_{n=}$ 14, $N_{f=}$ 17, $N_{w=}$ 14, F = 7.07, *P*<0.01). On average, winter bees had 87.3% Vg-positive hemocytes, while nurses

and foragers had 82.0% and 81.7%, respectively (Fig 4D). Post hoc analyses revealed significantly higher levels of Vg positive hemocytes in winter bees compared to both nurse and forager bees (Fisher LSD test; $P_{(n\ vs\ f)} = 0.84$, $P_{(n\ vs\ w)} < 0.01$, $P_{(f\ vs\ w)} < 0.001$).

Correlation analysis for phagocytic rate and Vg levels related to mitosis

We next studied possible correlations between phagocytosis and hemocyte recruitment through mitosis (Fig 5A; S3 Table), by using the same data set as for phagocytic rate assessments (compare Fig 3). For mature nurses, we found a significant negative correlation between phagocytic activity and mitotic activity (N = 15, $r^2 = 0.35$, $P < 0.05$). Foragers (N = 13, $r^2 = 0.08$, $P = 0.34$) and winter bees (N = 18, $r^2 = 0.06$, $P = 0.34$) showed no such correlation.

Lastly, we studied correlations between Vg levels and hemocyte mitosis (Fig 5B; S4 Table), using the same data set as for Vg in hemocytes assessments (compare Fig 4). For winter bees, we found a significant negative correlation between Vg levels and mitosis (N = 14, $r^2 = 0.63$, $P < 0.001$), while no relationships were detected in nurses (N = 15, $r^2 = 0.09$, $P = 0.28$) and foragers (N = 17, $r^2 = 0.13$, $P = 0.15$).

Discussion

Here we confirm that honey bee worker castes differ in hemocyte function. Nurses show two-fold higher levels of phagocytic activity compared to long-lived winter bees and foragers. Moreover, our data support tradeoffs between phagocytic activity and hemocyte recruitment through mitosis. Lastly, we demonstrate that the majority of hemocytes are immunopositive for Vg, and that Vg levels are negatively correlated with mitotic activity. However, links between phagocytic activity, hemocyte recruitment and Vg levels were only detected for specific worker castes.

To assess phagocytic activity, we adapted protocols for different phagocytosis markers, which were established in other insect species, e.g., *D. melanogaster* and *A. gambiae* [43–47]. Our simultaneous use of the respective markers allowed us compare their performance with quantitative assays. Our results show that both CM-DiI and latex beads are taken up at similar rates independent of protocol differences, including greatly different incubation times (latex beads = 2h, CM-Dil = 20min). In addition, we tested if prior bacterial infection can increase phagocytic immune response [55] as shown for *A. Aegypti and A. gambiae* [39, 42, 43]. However, our data do not support a priming-effect for honey bee nurses at 4h after injection.

Comparing phagocytosis levels among different study systems or species is complicated by the use of different quantification methods. Similarly, with microscopy, we found incorporation of the markers to be approximately 3%, for both CM-Dil and latex beads, while flow cytometry yielded a considerably higher phagocytic rate of 20% (in nurses). This could indicate that phagocytic levels might have been underestimated using microscopy due to different sample preparation approaches [50]. For microscopic sample preparations, a critical step is adherence of hemocytes to the poly-L-lysine coated glass slides, hence that they are not washed away during staining procedures. Nevertheless, based on our flow-cytometry data for phagocytosis we found no support for the notion that honey bees possess a generally less competent immune-defense than other insects, although it is hard to compare values between studies. Previous studies in other species report both higher [43, 46], similar [45] and lower phagocytic rates [46] than we established with flow cytometry (8–20%) for honey bees. Perhaps different phagocytic rates are linked to specie-specific body size, with the smallest insects having the highest phagocytic activity [46].

An interaction between Vg and hemocytes was proposed a decade ago [6]. We detected Vg in the hemocytes of all worker castes tested, in both the plasmatocytes and granulocytes. The

