An examination of stress resistance and lifespan extension in the honey bee, *Apis mellifera*

En undersøkelse av stresstoleranse og forlengelse av livsløpet hos bier, *Apis mellifera*

Philosophiae Doctor (PhD) Thesis

Brenda Rascón

Department of Chemistry, Biotechnology, and Food Science

Norwegian University of Life Sciences

Ås, 2013

Thesis number 2013:48

ISSN 1503-1667

ISBN 978-82-575-1148-7

Table of Contents

Acknowledgements

First, I would like to thank my advisor, Gro V. Amdam, who has held me to her own high standards for science throughout the years. Her clear-thinking, unwavering eagerness for research helped guide me through these formative years. Along the way, Gro unfailingly offered me opportunities for professional growth and believed in my potential as a scientist. I would also like to thank my collaborators: Navdeep S. Mutti, Christina Tolfsen, Erin Fennern, Nick Baker, Basil P. Hubbard, David A. Sinclair, and Kate E. Ihle who helped provide expertise and insight that strengthened my work. The members of the Amdam lab on both sides of the Atlantic were instrumental. In particular, I would like to note my appreciation for Daniel Muench, Margrethe Brynem, and Claus Kreibich in Norway. Without Daniel and Margrethe, working in both Norway and Arizona would have been nearly impossible. Many thanks to Ying Wang, Kirsten Traynor, Adam Siegel, Kevin Flores, and Adam Dolezal in Arizona for being great colleagues. I am indebted to Osman Kaftanoglu for his guidance and assistance with the bees. I am also grateful for the many talks I shared with Rick Overson and Sydella Blatch. I am lucky to have the support of Jon F. Harrison and Jennifer H. Fewell, two wonderful scientists and people who have always been my advocates and an essential source of encouragement over the years. Jon F. Harrison awakened my interest in research during my undergraduate years and has been pivotal in my personal and professional growth. My friends April Bojorquez, Anna Escobar, Matt Garcia, and Nate Mata offered laughter, encouragement, and perspective. To my loving parents, Linda and Armando, who started a new life in the United States so that my brother and I could have access to the educational opportunities that were unavailable to them. They imparted a desire to work hard, seize opportunity, and love deeply. I am incredibly grateful for my generous brother, Mando, for his reassurance and unconditional love. Lastly, I would like to thank my inexhaustible, loving husband, Florian Wolschin, for his continuous support, patience, and insight. Thank you to all of you.

Abstract

Many societies and health systems will soon face the unprecedented challenge of burgeoning aged populations. This demographic change will place pressure on social and medical systems, and give rise to questions concerning quality of life. Aging is often characterized by a decreased capacity for stress resistance and cognitive tasks. Thus, a longer life does not necessarily guarantee a prolonged health span, i.e., the time spent as a healthy individual. Additional research is needed to better understand and ultimately influence the connections between longevity, the decline of brain function, and health span.

Using the honeybee as a model system, this dissertation examines the relationship between stress and lifespan, the effects of oxidative stress on learning and sensory capacity, and the potential reversal of brain decline. By combining manipulative tools, including the alteration of oxygen environment, RNAi-mediated gene knockdown, and pharmacological intervention with behavioral assays, this dissertation demonstrates that key indicators of health and lifespan can be selectively modulated.

Specifically, hyperoxia negatively impacts survivorship and learning performance without compromising gustatory responsiveness. This indicates that peripheral and central brain functions are differentially affected by oxidative stress in honey bees. In addition, these differences in survivorship can be partially explained by vitellogenin, a yolk precursor protein with antioxidant properties that influences social behavior in honey bees.

Lastly, the pharmacological compound, resveratrol, extends honey bee lifespan and alters gustatory responsiveness and food consumption. Honey bees fed resveratrol eat less, suggesting that resveratrol-dependent life span extension may be driven by a mechanism related to caloric restriction.

The overall aim of these results is to inform research on future therapies focused on slowing or stalling age-related brain decline. Moreover, they illustrate that alternative model systems in aging research can also be informative.

Abstrakt

Mange samfunn og helsevesen vil I løpet av få år møte en helt ny utfordring med en økende andel eldre befolkning. Denne demografiske endringen vil legge press på sosiale og medisinske systemer og fremme spørsmål i forhold til livskvalitet. Aldring blir ofte karakterisert ved en redusert evne til å mestre stress og kognitive oppgaver. Et lenger livsløp korrelerer derfor ikke nødvendigvis med hvor stor del av livet man er frisk. Ytterligere forskning er nødvendig for å få en bedre forståelse og dermed mulighet til å påvirke sammenhengen mellom livsløp, reduksjon i hjernefunksjon og helse.

I denne avhandlingen brukes honningbien som modelldyr for å undersøke forholdet mellom stress og livsløp, effekten av oksidativt stress på læring og sanser, og potensialet for reversering av hjernens aldringsforfall. Ved å kombinere manipulasjonsteknikker som inkluderer endring av oksygennivå, RNAi-mediert gene-knockdown og farmakologiske tiltak i adferdstester viser denne avhandlingen at nøkkelindikatorer for helse og livsløp kan bli selektivt modulert. Spesielt hyperoxi innvirker negativt på overlevelse og læringsevne uten å kompromitere gustatorisk reaksjonsevne. Dette gir indikasjoner om at perifere og sentrale hjernefunksjoner er ulikt berørt av oksidativt stress hos honningbier. Disse forskjellene kan også forklares ved vitellogenin, et precursor protein med antioksidante egenskaper som påvirker sosial adferd hos honningbier.

Til slutt; den farmakologiske faktoren, resveratrol forlenger honningbiens livsløp og endrer gustatorisk respons og fôrforbruk. Honningbier som får resveratrol spiser mindre noe som antyder at resveratrol-avhengig økt livsløp kan være styrt av en mekanisme knyttet til kalorirestriksjon.

Det overordnede målet med disse resultatene er å opplyse forskning på fremtidig terapi som fokuserer på å bremse eller stagnere aldersrelatert redusert hjernefunksjon. I tillegg illustrerer disse resultatene at alternative modellsystemer i aldringsforskning også kan være informativ.

Norwegian translation provided by Margrethe Brynem.

List of Papers

I. Rascón B., Mutti, N., Tolfsen, C., Amdam G.V. (2011) Honey Bee Life-History Plasticity—Development, Behavior, and Aging. In *Mechanisms of Life History Evolution*, edited by Thomas Flatt & Andreas Heyland*.

II. Rascón B., Ihle K., Amdam G.V. Hyperoxia reveals a distinct resilience for central and peripheral brain functions in the honey bee. *Submitted*

III. Amdam G.V., Fennern E., Baker N., Rascón B. (2010) Honeybee associative learning performance and metabolic stress resilience are positively associated. *PLoS ONE* 5(3): e9740, doi:10.1371/journal.pone.0009740.

IV. Rascón B., Hubbard B.P., Sinclair D.A., Amdam G.V. (2012) The lifespan extension effects of resveratrol are conserved in the honey bee and may be driven by a mechanism related to caloric restriction. *Aging*, 4(7):499-508.

*The cover of this book was based on artwork designed by me and April Bojorquez.

List of Abbreviations

Vg	vitellogenin (protein)
vg	vitellogenin (gene)
JH	juvenile hormone
ROS	reactive oxygen species
SIRT1	Silent Information Regulator 1
Sir2	Silent Information Regulator 2
RNAi	ribonucleic acid interference
dsRNA	double-stranded ribonucleic acid
TOR	target of rapamycin
PER	proboscis extension reflex
GRS	gustatory responsiveness score
US	unconditioned stimulus
CS	conditioned stimulus
O_2	oxygen

1. Introduction

1.1 The Honey Bee

Honey bees have drawn the interest of scientists and non-scientists alike for decades. Their large, highly organized, cooperative societies are striking in their ecological success and complexity. Their specialized division of tasks within the colony and their level of cooperation have led some to characterize this social grouping as a "superorganism"(Hölldobler and Wilson, 2009). This particular metaphor conjures up images of a single organism composed of interdependent individuals that carry out information processing, reproductive, physiological, and communication tasks for the success of the collective. However, individual honey bees demonstrate complex brain functions (learning and memory), behavioral preferences for food collection, and aging patterns which can be linked to stage-specific physiological changes. During ontogeny, modifications in honey bee behavior lead to differences in task performance, altering the aging profile of the honey bee and sometimes offering the fascinating possibility for the reversal of aging.

1.2 Central Aim

This dissertation will focus on understanding how the physiology of individual worker bees changes during aging, with particular emphasis on learning behavior, sensory perception, and lifespan. I will address the following questions: Does metabolic stress accelerate aging in the honey bee? Can hyperoxia induce physiological changes that are reminiscent of aging patterns observed in free-flying honey bees? Is brain aging reversible? Can the phytochemical, resveratrol, alleviate functional decline in the honey bee and attenuate stress-induced mortality? The honey bee serves these goals well because it is a well-established neurobiological model, it is comparably large, and can be tracked throughout its entire life history, providing individual-level information (Menzel, 2012). Added to this, the natural behavioral plasticity of the honey bee brain provides a useful backdrop for the investigation of aging (Amdam, 2011).

1.3 Theories of Aging

Aging studies have generally focused on solitary model organisms and have given rise to aging theories that do not always apply to a eusocial species like the honey bee. For example, young honey bees (nurses) tend to perform the alloparental caregiving functions in the colony e.g., brood feeding and colony maintenance, while older, more mature honey bees (foragers) leave the nest in search of food (Winston, 1987). This high

level of ontogenic behavioral complexity and the compartmentalization of reproduction and caregiving render many of the well-known theories of aging inapplicable to the worker honey bee (Amdam and Page, 2005).

Life history theory, for instance, posits that the pressure of natural selection on survival favors fitness during the reproductive stage of life and then diminishes in power during the post-reproductive phase of life. However, worker bees are essentially sterile, so life history theory cannot adequately explain their patterns of aging. The classic evolutionary theories of aging e.g., Medawar's mutation accumulation theory (1952), Williams' antagonistic pleiotropy theory (1957), and Kirkwood's (1977) disposable soma theory rest on the concept of extrinsic mortality and postulate that natural selection will not favor further investment of resources into the soma when the risk of dying is high. However, the application of this idea to worker bees is problematic since they act as alloparental caregivers and are largely shielded from predators for part of their lives (Winston, 1987). Although these theories are generally regarded as dominant explanatory tools in the evolution of aging, their focus on reproduction limits their application to individual worker bees. Therefore, I used the free radical theory of aging as a framework for understanding the aging patterns of individual worker bees.

1.4 Free Radical Theory of Aging and Oxidative Stress

As worker bees age, it is possible to observe superficial changes such as wing wear and hair loss (Wolschin et al., 2009). These are, however, only a manifestation of the molecular and biochemical changes that lead to senescence. One of the concepts that may underlie senescence in honey bees is oxidative stress.

Molecular oxygen can serve as a source of reactive oxygen species (ROS) and ultimately cause aging. ROS are involved in normal cell respiration as by-products of aerobic mitochondrial metabolism, but can also inflict damage on proteins, lipids, and DNA if not successfully scavenged by cellular antioxidants. When the body's ability to properly quench excessive free radical production is compromised, this process can eventually lead to pervasive cellular damage that interferes with normal metabolism and causes aging (Harman, 1956).

1.5 Oxidative Stress and the Aging Honey Bee Brain

Oxidative stress has been widely implicated in aging and functional decline. During senescence, sensory and memory decline afflict a variety of organisms ranging from mammals to insects (Brown and Strausfeld, 2009; Flood and Morley, 1998; Grotewiel et

al., 2005; Mery, 2007; Tamura et al., 2003). In honey bees, this decline in central processing ability can be detected after over 15 days of flight. During this time, foraging honey bees show memory deficits and a reduction in associative learning performance (Behrends et al., 2007; Munch et al., 2010; Scheiner and Amdam, 2009). In the honey bee brain, this functional decline is associated with oxidative damage to lipids and proteins, an accumulation of proteins, and a reduction of proteins related to synaptic and neuronal growth (Seehuus et al., 2006a; Tolfsen et al., 2011; Wolschin et al., 2009).

In general, aging honey bees share many of the same symptoms of aging found in other animals (Munch and Amdam, 2010). However, in the honey bee, aging, like other characteristics of life history and behavior, exhibits remarkable plasticity which can be studied outdoors and in laboratory settings (Amdam, 2011; Munch et al., 2008; Page and Peng, 2001).

1.6 Resveratrol Effects on Lifespan and the Brain

Resveratrol is a plant polyphenol with reported lifespan extension effects in some (Howitz et al., 2003; Valenzano et al., 2006; Wood et al., 2004), but not all studies (Bass et al., 2007; Chen et al., 2013; Kaeberlein et al., 2005). Reports indicate that resveratrol elicits neuroprotective effects and prevents the decline of locomotory function (Bastianetto et al., 2000; Han et al., 2004; Jang and Surh, 2003; Marambaud et al., 2005; Valenzano et al., 2006). The beneficial effects of resveratrol also extend to cognitive performance. For instance, resveratrol can reverse cognitive deficits, maintain memory in aged rats (Joseph et al., 2008), and protect rats suffering from traumatic brain injury (Sonmez et al., 2007).

In addition, studies indicate that resveratrol may act as an antioxidant and confer protection against nervous system impairment and oxidative stress (Chanvitayapongs et al., 1997; Chen et al., 2013; de la Lastra and Villegas, 2007; Hung et al., 2002; Jang and Surh, 2003; Mahal and Mukherjee, 2006). For example, in the brain of healthy rats, resveratrol increases the activity of antioxidants such as superoxide dismutase and catalase, and decreases the level of oxidative stress (Mokni et al., 2007). In this dissertation, I examine the effects of resveratrol on the lifespan of honey bees in hyperoxic environments (Paper II), and test whether resveratrol can rescue or attenuate hyperoxia-induced deficits in learning performance (Paper IV). This work provides the first glimpse of resveratrol effects in a eusocial species.

1.7 Resveratrol and Caloric Restriction

The anti-aging effects of resveratrol may be regulated by the same pathways that govern caloric restriction. Caloric restriction is an evolutionarily conserved means of increasing lifespan and preventing the onset of diseases of age (Arking, 2006). Studies in several organisms indicate that sirtuins may mediate the beneficial outcomes of caloric restriction (Lin et al., 2000; Rogina and Helfand, 2004; Wang et al., 2006). In addition, resveratrol-dependent lifespan extension seems to depend on the activation of sirtuins (Frye, 2000). Sirtuins are a class of proteins that play leading roles in energy metabolism (Boily et al., 2008; Imai et al., 2000; Vaziri et al., 2001).

Many studies demonstrate that the overexpression of SIRT1 homologs extends lifespan (Kaeberlein et al., 2005; Rizki et al., 2011; Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001), but not all studies have replicated this finding (Burnett et al., 2011). Notably, the overexpression of SIRT1 in mice produces phenotypes reminiscent of caloric restriction (Bordone et al., 2007). In addition, a recent bioinformatics study that compared the gene expression profiles of species subjected to caloric restriction, Sir2 overexpression, and resveratrol administration discovered that 23 genes involved in stress, metabolism, and growth were conserved in response to caloric restriction and resveratrol (Antosh et al., 2011). This suggests that the responses to resveratrol and caloric restriction may share some common molecular responses. In the present dissertation, I examine the effects of resveratrol on lifespan and neurophysiological responses to hyperoxia.