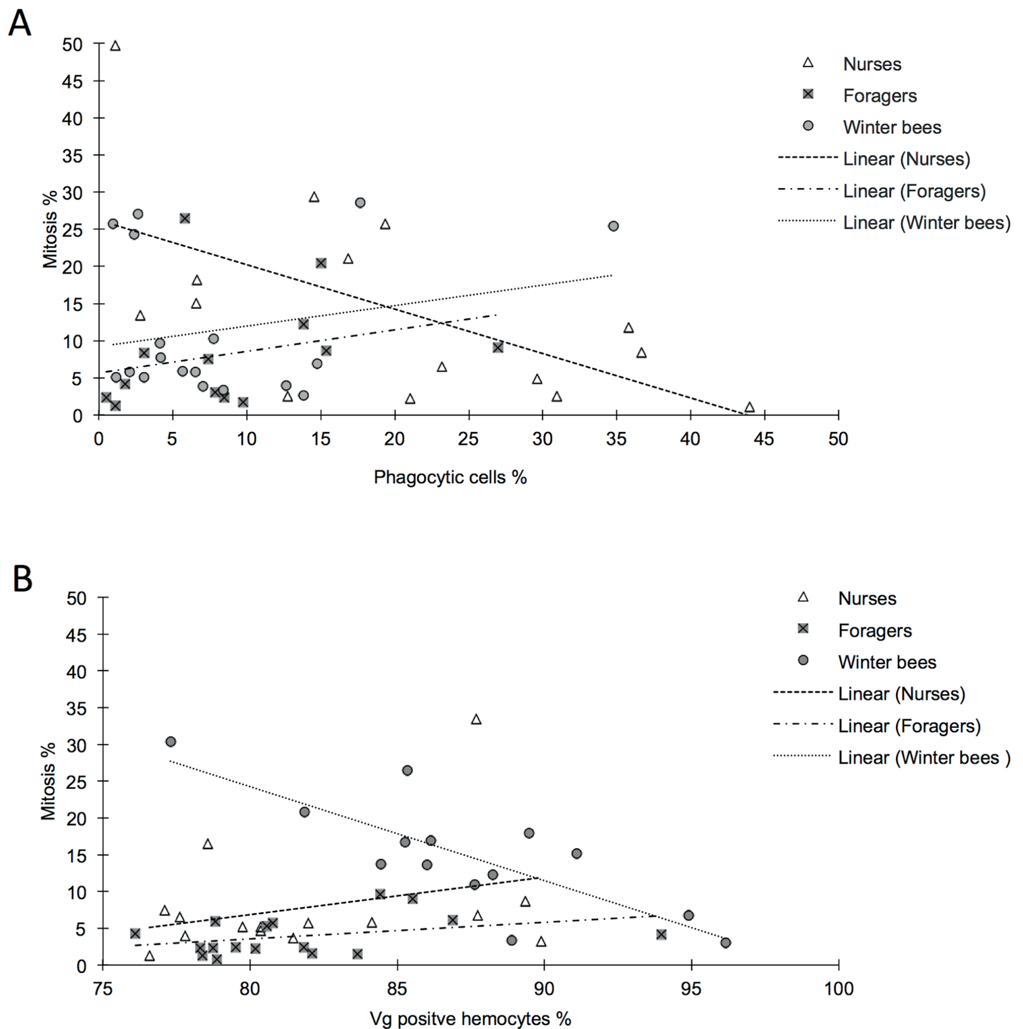

Fig 5. Correlation analysis, relation of mitosis and phagocytic hemocytes or vitellogenin positive hemocytes. A Phagocytic activity correlated to mitotic activity in nurses shows a significant negative correlation ($r^2 = 0.35$, $P<0.05$). This indicates that an individual has either a high phagocytic activity or a high mitotic activity. In forager and winter bees, no correlation was detectable. **B** Winter bees show a negative correlation between Vg levels and mitotic activity ($r^2 = 0.63$, $P<0.001$). This indicates that that the winter bees with a low number of vitellogenin positive hemocytes have more hemocytes that undergo mitosis. A significant correlation was not detected in nurse or forager bees.

https://doi.org/10.1371/journal.pone.0184108.g005

role of Vg in hemocytes might be related to its function as a zinc donor [6]. The zinc ion has important regulatory and catalytic roles in the immune system (reviewed by [56]). Another potential role for hemocyte-Vg is suggested by the finding that honey bee Vg acts as an opsonin that in the case of fish-Vg can make pathogens more susceptible to phagocytosis [32–34].

Specifically, honey bee Vg binds to surface molecules of pathogens, such as lipopolysaccharides [15], and this might activate hemocytes. Concerning worker-caste differences, we found highest levels of hemocyte-Vg for winter bees, reflecting the extreme Vg levels shown previously for total hemolymph fractions [57] reviewed by [13]. Despite the fact that foragers usually have lower Vg hemolymph levels than nurses [9], we found no difference in the number of Vg positive cells between these summer worker castes. A possible explanation for this could be that Vg, once internalized, does not degrade in persisting hemocytes.

The negative correlation between phagocytosis and mitosis we found for nurse bees, suggests a tradeoff, in which individuals with an already high phagocytic activity recruit less hemocytes. In turn, individuals with less phagocytic active hemocytes might need to recruit more hemocytes, and therefore increase mitotic activity. Yet, we could not find support for the latter, as reduced phagocytic activity in foragers did not correlate with mitotic activity. Likewise, winter bees showed no correlation between phagocytosis and mitosis, but a negative correlation between Vg levels and mitosis. This again may support that if protective mechanisms are already in place, like the abundant availability of Vg (compare [31]), there is less need to further increase immunocompetence, for example by mitotic hemocyte recruitment.

Based on available research data as well as on evolutionary theories on aging and cast-specific survival capacity (e.g., disposable soma theory), it has often been suggested that somatic maintenance is reduced in foragers. This view is supported by observations of reduced stress-resilience and faster aging in foragers [5, 10, 31, 58]. Along the same lines, we show that phagocytic levels are reduced in foragers as compared to nurse bees. This is in line with previous reports that found increased levels of pycnotic hemocytes in foragers [9], and that the nodulation response is abolished in forager bees [20]. Together, our data and the previous findings indicate a gradual loss of immune function integrity in foraging honey bees. Contrasting this conclusion are findings that show an increased phenoloxidase activity in older forager bees as compared to newly emerged bees [12, 41], or similar encapsulation responses in foragers and newly emerged bees [41]. A possible explanation for such contrasting findings is that newly emerged honey bees are immature. They perform poorly in many assays, including tests of sensory performance [59], learning and memory (discussed by [59]), flight ([60], for a general review on honey bee development see [61] and discussed by [62]), and worker physiology takes days to mature [61]. From this perspective, it may not be surprising that some aspects of honey bee immune function perform less well at the beginning of adult life.

Supporting information

S1 Fig. Vitellogenin is detectable within hemocytes. Both granulocytes and plasmatocytes were Vg positive. A-C Granulocyte morphology was identified by combined nuclear (DAPI, cyan) and F-actin (Alexa Flour 488 Phalloidin, green) staining. Superposition of the cell staining and the Vg (Alexa-647 conjugated goat-anti-rabbit antibody, red) signal suggests Vg to be localized within granulocytes (C). Scale bar for A-C = 10μm D-F Plasmatocyte morphology was labeled as in A-C and show co-localization with the Vg signal. Scale bar for D-F = 10μm.
(PDF)

S1 Table. Data for comparison of beads and CM-Dil injection, Fig 2A. Percentage of incorporation of markers.
(PDF)

S2 Table. Data for *E.coli* injection, Fig 2B. Percentage phagocytosis for the different individuals in the sample set.
(PDF)

S3 Table. Data for phagocytosis and mitosis, Figs 3 and 5. Percentage phagocytosis and mitosis for the different individuals in the sample set.
(PDF)

S4 Table. Data for vitellogenin levels and mitosis, Figs 4 and 5. Percentage of vitellogenin levels and mitosis for the different individuals in the sample set.
(PDF)

Acknowledgments

We thank Claus Kreibich managing beehives and taking care of the flight room. We thank Hilde Kolstad and Elin Ørmen at the Imaging centre at NMBU for support whenever needed. We thank Charlotte Kleiveland for help with the flow cytometry, Else Marie Aasen for sharing the *E.coli* strain and Dzung Diep for equipment for *E.coli* growth.

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Writing – original draft: Daniel Münch.

Writing – review & editing: Eva Marit Hystad, Heli Salmela, Gro Vang Amdam.

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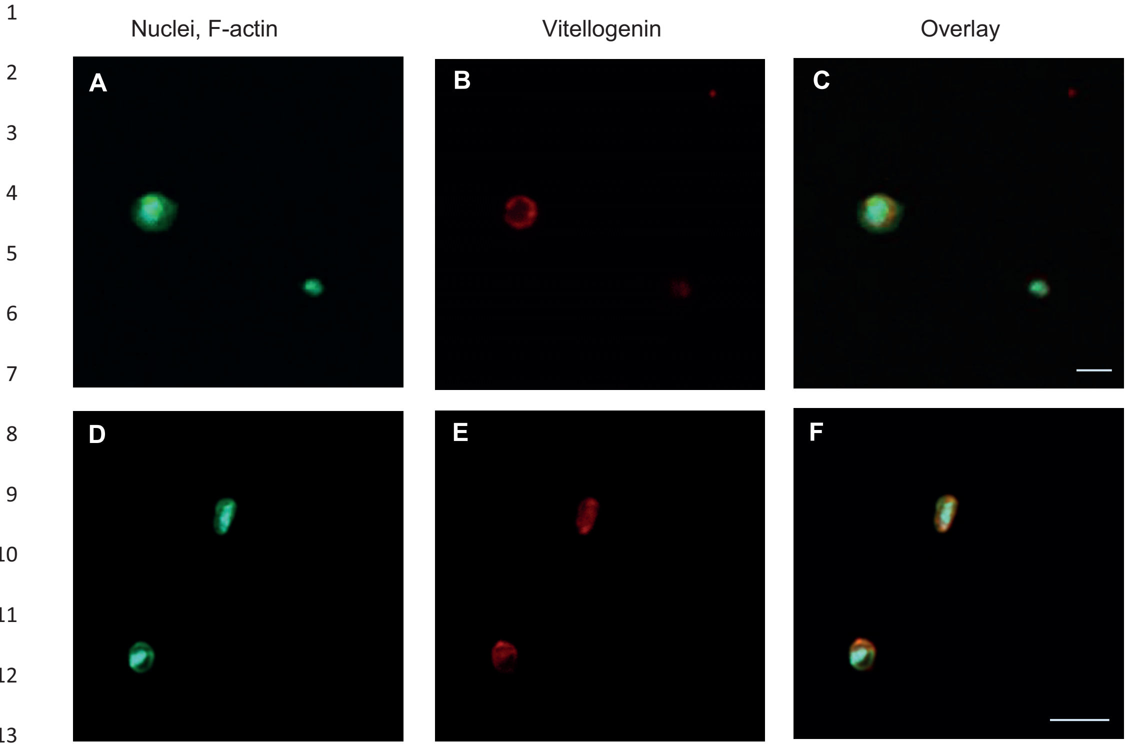
Supporting information

1



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14 **S1 Fig**

ID	Percentage
Bead 1	1,851851852
Bead 2	4,838709677
Bead 3	1,923076923
Bead 4	1,176470588
Bead 5	5,970149254
Bead 6	8,474576271
Bead 7	0
Bead 8	0
Bead 9	3,125
Bead 10	3,846153846
Bead 11	1,650165017
CM-Dil 1	4,87804878
CM-Dil 2	8,421052632
CM-Dil 3	4,47761194
CM-Dil 4	1,408450704
CM-Dil 5	13,63636364
CM-Dil 6	4
CM-Dil 7	1,612903226
CM-Dil 8	3,496503497
CM-Dil 9	0
CM-Dil 10	0
CM-Dil 11	0
CM-Dil 12	0
CM-Dil 13	1,515151515
CM-Dil 14	3,719008264
CM-Dil 15	2,127659574

Sample	% Phagocytic cells
Ec1	0
Ec3	4,109589041
Ec4	3,80952381
Ec5	1,639344262
Ec6	4,166666667
Ec7	0
Ec8	4,615384615
Ec9	3,968253968
Ec10	6,52173913
Ec12	0,760456274
Ec13	5,714285714
Ec14	2,941176471
Grace1	4,081632653
Grace2	0
Grace3	3,225806452
Grace4	0,492610837
Grace5	2,597402597
Grace6	14,70588235
Grace7	1,351351351
Grace8	4,761904762
Grace9	1,176470588
Grace10	8,379888268
Grace11	5,194805195
Grace12	0,892857143
Control 1	6,790123457
Control 2	13,63636364
Control 4	1,694915254
Control 5	0
Control 6	5,309734513
Control 7	1,986754967
Control 8	2,941176471
Control 9	3,448275862
Control 10	7,894736842

ID	% phagocytic cells	% mitotic cells	Group
1 nurse h1.012	44,00452489	1,131221719	N
2 nurse h1 .014	12,77161863	2,527716186	N
3 nurse h1 .015	23,17229585	6,512754738	N
4 nurse h1 .016	36,69631512	8,411689962	N
7 nurse h2 .017	21,04956268	2,274052478	N
8 nurse h2 .019	35,78750293	11,84179733	N
10 nurse h2 .020	29,63385541	4,849082045	N
13 forager h1.022	0,49045367	2,355929235	F
14 forager h1.023	7,364173512	7,493875198	F
15 forager h1.024	1,076320939	1,204275177	F
19 forager h2 .028	3,031578947	8,392982456	F
21 forager h2 .029	15,02093643	20,41872859	F
22 forager h2 .030	13,84998223	12,22182723	F
25 winter h3.033	34,79566523	25,42107325	W
26 winter h3.034	2,659848947	27,03945208	W
27 winter h3.035	0,966414126	25,70542266	W
28 winter h3.036	17,63929768	28,53445651	W
31 winter h4 .039	6,519224541	5,792809883	W
32 winter h4 .040	7,006058833	3,875458679	W
33 winter h4 .041	8,416626192	3,387100897	W

34 winter
h4 .042 13,82427223 2,635251894 W
37 nurse
h2.045 19,3269668 25,69349704 N
39 nurse
h1 .047 30,93795888 2,560572687 N
40 nurse
h1 .048 1,07809131 49,74219556 N
42 nurse
h1 .050 14,56568676 29,38050158 N
43 nurse
h1 .051 2,797001153 13,45155709 N
45 nurse
h2 .053 6,558122612 15,03547389 N
46 nurse
h2 .054 16,83753258 21,04257168 N
47 nurse
h2 .055 6,630921678 18,16207411 N
49
forager
h1 .057 9,726463104 1,692111959 F
51
forager
h1.059 5,791106515 26,4506492 F
52
forager
h1.060 15,38082933 8,687092408 F
53
forager
h1.061 7,836485455 3,048935629 F
54
forager
h1.062 1,741877642 4,216653147 F
55
forager
h1.063 8,478292647 2,368074843 F
56
forager
h1.064 26,96502058 9,063786008 F
58 winter
h3.066 12,67605634 3,979031137 W
59 winter
h3.067 3,049488235 5,070169885 W
60 winter
h3.068 2,380170716 24,35160867 W
61 winter
h3.069 7,741303283 10,32990364 W
62 winter
h3.070 5,669761273 5,868700265 W