2. Results

2.1 Paper I

In this book chapter, I and my co-authors discussed the current ideas that underlie the life history of the honey bee. First, we examined honey bee development and how environmental variation shapes the social role of female honey bees. We focused our mechanistic insights on important metabolic networks, e.g., insulin-insulin-like signaling (IIS), target of rapamycin (TOR), hormonal titers (vitellogenin and juvenile hormone), and DNA methylation, which are thought to direct these differences in caste formation. Thereafter, we examined behavioral maturation, foraging specialization, and aging via the Vg-JH axis lens. We also considered behavioral maturation of worker bees and the physiological, anatomical, and biochemical changes that prepare the bee for metabolically-demanding activities such as flight during foraging. These complex

behaviors require the acquisition and recall of spatial memories, and the expansion of oxidative capacity, which we also closely examined. Lastly, we discussed the aging patterns observed in honey bees, some of which encompass classic signs of aging e.g., oxidative stress, functional decline, morphological changes, along with other characteristics which are particularly unique to this model system. For instance, the fact that senescence in honey bees is tied to social task rather than simple chronological age makes the honey bee an interesting, new promising model for aging.

2.2 Paper II

In this published research article, I contributed the hyperoxia method that was used throughout and assisted in writing the publication. Paper II examined the link between learning performance and mortality in honey bees, a correlation that was previously observed in humans. Environmental and socioeconomic background strongly influence markers of health, survival, and educational achievement in humans. But despite their strong impact on health and survival, psychometric tests can still predict the length of life in some human populations. These relationships, however, are difficult to explore in humans due to the high degree of covariance between these variables. Furthermore, these types of studies are controversial due to the historical misuse of psychometric testing against members of certain cultural groups. Nevertheless, in this study, we controlled for social background and found a positive correlation between olfactory learning performance and stress resistance in individual honey bees. The outcomes documented in this honey bee study, however, do not provide evidence of shared functional principles between honey bees and humans. Nonetheless, we hope our findings can help delineate how metabolic resistance can influence life outcomes.

2.3 Paper III

Paper III is a research article in which I examined how oxidative stress affects lifespan, and the peripheral and central brain functions of the honey bee. In this study, we measured gustatory responsiveness (GRS) and associative learning performance in response to hyperoxia. I chose hyperoxia to prematurely induce pathologies that are often present in aged individuals to test oxidative stress resistance. Stress resistance is thought to contribute to longer lifespans in insects such as fruit flies (Lin et al., 1998; Orr and Sohal, 1992). Moreover, because worker honey bee susceptibility to oxidative stress may be explained by differences in vitellogenin expression (Seehuus et al., 2006b), we investigated the effects of hyperoxia on vitellogenin expression. With the

assistance of a co-author, we reduced *vitellogenin* expression via RNAi in worker honey bees, and compared survivorship between knockdowns and controls. We used two genetic honey bee stocks that exhibit consistent differences in gustatory responsiveness, associative learning performance, and lifespan (Amdam et al., 2004; Page et al., 1998; Pankiw et al., 2001; Scheiner et al., 2001a; Scheiner et al., 2001b) to examine whether a genetic component exists for metabolic stress resistance.

In this article, our data reveal that oxidative stress negatively impacts survivorship and learning ability without compromising gustation. This differential susceptibility of peripheral and central brain functions in response to hyperoxia is consistent with the effects of aging in free-flying honey bees. In addition, our data indicate that differences in survival can be partially explained by vitellogenin.

2.4 Paper IV

Paper IV is a published research article in which I examined the effect of resveratrol on lifespan, learning performance, and gustatory responsiveness in hyperoxia- and normoxia-reared individuals. Resveratrol is thought to act as an antioxidant and confer protection against nervous system impairment and oxidative stress (Chanvitayapongs et al., 1997; Chen et al., 2013; de la Lastra and Villegas, 2007; Hung et al., 2002; Jang and Surh, 2003; Mahal and Mukherjee, 2006). In addition, various reports indicate that resveratrol elicits neuroprotective effects and prevents the decline of locomotory function (Bastianetto et al., 2000; Han et al., 2004; Jang and Surh, 2003; Marambaud et al., 2005; Valenzano et al., 2006). In Paper III, we observed high mortality rates and deficits in learning performance due to oxidative stress. Therefore, I sought to investigate whether these features of age-related decline in the honey bee could be attenuated by resveratrol. We discovered that resveratrol treatment lengthened lifespan (average, maximum, and median) in wild-type honey bees under normoxic conditions. In contrast to the purported antioxidant effects of resveratrol, hyperoxia abolished the resveratrol life-extension response. Lastly, we observed that resveratrol alters sugar sensitivity and food consumption. For example, honey bees supplemented with resveratrol were less responsive to sugar and ingested fewer quantities of food under *ad libitum* feeding conditions in comparison to controls, which exhibited just the opposite.

3. Methods

3.1 Methods Overview

In the present work, I used hyperoxia to examine the robustness of central and peripheral brain functions (Paper II), the connections between learning performance and metabolic stress (Paper III), and the antioxidant capacity of resveratrol (Paper IV). In Papers II and III, we used high and low genotypes to test the robustness of stress resistance. High and low genotypes represent the two behavioral and physiological extremes of wild-type honey bee populations (Hellmich et al., 1985; Page and Fondrk, 1995). Thus, we reasoned that if patterns that are comparable to those seen in more natural settings persisted in response to laboratory tests, then these genotypes would allow us to generalize about the trait associations of aging in wild-type honey bees. In Papers II-V, I used a gustatory responsiveness assay to examine the sensory response to hyperoxic stress. In addition, I used olfactory conditioning to study the learning capability of honey bees exposed to either hyperoxia and/or resveratrol (Papers II and III). I applied the survivorship method throughout with the goal of assessing the impact of treatment on lifespan (Papers II-V). Lastly, we used RNAi to downregulate vitellogenin to investigate whether it could neutralize the effects of hyperoxia.

3.2 Genotypes

In nature, a forager bee collects both pollen and nectar, but it may bias its collection of food towards one or the other (Amdam et al., 2009). In a previous breeding program, honey bees from wild-type populations were selected based on the amount of pollen stored in their colonies (referred to as high and low genotype) (Page and Fondrk, 1995). Individual worker bees of high and low genotypes vary dramatically in physiology, behavior, and lifespan. For example, high genotype bees possess higher levels of vitellogenin (Vg) and juvenile hormone (JH). Vg and JH are important hormonal regulators that influence honey bee development and are thought to suppress one another (Amdam and Page, 2005). Vg can affect immunity, oxidative stress resistance, and longevity (Seehuus et al., 2006a; Seehuus et al., 2006b), and JH is sensitive to environmental conditions e.g., social environment, nutrition (Hartfelder and Engels, 1998). Moreover, in comparison to low genotype bees, highs tend to return to the colony with water (Page et al., 1998) and sucrose of lower concentration (Page et al., 1998; Pankiw and Page, 2001).

3.3 Hyperoxia as a Tool in Aging Research

Hyperoxia is an essential tool for the manipulation of oxidative stress *in vivo*. Increased oxygen tension is known to augment the rate of ROS, which can lead to aging. Therefore, hyperoxia can be used as a metabolic stressor in insect model systems to prematurely induce age-related pathologies (von Zglinicki and Sitte, 2003). In *Drosophila*, hyperoxia reliably produces gradual changes in lifespan and protein oxidative stress (Rascón and Harrison, 2010; Sohal et al., 1993; Sohal and Dubey, 1994), neural system deterioration (Miquel et al., 1975), and mitochondrial deformations (Walker and Benzer, 2004). Moreover, 38% of the genes implicated in normal aging are altered in the same direction during hyperoxia (Landis et al., 2004).

Previously, I reared *Drosophila melanogaster* in hyperoxia and examined its effects on lifespan and protein oxidative stress (Rascón and Harrison, 2011). In the present dissertation, I applied the concept of hyperoxia rearing to the honey bee with the intention of replicating aging-related changes normally observed in more natural settings for this species (Behrends et al., 2007). This method required rearing honey bees in the laboratory under hyperoxia for long periods, which is a new empirical approach to handling an organism commonly studied outdoors.

In Papers II, III, and IV, I reared adult honey bees in an incubator (Heracell, Thermo Scientific, MA, USA) that maintained the following controlled conditions: 75-80% or 21% O_2, 34 °C, 63±2% relative humidity. Relative humidity was monitored by Hobo data loggers (Onset Computer Corporation, MA, USA). Honey bees were individually housed in 1.5 mL Eppendorf tubes (Figure 1), each outfitted with a feeding port, breathing hole, and an opening for waste and defecation, as previously described (Amdam et al., 2010). Honey bees were fed 25 μL of a standard diet consisting of 1.5 g of ground pollen per 30 mL of 30% sucrose solution. Bees were allowed to feed *ad libitum* through an easily accessible food-containing pipette tip. Feeding was verified to prevent starvation and/or caloric restriction, and thereby minimize survivorship effects not associated with oxygen treatment.

Figure 1. An adult honey bee housed in a plastic tube that served as a rearing chamber for hyperoxia experiments.

3.4 Gustatory Responsiveness

I used the proboscis extension reflex (PER) to measure the gustatory responsiveness of honey bees. PER is a natural component of honey bee feeding behavior in response to stimulation of the antennae. The criterion for PER was complete extension of the proboscis upon stimulation of the antennae with water and six sucrose solutions in the following order: 0.1, 0.3, 1, 3, 10, 30%. If the honey bee exhibited full extension of the proboscis, I recorded a number one in my notes. In contrast, if the honey bee did not fully extend its proboscis, I recorded a zero. To prevent sensitization and habituation, I adhered to an inter-stimulus interval of two minutes. After I presented the seven stimuli (water and six sucrose solutions) to each honey bee, I calculated an overall index of performance—gustatory response score (GRS)—by using the sum of all seven PER responses. A honey bee with a total score of seven showed the highest level of sensory responsiveness, while a score of zero indicated no responsiveness. Bees that failed to respond to the 30% sucrose stimulus were not included in the olfactory conditioning trials because this sucrose concentration was used as an unconditioned stimulus (US) in the olfactory conditioning experiments.

3.5 Olfactory Conditioning

Olfactory conditioning is a Pavlovian, classical conditioning procedure in which individually harnessed honey bees are trained to associate an odor with a sucrose

reward. In classical conditioning, the animal is presented with the two stimuli: 1) the unconditioned stimulus (US) and the 2) conditioned stimulus (CS). Pavlov described the US as an inborn reflex, whereas the CS is learned and acquired (Pavlov, 1927). In olfactory conditioning of honey bees, the sucrose reward represents the US, while the odor is the CS. Through the association or pairing of the US to the CS, the honey bee learns to anticipate a sugar reward by extending its proboscis when the CS is presented alone (Figure 2) (Bitterman et al., 1983; Takeda, 1961).

To test for hyperoxia-induced performance deficits, I measured associative olfactory learning in honey bees that responded to at least 30% sucrose (GRS ≥1). Prior to training, I tested honey bees for spontaneous PER to carnation odor and cineole. Thereafter, I only conditioned bees that did not exhibit a spontaneous response to either odor, as in previous studies (Amdam et al., 2010; Tolfsen et al., 2011). For odor preparation, I applied 2 µl of carnation oil to a piece of filter paper, which I then placed into a capped 20 ml syringe. I fixed honey bees onto plastic holders using thin strips of duct tape, which I placed just underneath each honey bee's neck. I then placed honey bees into a Plexiglas vacuum enclosure that neutralized the airstream. Throughout each of the six conditioning trials, I delivered controlled puffs of odorant air (5 ml of carnation) to honey bee antennae for five seconds. During the last three seconds of odor presentation, I administered 1 µl of the US (30% sucrose in H_2O) to form a paired stimulus-reward association. The inter-trial interval was 5 min to prevent sensitization and habituation effects. After each conditioning trial, I scored the bee's response as a binary variable via PER (i.e., response or no response). Once all conditioning trials took place, I tested for odor generalization by presenting honey bees with cineole. This allowed us to test the discrimination ability of each bee as it should only respond to the CS (carnation). Lastly, I calculated a learning acquisition score based on the conditioned responses. The score, with a numerical value between zero and five, was based on five conditioning trials and an additional trial that tested reaction spontaneity.

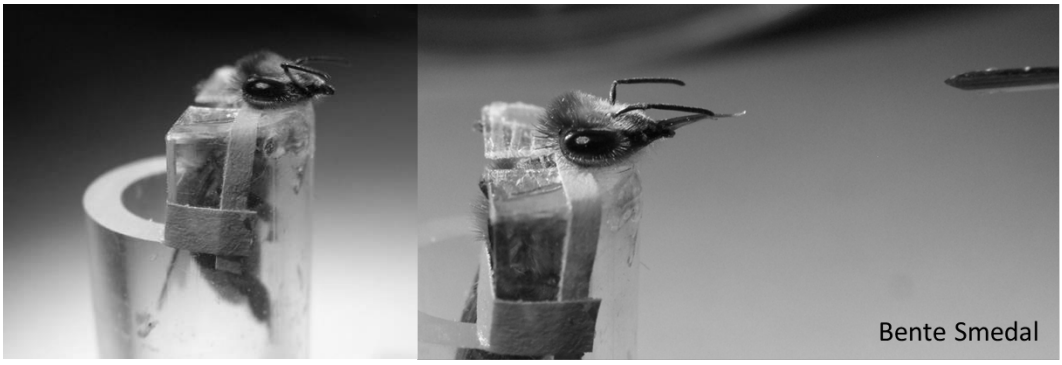


Figure 2. In the left photograph, a honey bee is harnessed to a plastic holder for gustatory responsiveness and olfactory conditioning trials. In the right photo, a honey bee extends its proboscis (tongue) in response to a conditioned stimulus during olfactory conditioning.

3.6 Survivorship measurements

Survivorship censuses took place two or three times per day (four to five times in the resveratrol study) at similar times until the last bee was observed dead. During these observation periods, bees were either observed dead or alive, and remaining live bees were transferred to fresh tubes to prevent bacterial and/or fungal growth. Individuals that appeared to have died due to accident (e.g. killed during routine transfers) were not included in the data analysis. Individual life spans were calculated using the frequency of bees alive at each temporal observation. We chose three oxygen exposure times (17, 40, 64 hr) based on pilot experiments. These oxygen exposure times formed the basis of our observations for the gustatory and learning performance assessments in Paper II. I reasoned that progressive performance deterioration would be detected the longer honey bees spent in hyperoxic treatment.

3.7 Vitellogenin downregulation by RNA interference (RNAi)

3.7.1 dsRNA preparation for vitellogenin gene downregulation

We prepared double-stranded RNA (dsRNA) toward the *vitellogenin* gene as previously described (Amdam et al., 2006; Amdam et al., 2003). Briefly, we used cDNA clone AP4a5 as a template (GenBank accession #: AJ517411) and fused primers to a T7 promoter sequence, which is underlined in the sequence below:

Fw:5'-TAATACGACTCACTATAGGGCGAACGACTCGACCAACGACTT-3'

Re:5'-TAATACGACTCACTATAGGGCGAAACGAAAGGAACGGTCAATTCC-3'

We purified the polymerase chain reaction (PCR) product using the QIAquick PCR purification kit (Qiagen, Valencia, California, USA), and prepared the RNA with the Promega RiboMax T7 system (Promega, Madison, Wisconsin, USA). We extracted RNA using the TRIzol LS reagent (GIBCO-BRL, San Diego, California, USA). Subsequently, we diluted dsRNA products with nuclease-free H_2O (Qiagen) (Amdam et al., 2003; Nelson et al., 2007; Seehuus et al., 2006b). As in a previous study, we used nuclease-free water as a control, (Ihle et al., 2010).