65 winter

h4 .073	4,183311977	7,786236527	W

66 winter

h4 .074	14,7611486	6,894270213	W

67 winter

h4 .075	2,06779998	5,835570136	W

68 winter

h4 .076	1,154912227	5,043116723	W

69 winter

h4 .077	4,107142857	9,62406015	W

Name	% Vg positive cells	% Mitotic cells	Group
_1 N.014	89,87730061	3,269754768	N
_2 N.015	81,4516129	3,645833333	N
_3 N.016	87,71929825	6,707317073	N
_4 N.017	81,97278912	5,685618729	N
_7 N.018	89,33717579	8,707865169	N
_8 N.019	76,57657658	1,307189542	N
_9 N.020	77,77777778	3,95480226	N
_10 N.021	79,75206612	5,185185185	N
_13 F.022	84,40860215	9,578544061	F
_14 F.023	79,50819672	2,43902439	F
_15 F.024	80,56338028	5,292479109	F
_16 F.025	93,97260274	4,146341463	F
_19 F.026	80,76923077	5,703422053	F
_20 F.027	78,31325301	2,325581395	F
_21 F.028	86,8852459	6,111111111	F
_22 F.029	82,11382114	1,621621622	F
_25 W.030	86,14457831	16,91729323	W
_26 W.031	91,08910891	15,15151515	W
_27 W.032	94,90333919	6,692913386	W
_28 W.033	86,02150538	13,63636364	W
_31 W.034	88,23529412	12,32876712	W
_32 W.035	89,47368421	17,92828685	W
_33 W.036	96,16724739	3,006329114	W
_34 W.037	88,88888889	3,344481605	W
_37 N.054	87,67908309	33,43108504	N
_38 N.055	77,09251101	7,5	N
_39 N.056	84,14096916	5,853658537	N
_43 N.057	78,57142857	16,52542373	N
_44 N.058	80,3652968	5,240174672	N
_45 N.059	80,35320088	4,705882353	N
_50 F.061	78,86178862	0,787401575	F
_51 F.062	78,37837838	1,276595745	F
_55 F.063	85,5227882	9,034267913	F
_56 F.064	78,82736156	5,94795539	F
_57 F.065	78,74015748	2,272727273	F
_61 W.066	77,28842832	30,42328042	W
_62 W.067	85,2739726	16,72473868	W
_63 W.068	85,34883721	26,50918635	W
_67 W.069	87,6146789	10,93023256	W
_68 W.070	81,84931507	20,81784387	W
_69 W.071	84,43579767	13,75	W
_FA H1.038	80,17241379	2,162162162	F
_FB H1.039	83,65384615	1,449275362	F
_FC H1.041	76,10619469	4,301075269	F
_FD H1.042	81,81818182	2,4	F
_NA H1.072	77,6119403	6,52173913	N

Paper III

Title: Different activation of immune-related genes in honey bee nurses and foragers (*Apis mellifera*).

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Short title: Immune-related genes in honey bee

Abstract

Honey bee workers show very flexible aging patterns. Their typical behavioral progression, from nurse bees to forager bees, is associated with an onset of aging symptoms. Here, we studied how immune-activation can affect nurse and forager bees differently. Nurse and forager bees of identical chronological age were injected with vehicle control or the bacterial cell walls components lipopolysaccharide (LPS) or peptidoglycan (PGN). Next, we monitored survival and tested the expression of three immune-related genes. We confirm that forager bees die sooner than same-aged nurse bees, independent of the type of immune challenge. The relative gene expression patterns of two immune-related genes differed between the nurse and forager honey bees as well as between the treatment groups. Our findings support that nurse and forager honey bees can respond differently to immune challenge, despite a lack of detectable differences in mortality between treatments. This response was specifically associated with the workers' behavioral caste, as the chronological age was similar among all tested animals.

Key words

honeybee/ aging/ immune activation/ *vitellogenin*/ *defensin-2*

1. Introduction

Honey bee colonies are maintained by elaborate social interactions between worker bees. Workers collect, process and pass on food, engage in hygienic behaviors, nest defense and thermoregulation (Seeley 1995). During adult life, most honey bee workers transition from one social task to another. Younger adult bees typically carry out in-nest tasks, such as food jelly production and nursing (nurse bees). Successively, they shift to outside tasks, mostly foraging for nectar and pollen (forager bees). Forager bees rapidly develop aging symptoms, including increased mortality, impaired behavioral performance, accumulation of lipofuscin (cellular 'waste'), oxidative stress damage, and reduced numbers of functional hemocytes (circulating immune cells) (reviewed by (Münch and Amdam 2013)). A decline in abundance of intact, circulating immune cells in forager bees could imply that honey bee immunity is a function of behavioral caste (Amdam, Aase et al. 2005). However, apparently contradicting this proposed negative association between immunity and foraging behavior is the upregulation of humoral immunity and phenol oxidase activity in foragers (Schmid, Brockmann et al. 2008, Wilson-Rich, Dres et al. 2008, Laughton, Boots et al. 2011).

After immune activation, humoral effector molecules are released into the hemolymph (insect blood) (reviewed by (Hillyer 2016)) from the fat body (analogous to the mammalian

liver and white adipose tissue). The fat body is central to the insect innate immune system, and it is, e.g., the production-site of various antimicrobial proteins and peptides (AMPs) (reviewed by (Hillyer 2016)). Honey bees feature the four signaling pathways that are typically associated with insect immune responses (i.e. Toll, Immune deficiency (IMD), Janus kinase- Signal Transducer and Activator of Transcription (JAK-STAT), and Jun N-terminal kinases (JNK)). However, compared to established model insects such as *Drosophila melanogaster* and *Anopheles gambiae*, the number of immune-related genes in these pathways is only one third in honey bees (Evans, Aronstein et al. 2006). This reduction might confer a more limited capability to fight pathogens, or suggest that less explored molecular mechanisms play a role in honey bee immunity (Evans, Aronstein et al. 2006).

One such less explored pathway involves Vitellogenin (Vg). Vg is a lipoglycophosphoprotein that is more widely known for its role in egg-yolk formation. Vg is produced in the fat body of most female insects, including the functionally sterile honey bee workers. Honey bee Vg binds to dead bacteria (Salmela, Amdam et al. 2015), supports immune cell functions (Amdam, Aase et al. 2005), and reduces oxidative stress (Seehuus, Norberg et al. 2006). Vg hemolymph levels are higher in nurse bees compared to foragers. Hence, Vg levels are positively correlated with worker stress-resilience and life-span potential (Fluri, Wille et al. 1977, Amdam, Aase et al. 2005, Bordier, Suchail et al. 2017). Causal roles of Vg have been verified via RNA interference-mediated gene knockdown of the *vg* gene. This knockdown reduces honey bee stress resistance and shortens lifespan, in addition to effects on behavior (reviewed by (Münch and Amdam 2013)).

Recent experiments have contrasted the susceptibility of nurse and forager honey bees to multiple stressors, including starvation (Speth and others 2015), heat, pesticides, and mechanical wounding (Bordier and others 2016). Mechanical wounding represents an immune challenge that results in reduced *vg* gene expression in both nurses and foragers in some studies but not in others (discussed by (Bordier, Suchail et al. 2017)). Overall, there is considerable interest in effects of immune challenge in honey bee workers due to roles of diseases in the syndrome of Colony Collapse Disorder (Laughton, Boots et al. 2011, Kazlauskas, Klappenbach et al. 2016, Bordier, Suchail et al. 2017, Li, Chen et al. 2018).

Here, we ask how immune challenge with lipopolysaccharide (LPS) or peptidoglycan (PGN) affects worker honey bees, and whether bees representing different castes (nurse and forager bees) respond differently. We performed an experiment where we compared survival and immune-related gene expression in nurses and foragers. We used a standard protocol to produce worker bees of the same chronological age but with different behavioral task. This set-up, in which nurse and forager bees are of the same age, is a gold-standard for comparing task-dependent differences in this species (Huang and Robinson 1992).