3.7.2 Knockdown verification

Previously, the efficacy of this particular *vitellogenin* RNAi method was confirmed in honeybees of diverse commercial origins, in high and low genotypes (Amdam et al., 2007; Amdam et al., 2003; Marco Antonio et al., 2008; Nelson et al., 2007), and in Paper II of the present dissertation. To verify the *vitellogenin* knockdown, we isolated RNA from the honey bee abdominal fat body (site of vitellogenin synthesis) using TRIzol phenol-chloroform extraction combined with the RNeasy kit (Qiagen), as previously described (Ihle et al., 2010; Nelson et al., 2007). Thereafter, we used reverse transcriptase real-time PCR (Applied Biosciences, Foster City, CA, USA) to validate knockdown of *vitellogenin* mRNA levels. Relative gene expression levels were obtained against β-actin expression (Nelson et al., 2007). β-actin is an effective control gene when measuring gene expression in adult honey bee fat bodies (Lourenco et al., 2008; Scharlaken et al., 2008). Primers for *vitellogenin:* 5'-GTTGGAGAGCAACATGCAGA-3' and 5'-TCGATCCATTCCTTGATGGT-3'. Primers for actin: 5'-TGCCAACACTGTCCTTTCTG-3' and 5'-AGAATTGACCCACCAATCCA-3' (Amdam et al., 2004).

4. Conclusion

4.1 Is hyperoxia a suitable tool to investigate aging in the honey bee?

The honey bee has a long-standing history as a behavioral model and has generally been studied outdoors in more natural settings. An early aim of this dissertation was to replicate patterns of honey bee aging, which were previously observed in the field, in the laboratory under hyperoxic conditions. These patterns included the decline of learning performance, increased mortality, and the preservation of gustation.

My dissertation reveals that hyperoxia negatively impacts the survival and learning ability of the honey bee, without influencing gustatory responsiveness. This demonstrates that peripheral and central brain functions in the honey bee respond differently to hyperoxia. These findings match the changes observed in free-flight studies for this animal (Behrends et al., 2007) and thus underline the potential of hyperoxia treatment as a proxy for aging. It is possible that the differential response of peripheral and central brain functions to hyperoxia may reflect distinct spatial and tissue-specific thresholds for aging. If so, then it is corollary that these distinct thresholds could ultimately influence behavioral indicators of neurophysiological function, such as associative learning performance and gustatory responsiveness. Paper II shows that the survival differences in hyperoxia can be partially explained by vitellogenin—a finding that is comparable to previous reports on oxidative damage and longevity in the free-flying honey bee (Seehuus et al., 2006a; Seehuus et al., 2006b). Thus, hyperoxia is a suitable tool to mimic and/or accelerate signs of aging in the honeybee. This finding set the stage for additional manipulation experiments in the laboratory.

4.2 Insights from resveratrol

The pharmaceutical resveratrol, which has been linked to life extension in other species, was unable to rescue lifespan and functional deficits in hyperoxic environments. This suggests that resveratrol may not be a potent antioxidant or that the oxygen tension used in these studies represents a limit for the antioxidant capacity of resveratrol. However, my observation that resveratrol prolongs honey bee lifespan under normal oxygen conditions, strengthens the supposition that the resveratrol-dependent lifespan extension response may be conserved across species. Furthermore, the experiments that combine gustatory responsiveness with food consumption measurements illustrate that resveratrol influences gustation and leads to a satiety effect in honey bees. This finding indicates

that resveratrol may be driven by a mechanism related to caloric restriction. In conclusion, the results documented here illustrate and emphasize that hyperoxia and the honeybee can be useful tools in the quest for a better understanding the phenomenon of aging.

References

Amdam, G. V. (2011). Social context, stress, and plasticity of aging. *Aging Cell* **10**, 18-27.

Amdam, G. V., Fennern, E., Baker, N. and Rascón, B. (2010). Honeybee associative learning performance and metabolic stress resilience are positively associated. *PLoS One* **5**, e9740.

Amdam, G. V., Nilsen, K. A., Norberg, K., Fondrk, K. and Hartfelder, K. (2007). Variation in Endocrine Signaling Underlies Variation in Social Life History. *American Naturalist* **170**, 37-46.

Amdam, G. V., Norberg, K., Fondrk, M. K. and Page, R. E., Jr. (2004). Reproductive ground plan may mediate colony-level selection effects on individual foraging behavior in honey bees. *Proc Natl Acad Sci U S A* **101**, 11350-5.

Amdam, G. V., Norberg, K., Page, R. E., Jr., Erber, J. and Scheiner, R. (2006). Downregulation of vitellogenin gene activity increases the gustatory responsiveness of honey bee workers (Apis mellifera). *Behav Brain Res* **169**, 201-5.

Amdam, G. V. and Page, R. E., Jr. (2005). Intergenerational transfers may have decoupled physiological and chronological age in a eusocial insect. *Ageing Res Rev* **4**, 398-408.

Amdam, G. V., Rueppell, O., Fondrk, M. K., Page, R. E. and Nelson, C. M. (2009). The nurse's load: early-life exposure to brood-rearing affects behavior and lifespan in honey bees (Apis mellifera). *Exp Gerontol* **44**, 467-71.

Amdam, G. V., Simoes, Z. L., Guidugli, K. R., Norberg, K. and Omholt, S. W. (2003). Disruption of vitellogenin gene function in adult honeybees by intra-abdominal injection of double-stranded RNA. *BMC Biotechnol* **3**, 1.

Antosh, M., Whitaker, R., Kroll, A., Hosier, S., Chang, C., Bauer, J., Cooper, L., Neretti, N. and Helfand, S. L. (2011). Comparative transcriptional pathway bioinformatic analysis of dietary restriction, Sir2, p53 and resveratrol life span extension in Drosophila. *Cell Cycle* **10**, 904-11.

Arking, R. (2006). Altering Aging: Interventions That Modify Longevity and Aging. In *The Biology of Aging: Observations and Principles*, (ed. R. Arking), pp. 202-236. New York: Oxford University Press.

Bass, T. M., Weinkove, D., Houthoofd, K., Gems, D. and Partridge, L. (2007). Effects of resveratrol on lifespan in Drosophila melanogaster and Caenorhabditis elegans. *Mech Ageing Dev* **128**, 546-52.

Bastianetto, S., Zheng, W. H. and Quirion, R. (2000). Neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide-related toxicity in cultured hippocampal neurons. *Br J Pharmacol* **131**, 711-20.

Behrends, A., Scheiner, R., Baker, N. and Amdam, G. V. (2007). Cognitive aging is linked to social role in honey bees (Apis mellifera). *Exp Gerontol* **42**, 1146-53.

Bitterman, M. E., Menzel, R., Fietz, A. and Schafer, S. (1983). Classical conditioning of proboscis extension in honey bees (Apis mellifera). *Journal of Comparative Psychology* **97**, 107-119.

Boily, G., Seifert, E. L., Bevilacqua, L., He, X. H., Sabourin, G., Estey, C., Moffat, C., Crawford, S., Saliba, S., Jardine, K. et al. (2008). SirT1 regulates energy metabolism and response to caloric restriction in mice. *PLoS One* **3**, e1759.

Bordone, L., Cohen, D., Robinson, A., Motta, M. C., van Veen, E., Czopik, A., Steele, A. D., Crowe, H., Marmor, S., Luo, J. et al. (2007). SIRT1 transgenic mice show phenotypes resembling calorie restriction. *Aging Cell* **6**, 759-67.

Brown, S. and Strausfeld, N. (2009). The effect of age on a visual learning task in the American cockroach. *Learn Mem* **16**, 210-23.

Burnett, C., Valentini, S., Cabreiro, F., Goss, M., Somogyvari, M., Piper, M. D., Hoddinott, M., Sutphin, G. L., Leko, V., McElwee, J. J. et al. (2011). Absence of

effects of Sir2 overexpression on lifespan in C. elegans and Drosophila. *Nature* **477**, 482-5.

Chanvitayapongs, S., Draczynska-Lusiak, B. and Sun, A. Y. (1997). Amelioration of oxidative stress by antioxidants and resveratrol in PC12 cells. *NeuroReport* **8**, 1499-1502.

Chen, W., Rezaizadehnajafi, L. and Wink, M. (2013). Influence of resveratrol on oxidative stress resistance and life span in Caenorhabditis elegans. *J Pharm Pharmacol* **65**, 682-8.

de la Lastra, C. A. and Villegas, I. (2007). Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. *Biochemical Society transactions* **35**, 1156-60.

Flood, J. F. and Morley, J. E. (1998). Learning and memory in the SAMP8 mouse. *Neurosci Biobehav Rev* **22**, 1-20.

Frye, R. A. (2000). Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem Biophys Res Commun* **273**, 793-8.

Grotewiel, M. S., Martin, I., Bhandari, P. and Cook-Wiens, E. (2005). Functional senescence in Drosophila melanogaster. *Ageing Res Rev* **4**, 372-97.

Han, Y. S., Zheng, W. H., Bastianetto, S., Chabot, J. G. and Quirion, R. (2004). Neuroprotective effects of resveratrol against beta-amyloid-induced neurotoxicity in rat hippocampal neurons: involvement of protein kinase C. *Br J Pharmacol* **141**, 997-1005.

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *J Gerontol* **11**, 298-300.

Hartfelder, K. and Engels, W. (1998). Social insect polymorphism: hormonal regulation of plasticity in development and reproduction in the honeybee. *Curr Top Dev Biol* **40**, 45-77.

Hellmich, R. L., Kulincevic, J. M. and Rothenbuhler, W. C. (1985). Selection for high and low pollenhoarding honey bees. *J Hered* **76** 155-158.

Hölldobler, B. and Wilson, E. O. (2009). The Superorgansim: The Beauty, Elegance and Strangeness of Insect Societies. New York, N.Y.: W.W. Norton & Company, Inc.

Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., Zipkin, R. E., Chung, P., Kisielewski, A., Zhang, L. L. et al. (2003). Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature* **425**, 191-6.

Hung, L.-M., Su, M.-J., Chu, W.-K., Chiao, C.-W., Chan, W.-F. and Chen, J.-K. (2002). The protective effect of resveratrols on ischaemia-reperfusion injuries of rat hearts is correlated with antioxidant efficacy. *British Journal of Pharmacology* **135**, 1627-1633.

Ihle, K. E., Page, R. E., Frederick, K., Fondrk, M. K. and Amdam, G. V. (2010). Genotype effect on regulation of behaviour by vitellogenin supports reproductive origin of honeybee foraging bias. *Anim Behav* **79**, 1001-1006.

Imai, S., Armstrong, C. M., Kaeberlein, M. and Guarente, L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* **403**, 795-800.

Jang, J. H. and Surh, Y. J. (2003). Protective effect of resveratrol on beta-amyloid-induced oxidative PC12 cell death. *Free Radic Biol Med* **34**, 1100-10.

Joseph, J. A., Fisher, D. R., Cheng, V., Rimando, A. M. and Shukitt-Hale, B. (2008). Cellular and behavioral effects of stilbene resveratrol analogues: implications for reducing the deleterious effects of aging. *J Agric Food Chem* **56**, 10544-51.

Kaeberlein, M., McDonagh, T., Heltweg, B., Hixon, J., Westman, E. A., Caldwell, S. D., Napper, A., Curtis, R., DiStefano, P. S., Fields, S. et al. (2005). Substrate-specific activation of sirtuins by resveratrol. *J Biol Chem* **280**, 17038-45.

Landis, G. N., Abdueva, D., Skvortsov, D., Yang, J., Rabin, B. E., Carrick, J., Tavare, S. and Tower, J. (2004). Similar gene expression patterns characterize aging and oxidative stress in Drosophila melanogaster. *Proc Natl Acad Sci U S A* **101**, 7663-8.

Lin, S. J., Defossez, P. A. and Guarente, L. (2000). Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. *Science* **289**, 2126-8.

Lin, Y. J., Seroude, L. and Benzer, S. (1998). Extended life-span and stress resistance in the Drosophila mutant methuselah. *Science* **282**, 943-6.

Lourenco, A. P., Mackert, A., Cristino, A. D. and Simoes, Z. L. P. (2008). Validation of reference genes for gene expression studies in the honey bee, Apis mellifera, by quantitative real-time RT-PCR. *Apidologie* **39**, 372-U33.

Mahal, H. and Mukherjee, T. (2006). Scavenging of reactive oxygen radicals by resveratrol: antioxidant effect. *Research on Chemical Intermediates* **32**, 59-71.

Marambaud, P., Zhao, H. and Davies, P. (2005). Resveratrol promotes clearance of Alzheimer's disease amyloid-beta peptides. *J Biol Chem* **280**, 37377-82.

Marco Antonio, D., Guidugli-Lazzarini, K., do Nascimento, A., Simões, Z. and Hartfelder, K. (2008). RNAi-mediated silencing of vitellogenin gene function turns honeybee (Apis mellifera) workers into extremely precocious foragers. *Naturwissenschaften* **95**, 953-961.

Menzel, R. (2012). The honeybee as a model for understanding the basis of cognition. *Nat Rev Neurosci* **13**, 758-68.

Mery, F. (2007). Aging and its differential effects on consolidated memory forms in Drosophila. *Exp Gerontol* **42**, 99-101.

Miquel, J., Lundgren, P. R. and Bensch, K. G. (1975). Effects of exygen-nitrogen (1:1) at 760 Torr on the life span and fine structure of Drosophila melanogaster. *Mech Ageing Dev* **4**, 41-57.

Mokni, M., Elkahoui, S., Limam, F., Amri, M. and Aouani, E. (2007). Effect of Resveratrol on Antioxidant Enzyme Activities in the Brain of Healthy Rat. *Neurochemical Research* **32**, 981-987.

Munch, D. and Amdam, G. V. (2010). The curious case of aging plasticity in honey bees. *FEBS Lett* **584**, 2496-503.

Munch, D., Amdam, G. V. and Wolschin, F. (2008). Ageing in a eusocial insect: molecular and physiological characteristics of life span plasticity in the honey bee. *Funct Ecol* **22**, 407-421.

Munch, D., Baker, N., Kreibich, C. D., Braten, A. T. and Amdam, G. V. (2010). In the laboratory and during free-flight: old honey bees reveal learning and extinction deficits that mirror mammalian functional decline. *PLoS One* **5**, e13504.

Nelson, C. M., Ihle, K. E., Fondrk, M. K., Page, R. E. and Amdam, G. V. (2007). The gene vitellogenin has multiple coordinating effects on social organization. *PLoS Biol* **5**, e62.

Orr, W. C. and Sohal, R. S. (1992). The effects of catalase gene overexpression on life span and resistance to oxidative stress in transgenic Drosophila melanogaster. *Arch Biochem Biophys* **297**, 35-41.

Page, R. E., Jr., Erber, J. and Fondrk, M. K. (1998). The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (Apis mellifera L.). *J Comp Physiol A* **182**, 489-500.

Page, R. E., Jr. and Fondrk, M. K. (1995). The Effects of Colony-Level Selection on the Social Organization of Honey Bee (Apis mellifera L.) Colonies: Colony-Level Components of Pollen Hoarding. *Behavioral Ecology and Sociobiology* **36**, 135-144.

Page, R. E., Jr. and Peng, C. Y. (2001). Aging and development in social insects with emphasis on the honey bee, Apis mellifera L. *Exp Gerontol* **36**, 695-711.

Pankiw, T. and Page, R. E., Jr. (2001). Genotype and Colony Environment Affect Honeybee (Apis mellifera L.) Development and Foraging Behavior. *Behavioral Ecology and Sociobiology* **51**, 87-94.

Pankiw, T., Waddington, K. D. and Page, R. E., Jr. (2001). Modulation of sucrose response thresholds in honey bees (Apis mellifera L.): influence of genotype, feeding, and foraging experience. *J Comp Physiol A* **187**, 293-301.

Pavlov, I. P. (1927). Lecture II. In *Conditioned reflexes*, pp. 16-32. New York: Courier Dover Publications.

Rascón, B. and Harrison, J. F. (2010). Lifespan and oxidative stress show a non-linear response to atmospheric oxygen in Drosophila. *J Exp Biol* **213**, 3441-8.

Rascón, B. and Harrison, J. F. (2011). Lifespan and oxidative stress show a non-linear response to atmospheric oxygen in Drosophila. *J Exp Biol* **213**, 3441-8.

Rizki, G., Iwata, T. N., Li, J., Riedel, C. G., Picard, C. L., Jan, M., Murphy, C. T. and Lee, S. S. (2011). The evolutionarily conserved longevity determinants HCF-1 and SIR-2.1/SIRT1 collaborate to regulate DAF-16/FOXO. *PLoS Genet* **7**, e1002235.

Rogina, B. and Helfand, S. L. (2004). Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc Natl Acad Sci U S A* **101**, 15998-6003.

Scharlaken, B., de Graaf, D. C., Goossens, K., Brunain, M., Peelman, L. J. and Jacobs, F. J. (2008). Reference gene selection for insect expression studies using quantitative real-time PCR: The head of the honeybee, Apis mellifera, after a bacterial challenge. *Journal of Insect Science* **8**.

Scheiner, R. and Amdam, G. V. (2009). Impaired tactile learning is related to social role in honeybees. *J Exp Biol* **212**, 994-1002.

Scheiner, R., Page, R. E., Jr. and Erber, J. (2001a). The effects of genotype, foraging role, and sucrose responsiveness on the tactile learning performance of honey bees (Apis mellifera L.). *Neurobiol Learn Mem* **76**, 138-50.

Scheiner, R., Page, R. E., Jr. and Erber, J. (2001b). Responsiveness to sucrose affects tactile and olfactory learning in preforaging honey bees of two genetic strains. *Behav Brain Res* **120**, 67-73.

Seehuus, S. C., Krekling, T. and Amdam, G. V. (2006a). Cellular senescence in honey bee brain is largely independent of chronological age. *Exp Gerontol* **41**, 1117-25.

Seehuus, S. C., Norberg, K., Gimsa, U., Krekling, T. and Amdam, G. V. (2006b). Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proc Natl Acad Sci U S A* **103**, 962-7.

Sohal, R. S., Agarwal, S., Dubey, A. and Orr, W. C. (1993). Protein oxidative damage is associated with life expectancy of houseflies. *Proc Natl Acad Sci U S A* **90**, 7255-9.

Sohal, R. S. and Dubey, A. (1994). Mitochondrial oxidative damage, hydrogen peroxide release, and aging. *Free Radic Biol Med* **16**, 621-6.

Sonmez, U., Sonmez, A., Erbil, G., Tekmen, I. and Baykara, B. (2007). Neuroprotective effects of resveratrol against traumatic brain injury in immature rats. *Neurosci Lett* **420**, 133-7.

Takeda, K. (1961). Classical conditioned response in the honey bee. *J Insect Physiol* **6**, 168-179.

Tamura, T., Chiang, A. S., Ito, N., Liu, H. P., Horiuchi, J., Tully, T. and Saitoe, M. (2003). Aging specifically impairs amnesiac-dependent memory in Drosophila. *Neuron* **40**, 1003-11.

Tissenbaum, H. A. and Guarente, L. (2001). Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. *Nature* **410**, 227-30.

Tolfsen, C. C., Baker, N., Kreibich, C. and Amdam, G. V. (2011). Flight restriction prevents associative learning deficits but not changes in brain protein-adduct formation during honeybee ageing. *J Exp Biol* **214**, 1322-32.

Valenzano, D. R., Terzibasi, E., Genade, T., Cattaneo, A., Domenici, L. and Cellerino, A. (2006). Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. *Curr Biol* **16**, 296-300.

Vaziri, H., Dessain, S. K., Ng Eaton, E., Imai, S. I., Frye, R. A., Pandita, T. K., Guarente, L. and Weinberg, R. A. (2001). hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* **107**, 149-59.

von Zglinicki, T. and Sitte, N. (2003). Free Radical Production and Antioxidant Defense: A Primer. In *Aging at the Molecular Level* (ed. T. Von Zglinicki): Kluwer Academic Publishers.

Walker, D. W. and Benzer, S. (2004). Mitochondrial "swirls" induced by oxygen stress and in the Drosophila mutant hyperswirl. *Proc Natl Acad Sci U S A* **101**, 10290-5.

Wang, C., Chen, L., Hou, X., Li, Z., Kabra, N., Ma, Y., Nemoto, S., Finkel, T., Gu, W., Cress, W. D. et al. (2006). Interactions between E2F1 and SirT1 regulate apoptotic response to DNA damage. *Nat Cell Biol* **8**, 1025-31.

Winston, M. L. (1987). The Biology of the Honey Bee: First Harvard University Press.

Wolschin, F., Munch, D. and Amdam, G. V. (2009). Structural and proteomic analyses reveal regional brain differences during honeybee aging. *J Exp Biol* **212**, 4027-32.

Wood, J. G., Rogina, B., Lavu, S., Howitz, K., Helfand, S. L., Tatar, M. and Sinclair, D. (2004). Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature* **430**, 686-9.

Paper I

Honey Bee Life-History Plasticity — Development, Behavior, and Aging

Brenda Rascón, Navdeep S. Mutti, Christina Tolfsen, Gro V. Amdam

Abstract

Honey bee (*Apis mellifera*) colonies are considered homeostatic superorganisms, in which the collective behavior of thousands of individuals regulates colony growth, reproduction, core temperature, and food storage. Honey bee societies are sensitive to environmental variation, and can respond to such variation by generating individuals with different developmental, behavioral, and aging phenotypes from largely similar genomes. Based on controlled variation in larval feeding, female honey bees develop into distinct female reproductive castes: fertile queens and functionally sterile workers (helpers). Workers are characterized by variation in complex social behavior. Behavior follows a regulated developmental schedule, but the schedule is rather flexible and can even be reversed in response to changes in the colony environment. Variation in task performance translates into different aging rates, and thereby, aging becomes a function of behavioral control.

CHAPTER 20

Honey bee life history plasticity: Development, behavior, and aging

Brenda Rascón, Navdeep S. Mutti, Christina Tolfsen, and Gro V. Amdam

20.1 Introduction

Honey bees exhibit a complex pattern of social organization that is embodied in their division of labor, making them some of the most ecologically successful insects. Recently, Hölldobler and Wilson (2008) resurrected the early 20th century metaphor of the insect society as a "superorganism" with physiological, reproductive, communication, and information-processing properties not unlike that of the single individual. The metaphor works well at the phenomenological level of the colony, but it is not always applicable to the study of the development, behavior, and aging of individuals in a society. Individual social insects display different biases in the kinds of behavioral tasks they perform, and these are often associated with changes in physiology that are correlated with age and adult morphological differences. A single "social genome" that is responsible for the ontogeny of development, and on which natural selection can act, does not exist. Instead, each individual is a product of development derived from its own genome. A challenge for scientists will be to understand how the regulation of development, behavior, and aging is achieved in such an advanced social group.

20.2 Development

The honey bee, *A. mellifera*, is characterized by complete metamorphosis (holometabolism). This developmental process is demarcated by four distinct stages, egg, larva, pupa, and adult, and is controlled by the endocrine regulators juvenile hormone (JH) and ecdysone (Winston 1987). Honey bees have a haploid–diploid sex determination system in which a fertilized egg develops into a female and an unfertilized egg develops into a drone (male bee) (Winston 1987). The embryo grows for three days and hatches into a larva. Honey bee larvae develop rapidly and proceed through five larval instars in about 5–6 days. At the end of the fifth larval instar, feeding ceases and pupation begins. During the pupal stage, which lasts about 14 days, the larval structures are broken down and adult anatomical features are formed. Thereafter, the bee emerges and metamorphosis is complete. The duration of development is caste and sex-specific, and ranges from 16 days for a queen to 21 days for a worker and 24 days for a drone (male honey bee).

20.2.1 Female caste morphology: Physiology, function, and reproduction

Honey bee females can develop into two castes: reproductive queens or essentially sterile workers. The behavioral and functional distinctions between queens and workers are primarily shaped during larval life. This is achieved through differential nutrition received by larvae that are largely genetically identical. Caste fate is determined by adult nurse bees, which control the amount and type of food provisioned to the larvae (Fig. 20-1). In honey bee society, queens are solely responsible for egg-laying. The queen has a well-developed reproductive system with more than 150 ovarioles (ovary filaments that produce eggs) per ovary and can lay

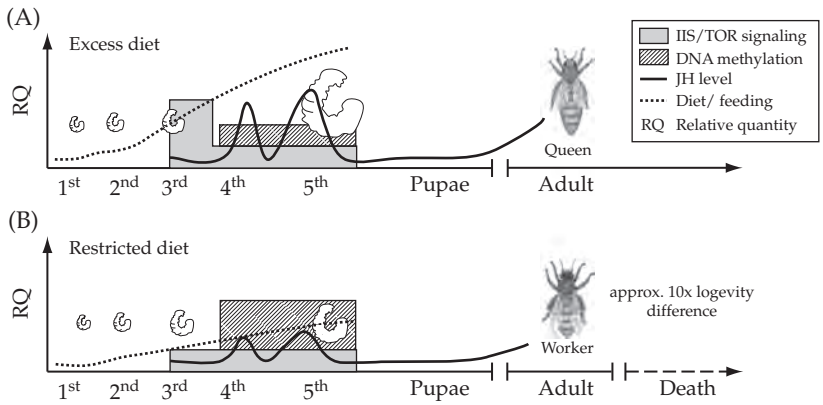


Figure 20-1 Female caste fate and longevity in the honey bee is determined by larval feeding. Molecular changes are depicted throughout larval development (five instars) and into adulthood. (A) Larvae fed a nutrient-rich diet (queen jelly, dotted line) early in life develop into reproductively active queens. Queen-destined larvae up-regulate IIS/TOR genes (relative quantities (RQ)), leading to enhanced IIS/TOR signaling followed by decreased DNA methylation in the fourth and fifth larval instar and a concomitant surge in JH titers. (B) Larvae fed a less nutrient-rich diet (worker jelly, dotted line) develop into workers and exhibit lowered IIS/TOR signaling (RQ) in the third and fourth larval instars relative to queen-destined larvae. These larvae show a higher degree of DNA methylation, and a less dramatic rise in JH titers in comparison to queen-destined larvae. The differences in IIS/TOR signaling cascade in the two female castes ensure more rapid growth in queen-destined larvae than in worker-destined larvae, which may underlie the differences in stress resistance and longevity in the adult stage. Interestingly, despite increased IIS/TOR signaling during development and high rates of reproduction, the queen can live markedly longer than her sibling worker bees.

up to 2000 eggs per day following a single or a few mating flights, which usually take place during the first weeks of her life (Winston 1987). In contrast, workers have reduced reproductive systems (only 2–20 ovarioles per ovary) and are functionally sterile. Instead of participating in direct reproduction, worker honey bees carry out necessary colony maintenance such as brood rearing and foraging for food resources, and take part in the reproductive swarm activities that are essential for colony-level reproduction. While worker honey bees have the potential to lay viable eggs, they will generally not do so under normal circumstances, as worker ovary development and egg-laying behavior are suppressed by pheromones secreted by the queen and brood (Ratnieks 1993). In the absence of the queen and young larvae, however, worker honey bees can lay unfertilized eggs that develop into haploid male drones. However, a colony with only laying worker bees is not sustainable and generally collapses within two months (Winston 1987).

As adults, queens, and workers are highly specialized in terms of morphology, physiology, and behavior. For instance, workers possess slim abdomens, corbiculae (a structure for carrying pollen) on their hind legs, and a well-developed proboscis (long tongue) for feeding, cleaning, and food collection. In contrast, the corpulent, full-bodied queen bee is fed and groomed by workers, but does not possess corbiculae or a long proboscis, nor does she take part in colony nourishment, construction, or maintenance activities (Winston 1987). Moreover, workers have hypopharyngeal head glands that synthesize nutritious brood food (jelly). Workers and queens both possess stingers, but they use them for different behaviors. Worker honey bees will use their barbed (unretractable) stingers to attack intruders as part of their suicidal aggressive response during colony defense. On the contrary, the queen does not engage in colony defense and seldom uses her smooth and retractable stinger except in cases of supersedure (to attack, kill, and supersede a competitor) (Winston 1987).

20.2.2 An integrative molecular model for caste development: Differential nutrition during larval development triggers caste differentiation

A major molecular player in this phenotypic switch is JH. JH is a major systemic lipophilic hormone that is

sensitive to ambient and social environment, nutrition, and physiology, and is an important transcriptional regulator in insects (Hartfelder and Engels 1998). The level of circulating JH is dynamic in both queen and worker-destined larvae throughout development. In fourth to fifth instar female larvae, JH levels in both whole-body extracts and in hemolymph are higher in queen-destined individuals than in worker-destined larvae of the same age (Rachinsky *et al.* 1990). Also, the application of synthetic JH causes worker-destined larvae to develop queen-like traits (Rembold *et al.* 1974, Barchuk *et al.* 2007). However, JH is only one of many factors that play a role during caste development.

Vertebrate studies show that environmental factors like food availability (affecting nutrient uptake) can influence gene expression by acting on transcription factors and the epigenome (Jaenisch and Bird 2003, Burdge *et al.* 2007). Because early-life social environment and nutrition are critical for the reliable segregation of honey bee castes, it has been postulated that caste differentiation may involve changes in the epigenome (Kucharski *et al.* 2008). In honey bees, a full complement of functional DNA cytosine-5-methyltransferases, similar to that of vertebrates, has been identified (Wang *et al.* 2006b). Interestingly, Kucharski *et al.* (2008) showed that cytosine–phosphate–guanosine (CpG) methylation by DNA methyltransferase 3 can be lower in queen-destined larvae than in developing worker-destined bees, supporting the hypothesis that DNA methylation may play an important role in caste development.

Large-scale transcript studies have identified hundreds of genes that are differentially expressed in queen- and worker-destined larvae (Evans and Wheeler 1999, Barchuk *et al.* 2007). Queen-destined larvae show up-regulation of genes involved in metabolism and nutrient sensing (Barchuk *et al.* 2007), including key components of the insulin/insulin-like signaling (IIS) and target of rapamycin (TOR) pathways (Wheeler *et al.* 2006). Recently, it was shown that decreasing the expression of TOR kinase (Patel *et al.* 2007) and insulin receptor substrate (IRS, a member of IIS) via RNA interference (RNAi) in young larvae, causes queen-destined individuals to develop worker-like traits (Wolschin *et al.* 2011). Moreover, Mutti and colleagues observed that suppression of IRS, TOR, and queen fate is accompanied by decreased JH titers and increased DNA methylation levels, consistent with the results that elevated JH and reduced DNA methylation are associated with normal queen development (Mutti *et al.* submitted). Taken together, this suggests that the honey bee caste-differentiation cascade may be organized with IIS and TOR as the upstream regulators of both DNA methylation and endocrine effectors like JH. These findings can be summarized in our model, which illustrates how nutritional input signal variation in genetically identical sisters can be canalized to produce two distinct female phenotypes (Fig. 20-1) (see also Chapter 25).

20.3 Behavioral maturation and specialization

In honey bees the division of labor is characterized by temporal polyethism, a maturational schedule in which worker bees move through an age-correlated series of tasks (Winston 1987). Young bees initially perform within-colony activities, such as nursing of brood, cleaning, and taking care of the queen. At two to three weeks of age, worker bees transition to more risky outdoor foraging tasks, which they usually carry out for the remainder of their lives. During this behavioral change, the physiology of the young bee is remodeled for foraging. Some of the gross physiological changes that take place include an overall drop in body weight of 40%, reduced innate immunity, reduced stress resistance, and altered hormonal and molecular profiles (Page *et al.* 2006, Amdam *et al.* 2009b, Whitfield *et al.* 2006).