We compared LPS and PGN injected nurse and forager groups with control groups injected with PBS (vehicle). Thereby, mechanical wounding was experienced by all the bees used in this study. We monitored worker survival and the expression of three immune-related genes: two AMPs and the *vg* gene. Our results support that worker survival is task dependent and not strongly influenced by the specific immunogen treatments we used in this study. However, we find that nurse and forager bees display different gene expression patterns in response to challenge with LPS or PGN.

This report can be of interest to the community that uses the honey bee as a model in aging research, and more generally in fields that focus on how the immune system tackles challenges across the lifespan.

2. Materials and methods

2.1 Bees

Honey bee foragers are usually chronologically older than nurse bees in natural colonies, which confounds behavior with age. To eliminate such age bias, we adopted an established method that allowed obtaining foragers and nurses of identical chronological age, thus avoiding this problem (Huang and Robinson 1992). Three single cohort colonies (SCC) of honey bees were established at Arizona State University, Tempe, USA. Each SCC was assembled by allowing worker bees to emerge from pupation in the laboratory, paint mark them within 24 h (Uni Posca, Japan), and placing them together (N = 3,000 workers) in a hive box containing one queen bee. Throughout the experiment, the three hives were monitored and managed to avoid that new workers emerged in the colonies. Thereby, the hives only contained workers of the same age. Under these conditions, the same-aged workers will divide labor between nursing and foraging (Huang and Robinson 1992).

2.2 Marking foragers in the SCC

After 15 to 17 days, we marked worker bees that were performing foraging tasks. These bees were painted with a new color, i.e. different from the color mark that the bees had already

received within 24 h of emerging from the pupal stage. Foraging behavior was confirmed when bees returned to the colonies with pollen on their hind legs, or with a nectar load in their distended abdomen (Huang and Robinson 1992, Behrends, Scheiner et al. 2007).

2.3 Collecting nurses and foragers

After 21-22 days, we collected nurses and foragers in the morning before foraging activity began. Nurses were identified with one mark (the original mark received within 24 h of emergence) combined with having their heads inserted into cells of the brood comb that contained larvae (Munch, Baker et al. 2013). Foragers were identified by having the additional color mark that they had received when returning from a foraging flight at least 5 days prior to the sample collection. This procedure ensured that we collected primarily established foragers that had been expressing foraging behavior for several days (Munch, Baker et al. 2013).

2.4 Immune challenge

Nurses and foragers were anaesthetized on ice in the laboratory and mounted on wax plates. They were injected abdominally with 1.5µl phosphate buffered saline (PBS, vehicle control), or two common immunogens using 701 LT Hamilton syringes and 31G needles (Sigma-Aldrich, Saint Louis, Missouri, USA). The immunogens were 1.5 µl lipopolysaccharide (LPS) derived from *Escherichia coli* serotype 055:B5 (0.5µg/µl/ PBS, Sigma- Aldrich, Saint Louis, Missouri, USA) or 1.5µl of peptidoglycan (PGN) derived from *Staphylococcus aureus*

(0.5µg/µl/ PBS, Sigma- Aldrich, Saint Louis, Missouri, USA) (Richard, Aubert et al. 2008, Laughton, Boots et al. 2011). Following injections, the worker bees were placed into small queen cages, with 7-9 bees per cage, and incubated at 33°C (CO_2 = 5%, relative humidity = 50-60 %). Each cage contained a combination of foragers and nurses representing all the three treatment groups. Cages were provided with bee candy (Apifonda, Südzucker AG, Ochenfurt, Germany) and a water source.

2.5 Survival experiment

Workers were collected from the SCC on day 22 of the experiment. Sampling, injection and incubation were performed as described in sections 2.3 and 2.4. Survival of the resulting bees in the queen cages was first monitored after 2, 4, 6, 8 h, followed by monitoring every 3 hours during the night and the following day, (11, 14, 17, 20, 23, 26, 29, 32 h), after which survival was recorded at 48 and 72 h post injection. Seventy-two hours after the injection, none of the bees survived across all three colonies. The specific sample sizes for each treatment were: $N(urse)_{control}$=68, N_{LPS}=75, N_{PGN}=61, $F(orager)_{control}$=67, F_{LPS}=70, and F_{PGN}=79 (Online resource table S1).

2.6 RNA extraction and cDNA synthesis

Worker bees were collected at day 21 and 22 of the experiment, immobilized on ice 8 h post injection. The gut and the stinger were removed, and the abdominal carcass was detached. The abdominal carcass, consisting mostly of cuticle and fat body, were placed in -80 °C.

RNA was extracted from 16 randomly picked samples in each treatment group by the general Trizol method (Wang, Brent et al. 2012). The quality and quantity of RNA was determined by spectrophotometry (Nanovue, GE Healthcare). DNase (RNase-free, DNase kit, Applied Biosystems, Bedford. MA, USA) was added to the total RNA extract to remove trace DNA contaminants, and 1 μg of such treated RNA was used for reverse transcription using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Bedford. MA, USA).

2.7 Quantitative Real-time PCR analyses

First-strand cDNA was used for real-time quantitative PCR (RT-qPCR) assays on an ABI Prism 7500 Real-Time PCR system (Applied Biosystems, Bedford. MA, USA). *vitellogenin* (AJ517411) (Wang, Brent et al. 2012), *defensin-2* (NM_001011638.1) (Richard, Aubert et al. 2008) and *hymenoptaecin* (NM_001011615.1) (Lourenco, Guidugli-Lazzarini et al. 2013) were chosen for gene expression analysis. The samples were organized on the optic plates (96 wells) to allow for powerful statistical comparison of treatment groups within each behavioral caste. With this strategy, we could make sound statistical inference about gene expression differences *within* the nurse bee caste and *within* the forager bee caste, but not *between* the two behavioral castes since nurses and foragers were represented on different optical plates. Each sample was run in technical triplicates for target genes and reference gene *actin* by means of the Delta-Delta Ct method (Wang, Brent et al. 2012). Studies have shown that actin is one of the most stable gene expressed across different stages, tissues and under stress condition (Lourenco, Mackert et al. 2008, Scharlaken, de Graaf et al. 2008) and is often used as a single reference gene for expression studies in honey bee (Alaux, Kemper

et al. 2012, Ihle, Rueppell et al. 2015, Rueppell, Yousefi et al. 2017, Li, Chen et al. 2018). One sample was lost during the procedures, leading to the final sample sizes of $N(urse)_{control}$=16, N_{LPS}=15, N_{PGN}=16, $F(orager)_{control}$=16, F_{LPS}=16, and F_{PGN}=16. By monitoring negative control samples (without reverse transcriptase) and melting curve analysis, we verified that the RT-qPCR assays were not confounded by DNA contamination or primer dimers (Wang, Brent et al. 2012).

2.8 Statistical analyses

Survial data was processed with Statistica's (Statistica 13, Dell Inc, Tulsa, USA) survival analysis tool. Specifically, survival curves were plotted using product-limited/Kaplan Meier estimates. Multiple-sample group testing was based on an extension of Gehan's as well as Peto and Peto's generalized Wilcoxon test, and the log-rank test. This approach was followed by Bonferroni-corrected (threshold p<0,003) post hoc Cox-Mantel pairwise comparisons between the treatment groups and for comparing survival between nurses and foragers independent of treatment. Post hoc Cox-Mantel multiple comparisons were also performed to contrast the tree SSC colonies of the experiment to determine possible colony effects. An additional analysis with ANOVA was used to explore confounding variables. In the ANOVA, we included colony as an independent variable in addition to treatment.

For the gene expression analysis, ANOVA was used to test overall effects of treatment, followed by Tukey post hoc test for multiple comparison of gene expression between

treatments. Tukey's test take multiple comparisons into account and no further correction of the *p*-values is required.

3. Results

3.1 Survival

None of the bees survived more than 72 h after treatment, which is consistent with a cost of immune system activation brought on by mechanical wounding during the injections of PBS, LPS and PGN. Nonetheless, we detected an overall survival effect in the experiment (Kaplan-Meier, sample sizes N(urse)$_{control}$=68, N_{LPS}=75, N_{PGN}=61, F(orager)$_{control}$=67 F_{LPS}=70 F_{PGN}=79, df=5, Chi^2=96.77, *p*<0.0001, fig 1). Bonferroni-corrected pairwise comparison by post hoc Cox- mantel test between groups (Online resource table S2), however, suggested that this overall effect was specifically explained by worker caste, with nurses surviving differently than foragers.

No specific immunogen treatment generated a detectable survival effect as compared to the control. In contrast, post hoc comparison between nurses and foragers confirmed a significant effect of caste on survival (Cox-Mantel, sample sizes Nurses=204, Foragers=216, U=82.25, *p*<0.0001) with nurses surviving longest (fig 1).

Our analysis of colony effects showed a significant survival difference between colonies (Cox-Mantel, sample sizes Colony1=140, Colony2=109, Colony3=171, df=2, Chi^2=19.54, *p*<0.0001). Therefore, we used ANOVA to explore whether the lack of a treatment effect in the previous survival analysis might be due to variance from this confounding factor

(colony). Reassuringly, ANOVA confirmed that treatment had no effect (ANOVA: $F=2.29$, $p=0.10$) while colony impacted survival significantly (ANOVA: $F=3.41$, $p=0.03$).

3.2 Gene expression in response to immune challenge

In nurse bees, expression of the AMP *defensin-2* was significantly upregulated after immunogen injection (ANOVA, sample sizes $N(urse)_{control}=16$, $N_{LPS}=15$, $N_{PGN}=16$, $p<0.0001$, fig 2A). Post hoc testing revealed significant differences between all individual treatments on *defensin-2* expression (Tukey: LPS vs Control: $p<0.05$, PGN vs Control: $p<0.001$, PGN vs LPS: $p<0.05$). In contrast, we did not detect treatment effects on the AMP *hymenoptaecin* (ANOVA, sample sizes $N(urse)_{control}=16$, $N_{LPS}=15$, $N_{PGN}=16$, $p=0.1$, fig 2B) or on *vg* (ANOVA, sample sizes $N(urse)_{control}=16$, $N_{LPS}=15$, $N_{PGN}=16$, $p=0.92$, fig 2C).

In forager bees, expression of *defensin-2* (ANOVA, sample sizes $F(orager)_{control}=16$, $F_{LPS}=16$, $F_{PGN}=16$, $p<0.01$, fig 3A) and *hymenoptaecin* (ANOVA: $F(orager)_{control}=16$, $F_{LPS}=16$, $F_{PGN}=16$, $p<0,0001$, fig 3B) was significantly upregulated after immunogen treatment. Post hoc analysis revealed a treatment effect for the gene *defensin-2* between PGN treated foragers compared to both LPS treated and control, but not between control and LPS injected workers (Tukey: PGN vs Control: $p=0.0014$, PGN vs LPS: $p=0.0125$, LPS vs Control: $p=0.71$). Expression of *hymenoptaecin* was upregulated in both, the PGN and LPS treatment compared to the control (Tukey: LPS vs Control: $p<0.001$, PGN vs Control: $p<0.001$, PGN vs LPS: $p=0.36$**).** There was, however, no significant treatment effect on *vg* (ANOVA: $F(orager)_{control}=16$, $F_{LPS}=16$, $F_{PGN}=16$, $p=0.27$, fig 3C).

4. Discussion

In this study, we exposed same-aged nurse and forager honey bees to two immunogens and control injections with PBS (vehicle control). We observed that forager bees died sooner than nurses, independent of the immune challenge they were exposed to. In contrast, nurse and forager bees appeared to regulate their immune response differently, depending on whether they received LPS or PGN challenge. Our results expand on the general finding that immunogen treatments, as well as mechanical wounding, are stressful events that impact worker bees in survival tests (Nelson, Ihle et al. 2007, Kohler, Pirk et al. 2012). Explicitly, we expand the list of immunogens to include injection of PGN, and we show that these survival results are valid when contrasting same-aged nurses and foragers, which is a gold-standard comparison in honey bee behavioral research. Yet, this standard may have some limitations relevant for experiments that include measurements of resilience or survival, because SCC have a larger representation of precocious foragers and over-aged nurses than normally age-structured colonies. These phenotypes might expose workers to elevated levels of physiological stress that could lead to susceptibilities and reduce lifespan (Guzmán-Novoa, Page et al. 1994, Rueppell, Bachelier et al. 2007). A recent study identified survival rates comparable to ours when contrasting 21-23 day-old nurses and foragers in a SCC. Also in their study, nurses survived significantly longer than foragers (Lourenco, Martins et al. 2019).

Our survival data and those of Lourenco, Martins et al. (2019) are consistent with previous research demonstrating that forager bees are generally more susceptible to stressors causing

a shortened survival as compared to nurses (Rueppell, Bachelier et al. 2007, Speth, Kreibich et al. 2015). Immune activation is often viewed as a cost that trades off with survival (Li, Chen et al. 2018). Yet, in the frailer and resource-limited worker group, foragers, we did not observe that survival was further reduced in response to immunogen injection as compared to the vehicle control, which did not contain an immunogen. In our experiment overall, variation in nurse and forager survival was not explained by immunogen exposure (vs. control). A possible explanation for this outcome is that LPS and PGN are nonpathogenic; i.e., they do not actively infect bees, while at the same time, our control treatment involved mechanical wounding which is an established (non-infections) protocol of immune challenge in bees (Bordier and others 2016). Thereby, our results are in accordance with previous studies that did not find specific survival differences in experiments involving LPS treated honey bees (Richard, Aubert et al. 2008, Alaux, Kemper et al. 2012, Kazlauskas, Klappenbach et al. 2016).