JH and vitellogenin (Vg) have been proposed as major endocrine regulators of behavioral maturation. Vg, an egg yolk-precursor and phospholipoglycoprotein, serves non-reproductive functions in worker honey bees, and elicits a positive influence on immunity, oxidative stress resistance, and longevity (see Seehuus *et al.* 2006a,b). Vg, which is mainly produced by young nurse bees before foraging initiation, is also an important transportable and transferable nutrient reserve for the colony as it serves as a source of protein for the brood and is distributed to other colony members by mouth (see Amdam *et al.* 2009b and references therein).

Vg synthesis occurs in the abdominal fat body (the functional homolog of vertebrate liver and white fat)

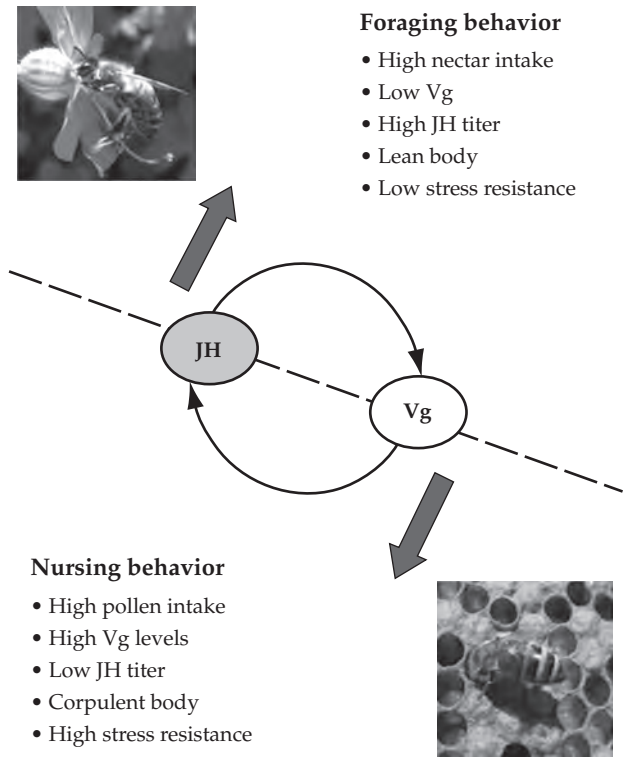


Figure 20-2 Behavioral maturation in worker bees is regulated by a negative feedback loop between Vg and JH. In worker bees, haemolymph Vg titres rise at emergence and remain high throughout the nursing developmental period. The transition to foraging is accompanied by a drop in Vg and an increase in JH levels. In bees, Vg may act as a free-radical scavenger and as a rich source of amino acids, lipids, and carbohydrate. The Vg–JH axis consequently also modulates stress sensitivity and nutrient status in the adult worker. Thus, while the bee is performing nursing tasks, her Vg levels and oxidative stress resistance are elevated. After transitioning to foraging, her Vg levels drop and JH titers rise. At foraging onset, the worker bee becomes more susceptible to oxidative stress. In the negative feedback loop, high Vg levels may block JH synthesis and delay foraging onset. JH may reciprocally inhibit Vg synthesis and induce early foraging onset.

and is released into the hemolymph. The production of Vg is initiated immediately prior to adult emergence and is detectable in the hemolymph of bees older than three days (Pinto *et al.* 2000). From this age on, Vg steadily increases and reaches a maximum level during the nursing stage of behavioral development. JH follows an inverse pattern: when Vg levels are high, JH titers are low. Prior to the foraging transition, Vg levels decline and JH increases.

At the molecular level, the temporal division of labor among worker bees appears to be orchestrated by a mutually antagonistic feedback loop between Vg and JH (Amdam and Omholt 2003). In the double repressor network proposed by Amdam and Omholt (2003), Vg suppresses JH to delay foraging in nurses (Fig. 20-2). The mutually antagonistic relationship between Vg and JH has been verified by RNAi-mediated knockdown of Vg, which causes an elevation of JH titer (Guidugli *et al.* 2005) and accelerates the transition to foraging (Nelson *et al.* 2007). Furthermore, the treatment of young bees with JH analogues such as methoprene or pyriproxyfen induces precocious foraging and lowers Vg levels (Pinto *et al.* 2000, Schulz *et al.* 2002 and references therein). These findings support the hypothesis of a feedback relationship in which high JH levels suppress Vg synthesis and/or accelerate its degradation (Amdam and Omholt 2003).

A mechanistic description for the causation route between Vg, JH, and onset of foraging remains elusive, but a putative Vg receptor has been localized to the head, fat body, and ovaries (Guidugli-Lazzarini

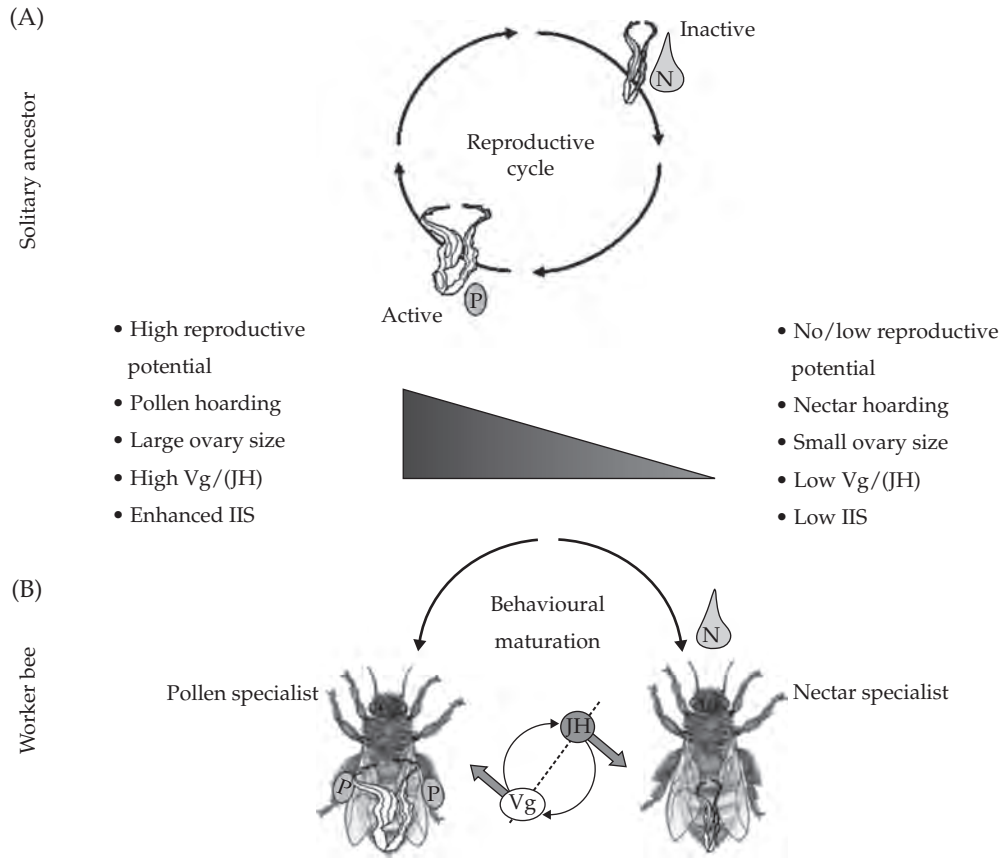


Figure 20-3 The modulation of foraging specialization by the negative feedback loop between Vg–JH as explained though the Reproductive Ground Plan Hypothesis of Amdam and colleagues (2004). This hypothesis posits that the reproductive physiology and reproductive genes of solitary ancestors were co-opted with foraging behavior during the social evolution of the honey bee. Pollen—a source of proteins, lipids, and vitamins—is hoarded by reproductively active individuals and required by brood for proper development. (A) In solitary insects, the reproductive state is characterized by large ovaries and many ovarioles, high Vg and JH titres, and a predisposition for pollen hoarding. In the non-reproductive state, ovary sizes are reduced, Vg and JH levels are low, and the solitary insect primarily forages for nectar. (B) Analogous to the solitary ancestor, high pollen-hoarding (high strain) honey bees forage earlier, possess larger ovaries (more, non-activated ovarioles), high Vg titres, and preferentially forage for pollen. On the other hand, low pollen-hoarding (low strain) bees forage later, possess smaller ovaries with fewer ovarioles, lower levels of Vg, and bias their foraging collection towards nectar. In worker bees, slow behavioral maturation and late foraging onset is typically associated with a longer life. Vg affects IIS signaling, which in turn, signals back to Vg via JH.

et al. 2008). This finding suggests that Vg could influence molecular networks in a variety of tissues, including those in which it is not expressed. Recently, Vg was also detected in brain tissue, and dynamic regulation has been localized to the central brain (Muench, Ihle and Amdam unpublished data).

20.3.1 Specialization of foraging behavior

A forager bee is capable of collecting both pollen and nectar in a single foraging trip, but she may bias her collection of food resources towards either nectar or pollen (Winston 1987). An explanatory framework for the evolution of foraging specialization is provided by the Reproductive Ground Plan Hypothesis (RGPH) of Amdam and colleagues (Amdam *et al.* 2004), which posits that gene networks that coordinated foraging behavior with reproductive physiology in ancestral solitary insects were co-opted to serve as a basis for behavioral specialization during the social evolution of the honey bee (Fig. 20-3).

The RGPH predicts that maternal reproductive traits such as Vg titer and ovary size, which are not normally geared toward actual reproduction in workers, are components of a suite of traits that influence foraging behavior. Support for the RGPH is evident in high- and low-pollen-hoarding honey bee strains that are bi-directionally selected for different foraging behavior toward pollen. Artificial selection resulted in bee colonies that collect and store low quantities (low sub-lines) versus higher (high sub-lines) quantities of pollen. These strains differ in several physiological traits that are generally associated with reproduction in insects (Page and Amdam 2007). Overall, the physiology of workers that collect pollen resembles the maternal or active reproductive stage of solitary insects (pollen hoarding is a specific maternal behavior in many solitary bees), including higher hemolymph levels of Vg, larger ovaries, and increased sensory sensitivity and motor activity (Page *et al.* 2006). The association between pollen hoarding and maternal behavior in solitary bees led to the proposition that honey bee foraging behavior is governed by the same ancestral molecular networks that have their roots in maternal care.

The link between reproductive traits and social behavior in workers has been corroborated by the mapping of major quantitative trait loci (QTL) for foraging behavior. The QTL architecture confirms that foraging behavior is influenced by a pleiotropic gene network, and that these genome regions show an over-abundance of IIS genes, which are central to nutritional regulation, reproduction, and food-related behavior in animals (Amdam *et al.* 2009a) (see Section 12.2). Ovarian factors may influence IIS (Flatt *et al.* 2008b), providing a potential explanation for the link between worker ovary size and behavior. These relationships are currently under investigation, but in the meantime the brain is generally regarded as the more autonomous pacemaker for behavior.

20.3.2 Central nervous system changes during behavioral maturation

Nurse bees mainly navigate in the darkness of the colony, where communication depends on odor- and mechanosensory perception (Winston 1987). Foraging, on the other hand, requires the processing of visual and olfactory stimuli for the learning and memorization of food sources and landmarks. In accordance with this, the most notable CNS changes during honey bee behavioral maturation occur within the olfactory glomeruli and the mushroom body (MB), higher order centers for olfactory perception and learning, respectively. The MB exhibits morphological plasticity through an increase in volume and outgrowth of brain neuropiles (Fahrbach *et al.* 1998), which cannot be explained by neurogenesis (Fahrbach *et al.* 1995) nor can these changes be correlated with the JH upregulation that is characteristic of the forager transition (Fahrbach *et al.* 1998).

Forager honey bee brains that show MB neuropil growth have higher expression levels of the transcription factor Krüppel homolog 1 (*Kr-h1*) (Fussnecker and Grozinger 2008). *Kr-h1* expression is induced by cGMP, and recent work on *Kr-h1* in *Drosophila melanogaster* implicates this transcription factor in ecdysone-mediated developmental MB plasticity (Hewes 2008). In honey bees, this relationship has not yet been confirmed, but it is possible that *Kr-h1* plays a role comparable to the one in *Drosophila*.

During behavioral development, honey bees also show an up-regulation of two candidate genes for *foraging* behavior: *malvolio* (Ammvel) and *foraging* (Amfor) (Ben-Shahar *et al.* 2002, 2004). *Malvolio* encodes a putative manganese transport protein that is associated with increased sucrose responsiveness in honey bees; *foraging* is an ortholog of the *D. melanogaster foraging* (for) gene that encodes a cGMP-dependent protein kinase (PKG) (Ben-Shahar *et al.* 2002). In bees, the manipulation of PKG levels causes precocious foraging. Foragers with increased levels of *foraging* navigate towards light, which suggests that *foraging* influences honey bee foraging behavior by stimulating phototaxis (Ben-Shahar *et al.* 2003). Since *Kr-h1* expression correlates with PKG activation and the Krh-1 promoter contains a putative cGMP-response element, *foraging*/PKG is believed to be a master regulator of a gene network for *foraging* behavior that includes *Kr-h1* (Fussnecker and Grozinger 2008).

Microarray studies suggest that there are additional independent molecular pathways that are correlated with honey bee behavioral maturation (Whitfield *et al.* 2006). Past molecular studies conducted on the honey bee brain show that the mRNA levels of many genes differ between nurse bees and foragers (Whitfield *et al.* 2003). Genes with alleged roles in signal transduction, glutamate biosynthesis, and chemical homeostasis are increased in foragers, whereas genes with presumed roles in structural development are up-regulated in nurse bees. Several genes involved in translation are up-regulated in foragers, while others of the same category are up-regulated in nurse bees (Whitfield *et al.* 2006).

Additional independent molecular pathways have been correlated with honey bee behavioral maturation. Biogenic amines, several protein kinases, and second messengers are all part of an intricate network that modulates sensory sensitivity, motor function, and learning in response to behavioral task and foraging specialization (Page *et al.* 2006, Amdam *et al.* 2009a). Biogenic amines such as dopamine, serotonin, octopamine, and tyramine modulate aspects of gustatory, olfactory, and visual sensitivity in honey bees. Foragers show elevated levels of dopamine, octopamine, and serotonin (Schulz and Robinson 1999). Specifically, octopamine has been implicated in the nurse bee to foraging transition and recently a study showed that this biogenic amine can increase the likelihood of waggle dancing, a behavioral display that signals food resource quality (Barron *et al.* 2007).

Further work is needed to decipher the events that take place in the CNS during behavioral maturation, but it is clear that multiple pathways may act in conjunction with one another to elicit behavioral changes in the honey bee.

20.3.3 Metabolic changes during behavioral maturation

The behavioral transition of the honey bee from within-colony labor to foraging duties is marked by metabolic changes that remodel the physiology of the bee to alter oxidative requirements during flight and foraging behavior. Honey bee foragers engage in extensive food hoarding that involves frequent and long flights, as well as other energy-demanding and complex behaviors such as navigation, recruitment dances, and associative learning, which are used for communication and the memorization of foraging sources (Winston 1987). As a result, the physiological demands of foraging depend on increased oxidative capacity and altered nutrient processing.

Flight induces a dramatic change in the basal metabolic rate of the honey bee. Overall, foragers have mass-specific oxygen consumption rates that are 50% higher than those of nurse bees (Harrison 1986). During behavioral ontogeny, honey bee flight metabolic rate is 10-fold higher than the resting rate and is paralleled by a 10-fold increase in flight muscle cytochrome and a significant rise in glycolytic and antioxidant protein levels (Harrison and Fewell 2002, Roberts and Elekonich 2005, Wolschin and Amdam 2007a). To fuel their flights, foragers utilize carbohydrates (Winston 1987) and have thoracic glycogen stores double that of nurse bees (Harrison 1986). Indicative of an elevated metabolism, foragers typically exhibit higher overall protein turnover levels than younger nurse bees (Crailsheim 1986).