While survival effects may differ in tests with active and non-pathogenic immunogens, previous studies corroborate that AMP production and expression of immune-related genes can be affected by LPS injection (Richard, Aubert et al. 2008, Alaux, Kemper et al. 2012). We found different transcription patterns in nurse and forager bees for *defensin-2* and *hymenoptaecin.* PGN upregulated *defensin-2* in both nurses and foragers, while LPS upregulate *defensin-2* in nurses only. Forager upregulated *hymenoptaecin* in response to both LPS and PGN, while the expression of *hymenoptaecin* was unchanged in nurses. Consequently, our results indicate that nurse and forager honey bees regulate expression of

immune related genes differently. *Defensin-2* and *hymenoptaecin* are proposed to be downstream genes of the Toll pathway and IMD pathway, respectively (Evans, Aronstein et al. 2006), suggesting different acute pathway activation in these two behavioral castes of workers. Data from the PBS-injected control group supports this conclusion. Injections with PBS are sufficient to activate the immune system, and thereby, we reveal antigen-specific effects when contrasting the control to the LPS and PGN treatments. In other words, the antigen-specific effects in worker bees appear to go beyond a general response to mechanical wounding.

In this study, we did not detect an up- or down-regulation of the *vg* gene when measuring expression 8 h after injection of immunogens vs. PBS control. It is possible that *vg* mRNA levels are consistently changed in either direction by all the three challenges, or alternatively, none of these manipulations impact *vg*. A previous experiment showed that PBS injections can up-regulate *vg* expression compared to non-injected bees (Salmela, Amdam et al. 2015), while a different experiment showed that *vg* is down-regulated after a "pin prick" challenge (mechanical wounding, Bordier and others 2016). Future experiment should use time-series with multiple treatment groups and including non-injected controls to resolve possible dynamic patterns in the response of *vg* to different immune challenges.

Acknowledgements

We thank Nick Baker for managing the colonies. We thank Claus Kreibich for valuable input. We thank Jane Ludvigsen for reading and commenting on the text.

Author contributions

EMHB, HS, GVA: Designed the study, Analyzed data; EMHB, HS, AV, YW: Performed research; EMHB, DM, GVA drafted paper. All authors provided text, read and approved the final manuscript.

Conflict of interests

The authors declare that they have no potential conflict of interest in relation to the study in this paper.

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Figure captions

Figure 1: The life span of honey bee workers after LPS, PGN or control (PBS vehicle) injections. Nurse bees (N) survived significantly longer than forager bees (F) in general (Cox-Mantel, sample sizes Nurses=204, Foragers=216, U=82.25, $p<0.0001$). By comparing immunogen treated bees with control-injected bees, we found that the challenge with immunogens did not affect survival significantly (statistics in online resource table S2).

Figure 2: Gene expression in nurse bees was calculated by delta delta ct method against the *actin* gene. Each bar present the mean ± SEM. Significant difference are denoted by different lowercase letters (a, b, c). **A)** LPS and PGN injections resulted in up-regulation of *defensin-2*. All treatment group where significantly different from each other (ANOVA, sample sizes

$N(urse)_{control}$=16, N_{LPS}=15, N_{PGN}=16, p<0.0001, Tukey's post hoc test: LPS vs Control: p<0.05, PGN vs Control: p<0.001, PGN vs LPS: p<0.05). **B)** No significant changes in *hymenoptaecin* expression were detected in response to treatment (ANOVA, sample sizes $N(urse)_{control}$=16, N_{LPS}=15, N_{PGN}=16, p=0.10). **C)** No significant changes in *vg* expression were detected in response to treatment (ANOVA, sample sizes $N(urse)_{control}$=16, N_{LPS}=15, N_{PGN}=16, p=0.92).

Figure 3: Gene expression in forager bees was calculated by delta delta ct method against the *actin* gene. Each bar present the mean ± SEM. Significant difference are denoted by different lowercase letters (a, b). **A)** PGN challenge resulted in up-regulation of *defensin-2* compared to the control and LPS-treated foragers, while the LPS treatment did not differ from the control (ANOVA, sample sizes $F(orager)_{control}$=16, F_{LPS}=16, F_{PGN}=16, p<0.01, Tukey: PGN vs Control: p=0.0014, PGN vs LPS: p=0.0125, LPS vs Control: p=0.71). **B)** *Hymenoptaecin* was upregulated by PGN and LPS compared to control (ANOVA, sample sizes $F(orager)_{control}$=16, F_{LPS}=16, F_{PGN}=16, p<0,0001, Tukey: LPS vs Control: p<0.001, PGN vs Control: p<0.001, PGN vs LPS: p=0.36). **C)** *Vg* expression did not respond to treatment (ANOVA, sample sizes $F(orager)_{control}$=16, F_{LPS}=16, F_{PGN}=16, p=0.27).

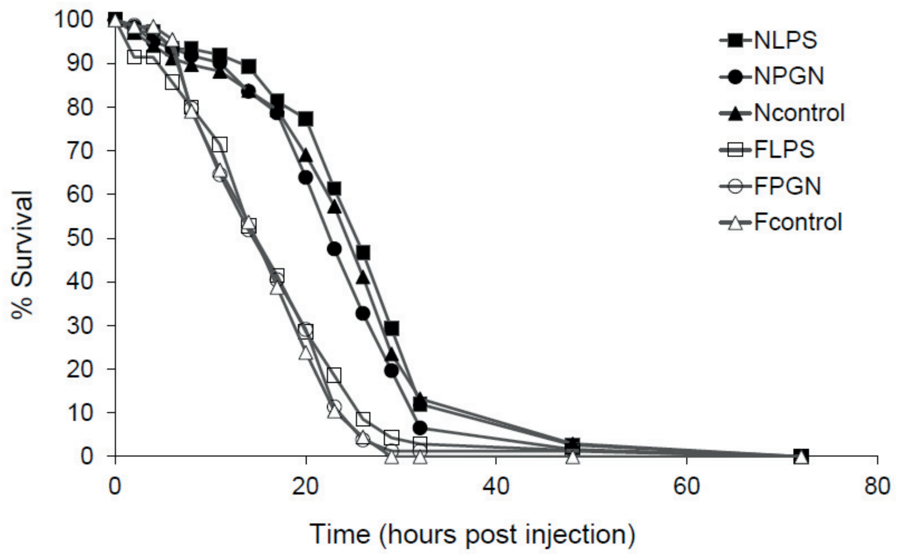

Figure 1: The life span of honey bee workers after LPS, PGN or control (PBS vehicle) injections.

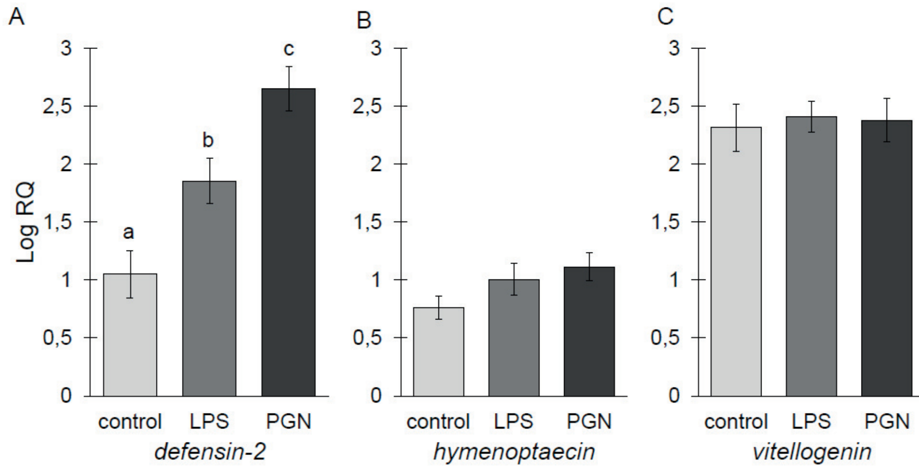

Figure 2: Gene expression in nurse bees was calculated by delta delta ct method against the *actin* gene.

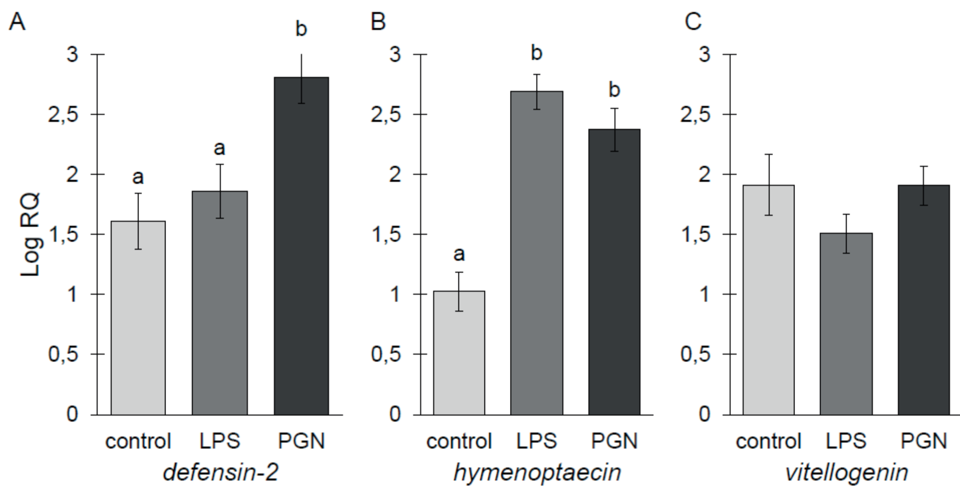

Figure 3: Gene expression in forager bees was calculated by delta delta ct method against the *actin* gene.

Supporting information

Title: Different activation of immune-related genes in honey bee nurses and foragers (*Apis mellifera*).

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Table S1: Number of bees included in the treatment groups of the survival experiment.

Treatment	**Colony 1**	**Colony 2**	**Colony 3**	**Total**
$N_{control}$[1]	25	15	28	68
N_{LPS}[2]	22	18	35	75
N_{PGN}[3]	20	17	24	61
$F_{control}$[4]	21	18	28	67
F_{LPS}[5]	25	18	27	70
F_{PGN}[6]	27	23	29	79

[1]Nurse PBS injected

[2]Nurse LPS injected

[3]Nurse PGN injected

[4]Forager PBS injected

[5]Forager LPS injected

[6]Forager PGN injected

Table S2: Post hoc Cox mantel test of survival. After Bonferroni correction for multiple testing *p*-values less than < 0,003 were considered significant.

	NPBS[1]	NLPS[2]	NPGN[3]	FPBS[4]	FLPS[5]	FPGN[6]
$N_{control}$	X	X	X	X	X	X
N_{LPS}	*p*= 0,21441 U= - 6,08246	X	X	X	X	X
N_{PGN}	*p*=0,45087 U=3,551747	*p*=0,04505 U=9,427568	X	X	X	X
$F_{control}$	***p*=0,00000 U=27,52505**	***p*=0,00000 U=33,28521**	***p*=0,00000 U=25,22109**	X	X	X
F_{LPS}	***p*= 0,00000 U= 22,39164**	***p*=0,00000 U=28,45455**	***p*=0,00003 U=19,65388**	*p*=0,32598 U=4,845546	X	X
F_{PGN}	***p*=0,00000 U=27,90076**	***p*=0,00000 U=34,49503**	***p*=0,00000 U=24,97405**	*p*=0,76850 U=1,470342	*p*=0,52288 U=3,310484	X

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Avhandlingens tittel:	Physiological resilience in honey bees (Apis mellifera)

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Ph.d.-kandidat:	Eva Marit H. Byhrø	Dato:	17.06.19

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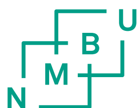
Errataliste

Ph.d.-kandidat: Eva Marit Hystad Byhrø
Avhandling: Physiological features of aging in honey bees (Apis mellifera)
Dato: 17.06.19

Side	Linje	Endret fra	Endret til
Tittel		Physiological resilience in honey bees (Apis mellifera)	Physiological features of aging in honey bees (Apis mellifera)
Tittel		Fysiologisk motstandsdyktighet hos honningbier (Apis mellifera)	Fysiologiske egenskaper ved aldring i honningbier (Apis mellifera)
Abstract		Aging and a decline in physiological resilience occur in most living organisms, including honey bee workers.	Aging and a change in physiological features occur in most living organisms, including honey bee workers.
Abstrakt		Aldring og nedgang i fysiologisk motstandsdyktighet inntreffer i de aller fleste levende organismer, inkludert honningbienes arbeidere.	Aldring og endring i fysiologiske egenskaper inntreffer i de aller fleste levende organismer, inkludert honningbienes arbeidere.
11		1.1 Physiological resilience and aging	1.1 Physiological features and aging
17		1.2 Honey bees as a research model for physiological resilience and aging	1.2 Honey bees as a research model for physiological features and aging
26		The main topic of this PhD thesis is aging and physiological resilience, and the methods used in the three papers	The main topic of this PhD thesis is aging and physiological features, and the methods used in the three papers
36		Overall, this thesis work highlights that physiological resilience and aging can be complex topics.	Overall, this thesis work highlights that physiological features and aging can be complex topics.

ISBN: 978-82-575-1611-6
ISSN: 1894-6402



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