Foragers also display changes in IIS, as evidenced by heightened levels of insulin-like peptide 1 (ilp1) in the head, and insulin receptor 1 and 2 (InR1 and InR2) in the abdomen (Ament *et al.* 2008). Evidence from Vg RNAi and protein injection experiments in honey bees suggests that Ilp1 and Ilp2 are part of two separate paracrine systems that control fat body metabolism and govern somatic resource allocation during behavioral maturation. The Ilp2–JH axis likely modulates fat body lipid/carbohydrate resources and Ilp1 regulates protein synthesis and storage (Nilsen *et al.*, submitted). Recently, Wang and co-workers demonstrated the effects of reduced peripheral IIS on honey bee foraging behavior by down-regulating IRS in the abdominal fat body. They showed that IRS knockdowns biased their foraging efforts towards the collection of pollen (protein source) rather than nectar (carbohydrate source) (Wang *et al.* 2010).

Collectively, these findings indicate that there is an ontogenetic shift in total metabolism and nutrient processing during behavioral maturation that can be mediated by IIS signaling.

20.4 Worker aging

In the past, many senescence-driven studies have utilized the ubiquitous *D. melanogaster* and the roundworm, *Caenorhabditis elegans*, due to their short, tractable lifespans and widely used genetic tools available for these species. Recently, the honey bee has emerged as a promising new model system for senescence research due to its remarkable aging plasticity and socio-behavioral repertoire (Munch *et al.* 2008). However, due to the high degree of complexity associated with the elastic, ontogenetic specialization of tasks and the compartmentalization of alloparental functions within the honey bee colony, many of the well-known theories of aging are not always applicable to the life history of the honey bee.

Life history theory postulates that the pressure of natural selection on survival, which favors fitness during the reproductive phase of life, decreases after reproductive capacity has been exhausted. However, because the worker bee is functionally sterile and behaviorally moves through a series of stage-dependent tasks (see Sections 20.2 and 20.3), established theories of aging may not adequately explain the honey bee pattern of senescence (Amdam and Page 2005).

Classic evolutionary theories of aging, such as Medawar's mutation accumulation theory (1952), Williams' antagonistic pleiotropy theory (1957), and Kirkwood's (1977) disposable soma theory, all attempt to explain why rising mortality rates accompany old age. All theories rest on the concept of extrinsic mortality, such that when hazard (risk of dying) is high, then natural selection will not favor further investment of resources into the soma. In contrast, if extrinsic mortality is low, then selection favors somatic maintenance and would act more weakly to reduce mortality rates at older ages. Although these theories are generally regarded as the dominant explanatory paradigms for the evolution of aging, the sole focus on reproduction limits the application of these ideas to sterile worker honey bees, which act as alloparental caregivers that experience low mortality risk for part of their lives (during nursing) before moving to more hazardous tasks outside the hive (foraging) (Winston 1987). However, a postulate that integrates social resource transfers (e.g., brood care) with classical evolutionary thinking on aging (Lee 2003) would center on parental investment and resource transfers between individuals of a multitude of ages. This approach could prove fruitful in describing the aging characteristics of honey bee workers, which, depending on age and environment, engage in different forms of social resource transfers throughout their lives. However, empirical evaluation of this theory will require measurement of a myriad of behaviors, such as guarding, food exchange, fanning, warming, and foraging (Amdam and Page 2005).

20.4.1 Plasticity of aging

Honey bee senescence appears to differ in certain aspects from the aging patterns of the solitary model organisms traditionally used in aging research, which are characterized by progressive and irreversible aging. In contrast, honey bee aging is largely related to social task performance, rather than to chronological age (Fig. 20-4). Bees do not exhibit the characteristics commonly associated with aging during the first 30 days of the nursing period, while aging accelerates after transition to foraging. Thus, the shift from nursing to foraging is the most crucial determinant of the overall lifespan expectancy for a honey bee (Rueppell *et al.* 2008, Amdam *et al.* 2009b).

The number of days honey bees spend within the colony performing nest tasks can vary depending on the season, and strongly influences lifespan. Nurse bees can survive for more than 130 days, while diutinus or "winter" bees, which develop in the absence of brood and nursing activity, can survive for more than 280 days (negligible senescence) before they segregate into nurse bees (slow aging) and foragers (rapid aging) (Seehuus *et al.* 2006a). Thus, senescence in honey bees can be remarkably plastic. This plasticity is tested by social environmental manipulations, where removal of nurse bees can cause forager bees to behaviorally and physiologically revert to nursing tasks (see Section 20.3 and Fig. 20-4 for details). This role-reversal alters many important biomarkers of senescence. Reverted nurse bees (former foragers) undergo a reversal of immunosenescence and exhibit some of

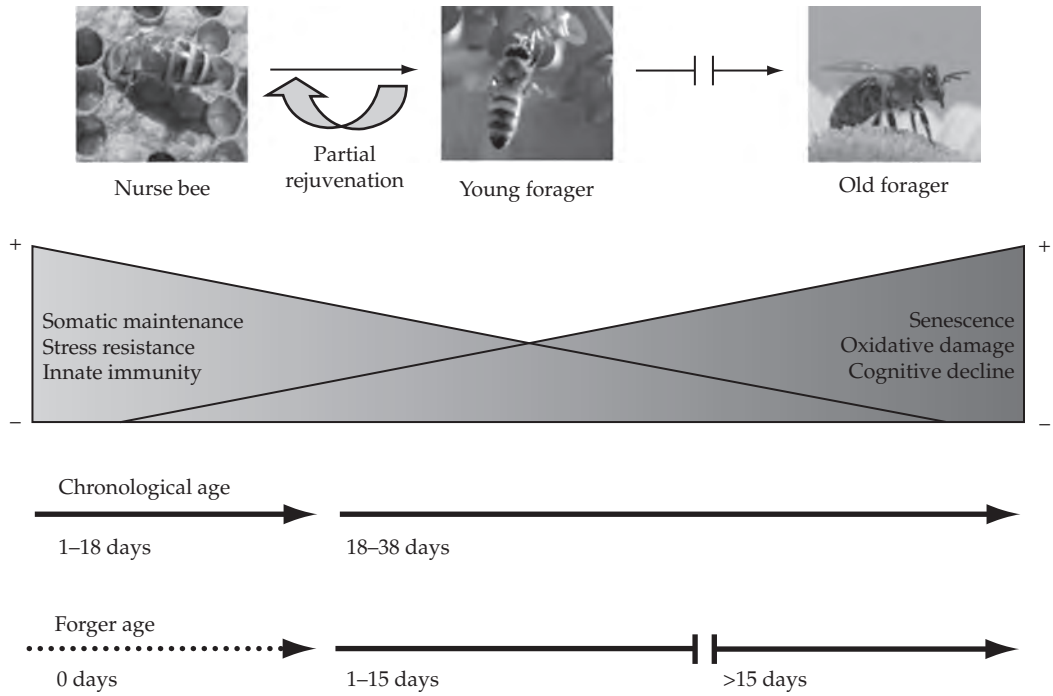


Figure 20-4 Honey bee worker senescence is a function of the behavioral task they perform within the colony. Senescence in workers is a plastic process that may be manipulated by changes in colony demography and/or environmental conditions. Nurse bees have high levels of Vg and are more resistant to oxidative and environmental stressors. During foraging onset, the somatic maintenance of the bee drops, and the worker bee experiences a dramatic decline in immune function. Young foragers are capable of a partial rejuvenation if they switch back to nursing tasks. As the forager bee becomes older, the cell repair and maintenance machinery becomes overwhelmed and the bee gradually accumulates protein oxidative damage in the brain. After approximately two active weeks of foraging, she experiences cognitive decline. While both nurses and young foragers perform well in associative learning, forager bees aged more than 15 days typically perform poorly. In the bee, as in many species, the accumulation of oxidatively modified proteins and a reduction in cognitive performance are unequivocal signs of senescence.

the biological hallmarks of the younger nurse bees they replace, for example elevation of Vg, and a suppression of JH (Amdam *et al.* 2005). However, reverted worker bees are not identical to normal nurse bees, but rather exhibit a mixed physiology that includes characteristics of the former forager state. Wolschin and Amdam (2007b) found that reverted worker bees have proteomic profiles that resemble both nurse bees and foragers. Moreover, there appear to be limits to aging reversal. Some aged foragers that have surpassed a "point of no return" appear unable to behaviorally revert and continue to progressively age.

In summary, although counterintuitive to our conventionally accepted axiom of aging, the revelation that chronological age can be decoupled from social task is intriguing, particularly for those interested in the reversal of the aging process.

20.4.2 Oxidative stress

One of the mechanisms that may underlie senescence in honey bees is oxidative stress. The free radical theory of aging proposes that molecular oxygen can serve as a source of reactive oxygen species (ROS), which can induce cumulative macromolecular damage and ultimately cause aging (Harman 1956). ROS are involved in normal cell respiration as by-products of aerobic mitochondrial metabolism, but they can also inflict damage on proteins, lipids, and DNA if not successfully scavenged by cellular antioxidants. The modulation of oxygen

tension in invertebrates has become an essential tool for manipulating oxidative stress *in vivo*. Increased oxygen levels are known to augment the rate of ROS production, reduce the lifespan of *C. elegans* and *D. melanogaster*, and induce physiological changes that are of relevance to senescence (von Zglinicki and Sitte 2003).

Aside from its previously described functions during behavioral maturation (see Section 20.3), Vg can also act as an antioxidant (Seehuus *et al.* 2006b). Using RNAi-mediated knockdown of Vg, Seehuus *et al.* (2006b) showed that Vg protects worker honey bees from oxidative stress. Moreover, a subsequent RNAi-mediated Vg knockdown study confirmed that Vg can extend life (Nelson *et al.* 2007). Collectively, these studies further reinforce the observation that foragers display a greater vulnerability to senescence and also highlight that Vg may have acquired new functions in the honey bee.

Honey bee foragers must expand oxidative capacity to accommodate the energetic demands of flight (see Section 20.3). The elevated oxygen consumption rates associated with flight presumably also augment ROS production and accelerate senescence. A honey bee study focused on examining metabolically active tissues—flight muscle and brain—found that flight behavior induces an up-regulation of antioxidants in young forager flight muscles in comparison to older foragers (Williams *et al.* 2008). In the same study it was reported that as foragers grew older, their antioxidant capacity diminished within the span of a day. The authors speculated that the reduction of antioxidant defenses in the bees likely led to an acceleration of senescence via an excessive production of ROS. Interestingly, the observed changes in antioxidant defense were only evident in the thorax and not in the heads, which suggests that senescence-related damage may be tissue-specific. However, it should be noted that Corona *et al.* (2005) did not observe a clear correlation between age and antioxidant mRNA in the multiple profiled tissues (abdominal, neural, and thoracic) of worker honey bees. This could be attributed, at least in part, to the fact that the authors did not control for behavioral task, but rather used chronological age as their main metric of senescence despite evidence that illustrates the importance of social role in honey bee aging (Behrends *et al.* 2007). Also, measurements of antioxidant activity or protein levels are often better indicators of cellular active state than transcripts. This may have also contributed to the lack of a correlation between antioxidant status and age.

The evidence for protein carbonylation, a marker of oxidative stress commonly used in aging experiments, is inconsistent and appears complex in honey bees. Although Seehuus *et al.* (2006b) showed that Vg in the brain of old workers is carbonylated in response to paraquat injections, a proxy for elevated ROS (Seehuus *et al.* 2006b), Williams *et al.* (2008) did not detect protein carbonylation differences in the thoraces or heads of honey bees of varying ages or activity levels. An explanation for this apparent discrepancy is that different stressors intended to mimic aging may preferentially target specific proteins, as shown in *D. melanogaster* (Das *et al.* 2001), or other macromolecules such as lipids. Also, oxidative damage may target proteins that are small and escape detection via common experimental methods. Collectively, the results of Seehuus *et al.* (2006b) and Williams *et al.* (2008) warrant further refinement of the current methods for detecting oxidative stress in honey bees.

Alternative strategies involving other molecular targets like lipids, DNA, and mitochondria should also be explored (Barja 2002, Kaneko 2003, Haddad *et al.* 2007). The measurement of proteasomal activity may also hold promise for oxidative stress detection in honey bees, particularly since it has been shown to change with age in *D. melanogaster* (Vernace *et al.* 2007).

20.4.3 Metabolic patterns of senescence

Global transcript profiling studies have shown that the mRNA levels of antimicrobial proteins and heat shock proteins, the latter of which are often involved in protein folding, are up-regulated during aging (reviewed by Munch *et al.* 2008). Interestingly, the accumulation of unfolded or misfolded proteins (proteotoxicity), and the subsequent lack of clearance by the proteasome, is thought to play a role in senescence and age-related neurodegeneration (Gray *et al.* 2003). Thus, the up-regulation of heat shock proteins could be a mechanism to counteract these processes. In contrast, mRNA for reproductive

proteins (e.g., yolk and fatty acid binding proteins) and ATP synthesis proteins are down-regulated with increasing age, which points to the often observed decrease of motility and fecundity that accompanies old age.

Tissue-specific profiling of *D. melanogaster* has revealed that distinct tissues of the fly have different propensities for aging (Zhan *et al.* 2007). In this study, the transcript reveals changes in important functional categories like energy metabolism, protein degradation, stress resistance, immunity, and neurotransmitter release. In honey bees, a recent transcriptional study of the abdominal, neural, and thoracic tissue of queens by Corona *et al.* (2005) revealed changes in antioxidant proteins with age. The researchers found decreased levels of mRNAs with presumed roles in longevity during aging. In contrast, worker honey bees, which generally live much shorter lives than queens, exhibited no clear pattern of decline in antioxidant protein mRNA. It is possible that the chronological ages that were chosen for the queen versus worker comparison in this study (one-month worker versus one-year queen) are not indicative of true physiological age as senescence is not a simple function of chronological age in honey bees (Seehuus *et al.* 2006a). Moreover, reports on queen longevity generally place "old" queens in the range of three to four years (Winston 1987). Corona *et al.* (2005) also reported no marked mRNA expression differences between queens and workers, despite vast lifespan dissimilarities. Overall, the results illustrate that antioxidant enzymes measured at the transcript level are not necessarily correlated with organismal longevity responses.

Proteomic and metabolomic data are regarded as more representative of current cellular conditions than transcript information, but few proteomic studies of aging focus on invertebrates. Those studies that do use invertebrates center on *D. melanogaster* and *A. mellifera* (Sowell *et al.* 2007, Wolschin and Amdam 2007a,b, Wolschin *et al.* 2009). Whole-body studies utilizing both proteomic and transcript methods have shown an up-regulation of proteins and peptides with antimicrobial properties with increased age (reviewed by Munch *et al.* 2008). Moreover, during aging, a down-regulation of prophenoloxidase, an enzyme implicated in immune defense, was also detected in fruit fly brains (Sowell *et al.* 2007). Sowell and colleagues found that, as indicated above, the affected proteins in the aged *Drosophila* brain not only segregate to categories of immunity, but have functions in the reproductive, developmental, metabolic, and cellular defense networks of the cell.

In honey bees, the behavioral maturational shift, a determinant of overall lifespan, is accompanied by changes in the abundance of proteins with roles in glycolysis, ATP synthesis, and free-radical defense (Schippers *et al.* 2006, Wolschin and Amdam 2007a) (see Section 20.3). Because honey bee senescence is tied to social task rather than chronological age, Wolschin and Amdam (2007b) used nest bees and foragers, before and after behavioral reversion, to profile their respective proteomes (see Section 20.4.1). The use of such a social–environmental technique enabled the researchers to study the proteome of bees of differing ages that performed identical tasks. This study showed significant alterations in the proteome due to age and behavioral task. Moreover, the study illustrated that worker bees of distinct life histories (nest bees versus foragers) and task-matched worker bees (nest bees and foragers) of differing ages show distinct protein expression profiles (Wolschin and Amdam 2007b). The proteomic profile of reverted nest bees resembled that of nest bees prior to the behavioral reversion, supporting the idea that behavioral tasks have a particular proteomic signature. Two proteins with putative roles in lipid and cholesterol metabolism, two α-glucosidases, and a malate dehydrogenase-like protein were associated with behavioral tasks, while others, including an odorant-binding protein, showed a clear age-dependent abundance.

Transcriptome and proteome studies can impart correlative information about the trajectory of aging and can provide valuable insight into the search for viable candidates for further research. Such large-scale profiling has spawned intervention into the aging process and has started to offer insight into the prolongation of cognitive span.

20.4.4 Cognitive senescence

Aging intervention studies have shown that lifespan extension is possible in vertebrates and invertebrate

models, but the quality of life (health span) question still remains unanswered. How do we achieve enhanced longevity that is not coupled to diminished mental capacity? This is inarguably one of the main questions of our time. The honey bee system boasts a rich marriage of naturally occurring quantifiable behaviors and a history as a neuroscience model. Added to this, its aging plasticity makes it a particularly compelling organism for the study of cognitive senescence.

In honey bees, some cognitive functions, but not all, decline in foragers with extended foraging experience (Behrends *et al.* 2007, Rueppell *et al.* 2007). More specifically, a study of nurses and foragers of identical chronological ages revealed that bees that foraged for more than 15 days, in comparison to those that foraged for 6–13 days, performed more poorly in olfactory learning trials. However, the ability to more accurately discern between two odors is enhanced in foragers of long foraging duration relative to younger foragers (Behrends *et al.* 2007), indicative of greater learning acuity. These findings highlight the importance of considering life history and workload when studying the impacts of aging on cognitive performance.

To explore whether differences in foraging duration are actually linked to biochemical and structural changes in the honey bee brain, Wolschin *et al.* (2009) used proteomics and immunocytochemistry to examine the central brain and MB calyces of bees with varying foraging experience. The calyx is usually regarded as the input region for the MB, the site of memory formation, whereas the central brain is thought to be the higher order integration center. The central brain of foragers with more than 15 days of foraging experience showed a down-regulation of kinases and synaptic/neuronal growth-related proteins in comparison to workers with less foraging experience. The calyx appeared to stay intact regardless of foraging duration (Wolschin *et al.* 2009). These findings point to a complex interaction between the central brain and the MB calyx, and suggest that regions other than the calyx are responsible for foraging-dependent decline. Senescence patterns that are characteristic of some but not all brain regions appear to be common in aging, since they have also been reported in chimpanzees (Fraser *et al.* 2005).

20.4.5 Impact of nutrition sensing pathways on lifespan

The conserved nutrient sensing IIS and TOR pathways that play a role in honey bee caste differentiation (see Section 20.2) have been connected to aging in *C. elegans*, *D. melanogaster*, and *M. musculus*. (Piper *et al.* 2005, Kaeberlein and Shamieh 2010). In model invertebrates such as *D. melanogaster* and *C. elegans*, decreased IIS and TOR signaling lengthen lifespan (Tatar *et al.* 2001b, Piper *et al.* 2005, Kaeberlein and Shamieh 2010). In honey bees, the same pathways have also been associated with major adult life history transitions. The switch from nest tasks to foraging can be accompanied by increased IIS (Ament *et al.* 2008) and causes increased mortality independent of predation (Neukirch 1982). Ament *et al.* (2008) showed that foragers and nurses differ in ilp1, InR1, and InR2 levels, integral components of the IIS pathway (see Section 20.3). This same study demonstrated that the onset of foraging could be delayed in worker bees fed rapamycin, a TOR inhibitor. Thus, IIS and TOR signaling are likely involved in the behavioral transition, which plays a preeminent role in the determination of lifespan in worker honey bees.

The IIS/TOR pathways influence the course of life history in both queens and workers during development and may govern some of the adult longevity differences seen between the two castes, which also vary greatly in fecundity. In general, the relationship between the IIS/TOR cascades and lifespan in honey bees parallels findings in *D. melanogaster*, except when fecundity is taken into account.

In a comparison between queens and workers, Corona *et al.* observed lower levels of InR expression in long-lived queens (2007), consistent with observations in *D. melanogaster* and *C. elegans* in which a downregulation of nutrient sensing genes or gene partners such as InR, IRS-1, TOR, FOXO (forkhead box, sub-group O), and others extend lifespan (see Chapters 11, 13, and 22). Low fecundity is known to confer longevity in classic models of aging (Flatt and Kawecki 2007). However, the highly fecund honey bee queen can live about ten times longer than essentially sterile short-lived workers, yet shows higher

levels of IIS expression as 3rd instar larvae (Wheeler *et al.* 2006, de Azevedo and Hartfelder 2008). Thus, queens depend on increased IIS during development to achieve an adult morphology geared toward reproduction, including large body size and ovaries, whereas adulthood lifespan *per se* may be extended via reduced IIS. This regulatory ontogeny has been mimicked in *C. elegans*, which confirms that IIS can be decoupled between life stages (Dillin *et al.* 2002).

The IIS and TOR signaling pathways are also thought to underlie the increased longevity response seen with caloric restriction, which is known to have a conserved effect on the reduction of aging rate (Bishop and Guarente 2007a). Two protein deacetylases, Sir2 (Silent information regulator 2) and Rpd3 (a transcriptional regulator), are thought to mediate the life-lengthening effect of caloric restriction and have spawned a variety of dietary mimetics and medical intervention therapies (Partridge *et al.* 2005b). For instance, resveratrol, which activates a class of conserved proteins (sirtuins) that includes Sir2, extends the lifespan of *C. elegans* and *D. melanogaster*, without sacrificing fecundity (Wood *et al.* 2004). It has been proposed that resveratrol's mechanism of action mimics that of caloric restriction and that Sir2 underlies this response (Wood *et al.* 2004), but its specific modes of interaction remain to be discovered.

Caloric restriction research remains highly promising for understanding the mechanistic underpinnings of aging, but such research remains unexplored in honey bees. In the future, honey bees could potentially add to this area of research because of their dynamic social nature, food provisioning strategies, and lifespan plasticity (see Chapter 14).

20.5 Concluding remarks

A unifying theory of life history progression and aging does not presently exist, but our current understanding warrants a holistic approach. Historically, aging has been depicted as a deregulated and unavoidable death sentence. This axiom gave rise to simple deterioration and preprogrammed theories that made the process of aging seem static and perhaps not investigation worthy to some scientists. Today, however, many studies of lifespan extension, genetic intervention, and caloric restriction have revealed that life history progression and aging are plastic, regulated processes that are governed by physiological and evolutionary trade-offs. These dynamics are broadly shared between taxa but some of the traits can have species-specific features. In advanced social insects, many species-specific traits result from colony-level selection. This context provides challenges, but it also provides unique opportunities for progress towards the goal of understanding how life history plasticity evolves.

20.6 Summary

1. Honey bees are social and possess a complex behavioral repertoire along with compensatory mechanisms that allow them to respond to environmental variation.
2. Female honey bees develop into two castes: long-lived reproductive queens or shorter-lived functionally sterile worker bees. Caste differentiation depends on nutritional variation, and is mediated by the action of large metabolic networks like IIS and TOR, hormonal titers (Vg and JH), and DNA methylation.
3. In the adult worker bee, the Vg–JH axis regulates behavioral maturation, foraging specialization, and longevity in a manner that suggests that foraging behaviors derived from an ancient solitary ancestor were co-opted during the social evolution of the honey bee.
4. Behavioral maturation in honey bee workers involves physiological, anatomical, and biochemical changes that prepare the bee for metabolically-demanding processes like flight and foraging. These behaviors require the expansion of oxidative capacity, and the formation and maintenance of complex spatial memories.
5. The honey bee is a new and promising model system for aging research because of its aging plasticity and socio-behavioral repertoire. Worker bees show classic features of aging, but these are partially reversible since senescence is tied to social task rather than chronological age.

20.7 Acknowledgments

We thank two anonymous reviewers for their constructive feedback and apologize to those colleagues whose work we could not cite due to space considerations. Many thanks to Svea Hohensee for her contributions to an early version of the manuscript, Bente Smedal and Adam Siegel for photographic material, and Jim Hunt, Øyvind Halskau, Heli Havukainen, and Florian Wolschin for their critical review of the manuscript. Financial support to GVA was provided by the Norwegian Research Council (#175413, 180504, 185306) and the PEW Foundation.

References

AMDAM, G. V., AASE, A. L., SEEHUUS, S. C., KIM FONDRK, M., NORBERG, K. & HARTFELDER, K. (2005) Social reversal of immunosenescence in honey bee workers. *Exp Gerontol,* 40**,** 939-47.

AMDAM, G. V., IHLE, K. E. & PAGE, R. E. (2009a) Regulation of honey bee (Apis mellifera) life histories by vitellogenin. IN PFAFF, D., ARNOLD, A., ETGEN, A., FAHRBACH, S. E. & RUBIN, R. (Eds.) *Hormones, Brains and Behavior.* 2nd ed. San Diego, CA, Elsevier Academic Press.

AMDAM, G. V., NORBERG, K., FONDRK, M. K. & PAGE, R. E., JR. (2004) Reproductive ground plan may mediate colony-level selection effects on individual foraging behavior in honey bees. *Proc Natl Acad Sci U S A,* 101**,** 11350-5.

AMDAM, G. V. & OMHOLT, S. W. (2003) The hive bee to forager transition in honeybee colonies: the double repressor hypothesis. *J Theor Biol,* 223**,** 451-64.

AMDAM, G. V. & PAGE, R. E., JR. (2005) Intergenerational transfers may have decoupled physiological and chronological age in a eusocial insect. *Ageing Res Rev,* 4**,** 398-408.

AMDAM, G. V., RUEPPELL, O., FONDRK, M. K., PAGE, R. E. & NELSON, C. M. (2009b) The nurse's load: early-life exposure to brood-rearing affects behavior and lifespan in honey bees (Apis mellifera). *Exp Gerontol,* 44**,** 467-71.

AMENT, S. A., CORONA, M., POLLOCK, H. S. & ROBINSON, G. E. (2008) Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. *Proc Natl Acad Sci U S A,* 105**,** 4226-31.

BARCHUK, A. R., CRISTINO, A. S., KUCHARSKI, R., COSTA, L. F., SIMOES, Z. L. & MALESZKA, R. (2007) Molecular determinants of caste differentiation in the highly eusocial honeybee Apis mellifera. *BMC Dev Biol,* 7**,** 70.

BARJA, G. (2002) Endogenous oxidative stress: relationship to aging, longevity and caloric restriction. *Ageing Res Rev,* 1**,** 397-411.

BARRON, A. B., MALESZKA, R., VANDER MEER, R. K. & ROBINSON, G. E. (2007) Octopamine modulates honey bee dance behavior. *Proc Natl Acad Sci U S A,* 104**,** 1703-7.

BEHRENDS, A., SCHEINER, R., BAKER, N. & AMDAM, G. V. (2007) Cognitive aging is linked to social role in honey bees (Apis mellifera). *Exp Gerontol,* 42**,** 1146-53.

BEN-SHAHAR, Y., DUDEK, N. L. & ROBINSON, G. E. (2004) Phenotypic deconstruction reveals involvement of manganese transporter malvolio in honey bee division of labor. *J Exp Biol,* 207**,** 3281-8.

BEN-SHAHAR, Y., LEUNG, H. T., PAK, W. L., SOKOLOWSKI, M. B. & ROBINSON, G. E. (2003) cGMP-dependent changes in phototaxis: a possible role for the foraging gene in honey bee division of labor. *J Exp Biol,* 206**,** 2507-15.

BEN-SHAHAR, Y., ROBICHON, A., SOKOLOWSKI, M. B. & ROBINSON, G. E. (2002) Influence of gene action across different time scales on behavior. *Science,* 296**,** 741-4.

BISHOP, N. A. & GUARENTE, L. (2007) Genetic links between diet and lifespan: shared mechanisms from yeast to humans. *Nat Rev Genet,* 8**,** 835-44.

BURDGE, G. C., HANSON, M. A., SLATER-JEFFERIES, J. L. & LILLYCROP, K. A. (2007) Epigenetic regulation of transcription: a mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life? *Br J Nutr,* 97**,** 1036-46.

CORONA, M., HUGHES, K. A., WEAVER, D. B. & ROBINSON, G. E. (2005) Gene expression patterns associated with queen honey bee longevity. *Mech Ageing Dev,* 126**,** 1230-8.

CORONA, M., VELARDE, R. A., REMOLINA, S., MORAN-LAUTER, A., WANG, Y., HUGHES, K. A. & ROBINSON, G. E. (2007) Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proc Natl Acad Sci U S A,* 104**,** 7128-33.

CRAILSHEIM, K. (1986) Dependence of Protein-Metabolism on Age and Season in the Honeybee (Apis-Mellifica-Carnica Pollm). *Journal of Insect Physiology,* 32**,** 629-634.

DAS, N., LEVINE, R. L., ORR, W. C. & SOHAL, R. S. (2001) Selectivity of protein oxidative damage during aging in Drosophila melanogaster. *Biochem J,* 360**,** 209-16.

DE AZEVEDO, S. V. & HARTFELDER, K. (2008) The insulin signaling pathway in honey bee (Apis mellifera) caste development - differential expression of insulin-like peptides and insulin receptors in queen and worker larvae. *J Insect Physiol,* 54**,** 1064-71.

DILLIN, A., CRAWFORD, D. K. & KENYON, C. (2002) Timing requirements for insulin/IGF-1 signaling in C. elegans. *Science,* 298**,** 830-4.

EVANS, J. D. & WHEELER, D. E. (1999) Differential gene expression between developing queens and workers in the honey bee, Apis mellifera. *Proc Natl Acad Sci U S A,* 96**,** 5575-80.

FAHRBACH, S. E., MOORE, D., CAPALDI, E. A., FARRIS, S. M. & ROBINSON, G. E. (1998) Experience-expectant plasticity in the mushroom bodies of the honeybee. *Learn Mem,* 5**,** 115-23.

FAHRBACH, S. E., STRANDE, J. L. & ROBINSON, G. E. (1995) Neurogenesis is absent in the brains of adult honey bees and does not explain behavioral neuroplasticity. *Neurosci Lett,* 197**,** 145-8.

FLATT, T. & KAWECKI, T. J. (2007) Juvenile hormone as a regulator of the trade-off between reproduction and life span in Drosophila melanogaster. *Evolution,* 61**,** 1980-91.

FLATT, T., MIN, K. J., D'ALTERIO, C., VILLA-CUESTA, E., CUMBERS, J., LEHMANN, R., JONES, D. L. & TATAR, M. (2008) Drosophila germ-line modulation of insulin signaling and lifespan. *Proc Natl Acad Sci U S A,* 105**,** 6368-73.

FRASER, H. B., KHAITOVICH, P., PLOTKIN, J. B., PAABO, S. & EISEN, M. B. (2005) Aging and gene expression in the primate brain. *PLoS Biol,* 3**,** e274.

FUSSNECKER, B. & GROZINGER, C. (2008) Dissecting the role of Kr-h1 brain gene expression in foraging behavior in honey bees (Apis mellifera). *Insect Mol Biol,* 17**,** 515-22.

GRAY, D. A., TSIRIGOTIS, M. & WOULFE, J. (2003) Ubiquitin, proteasomes, and the aging brain. *Sci Aging Knowledge Environ,* 2003**,** RE6.

GUIDUGLI-LAZZARINI, K. R., DO NASCIMENTO, A. M., TANAKA, E. D., PIULACHS, M. D., HARTFELDER, K., BITONDI, M. G. & SIMOES, Z. L. (2008) Expression analysis of putative vitellogenin and lipophorin receptors in honey bee (Apis mellifera L.) queens and workers. *J Insect Physiol,* 54**,** 1138-47.

GUIDUGLI, K. R., NASCIMENTO, A. M., AMDAM, G. V., BARCHUK, A. R., OMHOLT, S., SIMOES, Z. L. & HARTFELDER, K. (2005) Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect. *FEBS Lett,* 579**,** 4961-5.

HADDAD, L. S., KELBERT, L. & HULBERT, A. J. (2007) Extended longevity of queen honey bees compared to workers is associated with peroxidation-resistant membranes. *Exp Gerontol,* 42**,** 601-9.
HARMAN, D. (1956) Aging: a theory based on free radical and radiation chemistry. *J Gerontol,* 11**,** 298-300.
HARRISON, J. F. & FEWELL, J. H. (2002) Environmental and genetic influences on flight metabolic rate in the honey bee, Apis mellifera. *Comp Biochem Physiol A Mol Integr Physiol,* 133**,** 323-33.
HARRISON, J. M. (1986) Caste-Specific Changes in Honeybee Flight Capacity. *Physiological Zoology,* 59**,** 175-187.
HARTFELDER, K. & ENGELS, W. (1998) Social insect polymorphism: hormonal regulation of plasticity in development and reproduction in the honeybee. *Curr Top Dev Biol,* 40**,** 45-77.
HEWES, R. S. (2008) The buzz on fly neuronal remodeling. *Trends Endocrinol Metab,* 19**,** 317-23.
HÖLLDOBLER, B. & WILSON, E. O. (2008) *The Superorganism: The Beauty, Elegance, and Strangeness of Insect Societies*, W.W. Norton & Co.
JAENISCH, R. & BIRD, A. (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet,* 33 Suppl**,** 245-54.
KAEBERLEIN, M. & SHAMIEH, L. S. (2010) *The Role of TOR Signaling in Aging*, Springer Netherlands.
KANEKO, T. (2003) Aging and the accumulation of oxidative damage to DNA. *Journal of Clinical Biochemistry and Nutrition,* 34**,** 51-60.
KIRKWOOD, T. B. (1977) Evolution of ageing. *Nature,* 270**,** 301-4.
KUCHARSKI, R., MALESZKA, J., FORET, S. & MALESZKA, R. (2008) Nutritional control of reproductive status in honeybees via DNA methylation. *Science,* 319**,** 1827-30.
LEE, R. D. (2003) Rethinking the evolutionary theory of aging: transfers, not births, shape senescence in social species. *Proc Natl Acad Sci U S A,* 100**,** 9637-42.
MEDAWAR, P. B. (1952) *An Unsolved Problem in Biology,* London, Lewis.
MUNCH, D., AMDAM, G. V. & WOLSCHIN, F. (2008) Ageing in a eusocial insect: molecular and physiological characteristics of life span plasticity in the honey bee. *Funct Ecol,* 22**,** 407-421.
MUTTI, N. S., WOLSCHIN, F., DOLEZAL, A. G., MUTTI, J. S., GILL, K. S. & AMDAM, G. V. (submitted) IRS and TOR act on juvenile hormone and DNA methylation to mediate honeybee caste canalization.
NELSON, C. M., IHLE, K. E., FONDRK, M. K., PAGE, R. E. & AMDAM, G. V. (2007) The gene vitellogenin has multiple coordinating effects on social organization. *PLoS Biol,* 5**,** e62.
NEUKIRCH, A. (1982) Dependence of the Life-Span of the Honeybee (Apis-Mellifica) Upon Flight Performance and Energy-Consumption. *Journal of Comparative Physiology,* 146**,** 35-40.
NILSEN, K.-A., FREDERICK, M., FONDRK, K. M., SMEDAL, B. & HARTFELDER, G. V. (submitted) Dual role of fat body insulin/insulin-like growth factor signaling in honey bee behavioral physiology.
PAGE, R. E., JR. & AMDAM, G. V. (2007) The making of a social insect: developmental architectures of social design. *Bioessays,* 29**,** 334-43.

PAGE, R. E., JR., SCHEINER, R., ERBER, J. & AMDAM, G. V. (2006) 8. The development and evolution of division of labor and foraging specialization in a social insect (Apis mellifera L.). *Curr Top Dev Biol,* 74**,** 253-86.
PARTRIDGE, L., PIPER, M. D. & MAIR, W. (2005) Dietary restriction in Drosophila. *Mech Ageing Dev,* 126**,** 938-50.
PATEL, A., FONDRK, M. K., KAFTANOGLU, O., EMORE, C., HUNT, G., FREDERICK, K. & AMDAM, G. V. (2007) The making of a queen: TOR pathway is a key player in diphenic caste development. *PLoS One,* 2**,** e509.
PINTO, L. Z., BITONDI, M. M. G. & SIMÕES, Z. L. P. (2000) Inhibition of vitellogenin synthesis in *Apis mellifera* workers by a juvenile hormone analogue, pyriproxyfen. *J. Insect Physiol.,* 46**,** 153-160.
PIPER, M. D. W., SELMAN, C., MCELWEE, J. J. & PARTRIDGE, L. (2005) Models of insulin signalling and longevity. *Drug Discovery Today: Disease Models,* 2**,** 249-256.
RACHINSKY, A., STRAMBI, C., STRAMBI, A. & HARTFELDER, K. (1990) Caste and metamorphosis: hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. *Gen Comp Endocrinol,* 79**,** 31-8.
RATNIEKS, F. L. W. (1993) Egg-laying, egg-removal, and ovary development by workers in queenright honey bee colonies. *Behavioral Ecology and Sociobiology,* 32**,** 191-198.
REMBOLD, H., CZOPPELT, C. & RAO, P. J. (1974) Effect of juvenile hormone treatment on caste differentiation in the honeybee, Apis mellifera. *J Insect Physiol,* 20**,** 1193-202.
ROBERTS, S. P. & ELEKONICH, M. M. (2005) Muscle biochemistry and the ontogeny of flight capacity during behavioral development in the honey bee, Apis mellifera. *J Exp Biol,* 208**,** 4193-8.
RUEPPELL, O., CHRISTINE, S., MULCRONE, C. & GROVES, L. (2007) Aging without functional senescence in honey bee workers. *Curr Biol,* 17**,** R274-5.
RUEPPELL, O., LINFORD, R., GARDNER, P., COLEMAN, J. & FINE, K. (2008) Aging and demographic plasticity in response to experimental age structures in honeybees (Apis mellifera L). *Behav Ecol Sociobiol,* 62**,** 1621-1631.
SCHIPPERS, M. P., DUKAS, R., SMITH, R. W., WANG, J., SMOLEN, K. & MCCLELLAND, G. B. (2006) Lifetime performance in foraging honeybees: behaviour and physiology. *J Exp Biol,* 209**,** 3828-36.
SCHULZ, D. J. & ROBINSON, G. E. (1999) Biogenic amines and division of labor in honey bee colonies: behaviorally related changes in the antennal lobes and age-related changes in the mushroom bodies. *J Comp Physiol A,* 184**,** 481-8.
SCHULZ, D. J., SULLIVAN, J. P. & ROBINSON, G. E. (2002) Juvenile hormone and octopamine in the regulation of division of labor in honey bee colonies. *Horm Behav,* 42**,** 222-31.
SEEHUUS, S. C., KREKLING, T. & AMDAM, G. V. (2006a) Cellular senescence in honey bee brain is largely independent of chronological age. *Exp Gerontol,* 41**,** 1117-25.
SEEHUUS, S. C., NORBERG, K., GIMSA, U., KREKLING, T. & AMDAM, G. V. (2006b) Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proc Natl Acad Sci U S A,* 103**,** 962-7.
SOWELL, R. A., HERSBERGER, K. E., KAUFMAN, T. C. & CLEMMER, D. E. (2007) Examining the proteome of Drosophila across organism lifespan. *J Proteome Res,* 6**,** 3637-47.

TATAR, M., KOPELMAN, A., EPSTEIN, D., TU, M. P., YIN, C. M. & GAROFALO, R. S. (2001) A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science,* 292**,** 107-10.

VERNACE, V. A., ARNAUD, L., SCHMIDT-GLENEWINKEL, T. & FIGUEIREDO-PEREIRA, M. E. (2007) Aging perturbs 26S proteasome assembly in Drosophila melanogaster. *Faseb J,* 21**,** 2672-82.

VON ZGLINICKI, T. & SITTE, N. (2003) Free Radical Production and Antioxidant Defense: A Primer. IN VON ZGLINICKI, T. (Ed.) *Aging at the molecular level.* Kluwer Academic Publishers.

WANG, Y., JORDA, M., JONES, P. L., MALESZKA, R., LING, X., ROBERTSON, H. M., MIZZEN, C. A., PEINADO, M. A. & ROBINSON, G. E. (2006) Functional CpG methylation system in a social insect. *Science,* 314**,** 645-7.

WHEELER, D. E., BUCK, N. & EVANS, J. D. (2006) Expression of insulin pathway genes during the period of caste determination in the honey bee, Apis mellifera. *Insect Mol Biol,* 15**,** 597-602.

WHITFIELD, C. W., BEN-SHAHAR, Y., BRILLET, C., LEONCINI, I., CRAUSER, D., LECONTE, Y., RODRIGUEZ-ZAS, S. & ROBINSON, G. E. (2006) Genomic dissection of behavioral maturation in the honey bee. *Proc Natl Acad Sci U S A,* 103**,** 16068-75.

WHITFIELD, C. W., CZIKO, A. M. & ROBINSON, G. E. (2003) Gene expression profiles in the brain predict behavior in individual honey bees. *Science,* 302**,** 296-9.

WILLIAMS, G. C. (1957) Pleiotropy, natural selection and the evolution of senescence. *Evolution,* 11**,** 398-411.

WILLIAMS, J. B., ROBERTS, S. P. & ELEKONICH, M. M. (2008) Age and natural metabolically-intensive behavior affect oxidative stress and antioxidant mechanisms. *Exp Gerontol,* 43**,** 538-49.

WINSTON, A. L. (1987) *The biology of the honey bee,* Cambridge, MA, Harvard University Press.

WOLSCHIN, F. & AMDAM, G. V. (2007a) Comparative proteomics reveal characteristics of life-history transitions in a social insect. *Proteome Sci,* 5**,** 10.

WOLSCHIN, F. & AMDAM, G. V. (2007b) Plasticity and robustness of protein patterns during reversible development in the honey bee (Apis mellifera). *Anal Bioanal Chem,* 389**,** 1095-100.

WOLSCHIN, F., MUNCH, D. & AMDAM, G. V. (2009) Structural and proteomic analyses reveal regional brain differences during honeybee aging. *J Exp Biol,* 212**,** 4027-32.

WOOD, J. G., ROGINA, B., LAVU, S., HOWITZ, K., HELFAND, S. L., TATAR, M. & SINCLAIR, D. (2004) Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature,* 430**,** 686-9.

ZHAN, M., YAMAZA, H., SUN, Y., SINCLAIR, J., LI, H. & ZOU, S. (2007) Temporal and spatial transcriptional profiles of aging in Drosophila melanogaster. *Genome Res,* 17**,** 1236-43.

Paper II, *submitted manuscript*

Hyperoxia reveals a distinct resilience for central and peripheral brain functions in the honey bee

Brenda Rascón, Kate E. Ihle, Gro V. Amdam

Abstract

During senescence, sensory and memory decline are present in a variety of organisms ranging from mammals to insects. Several aging studies focused on interventional strategies reveal that lifespan extension is possible in model invertebrates. However, fewer surveys have probed the robustness of brain functions during the progression of aging. Furthermore, the understanding of how aging and stress are related to brain deterioration could be more complete. In this study, we used the honey bee to investigate how a metabolically challenging environment (hyperoxia)—which elicits pathophysiological symptoms of aging—affects peripheral (sensory) and central (olfactory conditioning) brain functions. Our study reveals that laboratory-induced hyperoxic stress reduced learning ability without impairing gustation. The negative effect of metabolic stress on learning ability was replicated in genetic strains with different survival in hyperoxia. We demonstrate that this differential survival can at least partly be explained by vitellogenin, a protein with antioxidant function in honey bees. The differential susceptibility of peripheral and central brain functions in response to hyperoxia is consistent with the effects of aging in free-flying honey bees. The agreement between these two results provides evidence for heterogeneity in how distinct brain functions senesce, and indicates that oxidative stress can cause central processing deficits in the honey bee.

Hyperoxia reveals a distinct resilience for central and peripheral brain functions in the honey bee

Brenda Rascón[1*], Kate E. Ihle[2, 3], and Gro V. Amdam [1, 2]

[1]Department of Chemistry, Biotechnology and Food Science (IKBM)

Norwegian University of Life Sciences (UMB), Ås N-1432, Norway

[2] School of Life Sciences

Arizona State University, Tempe, AZ 85287-4501, USA

[3] Smithsonian Tropical Research Institute

Apartado Postal 0843-03092, República de Panamá

*corresponding author: brenda.rascon@asu.edu

Running title: Aging resilience of the honey bee brain

Keywords: aging, brain, metabolism, learning, sensory perception, lifespan, stress

Abstract

During senescence, sensory and memory decline are present in a variety of organisms ranging from mammals to insects. Several aging studies focused on interventional strategies reveal that lifespan extension is possible in model invertebrates. However, fewer surveys have probed the robustness of brain functions during the progression of aging. Furthermore, the understanding of how aging and stress are related to brain deterioration could be more complete. In this study, we used the honey bee to investigate how a metabolically challenging environment (hyperoxia)—which elicits pathophysiological symptoms of aging—affects peripheral (sensory) and central (olfactory conditioning) brain functions. Our study reveals that laboratory-induced hyperoxic stress reduced learning ability without impairing gustation. The negative effect of metabolic stress on learning ability was replicated in genetic strains with different survival in hyperoxia. We demonstrate that this differential survival can at least partly be explained by vitellogenin, a protein with antioxidant function in honey bees. The differential susceptibility of peripheral and central brain functions in response to hyperoxia is consistent with the effects of aging in free-flying honey bees. The agreement between these two results provides evidence for heterogeneity in how distinct brain functions senesce, and indicates that oxidative stress can cause central processing deficits in the honey bee.

1. *Introduction*

During senescence, sensory and memory decline are present in a variety of organisms ranging from mammals to insects (Brown and Strausfeld 2009; Flood and Morley 1998; Grotewiel and others 2005; Mery 2007; Tamura and others 2003). In honey bees, this decline in central processing ability is visible after over 15 days of flight. At this time, foraging bees show a reduction in associative learning ability and memory deficits (Behrends and others 2007; Munch and others 2010; Scheiner and Amdam 2009). In the brain, this functional decline is paralleled by oxidative damage to lipids and proteins, an accumulation of proteins, and a reduction of proteins related to synaptic and neuronal growth (Seehuus and others 2006a; Tolfsen and others 2011; Wolschin and others 2009). In general, aging honey bees share many of the same symptoms of aging found in other animals (Munch and Amdam 2010). However, in the honey bee, senescence, like other aspects of life history and behavior, exhibits remarkable plasticity which can be studied both in the wild and in laboratory settings (Amdam 2011; Munch and others 2008; Page and Peng 2001). Moreover, the honey bee provides an attractive experimental system for studying the physiological consequences of senescence due to its large size, its commercial availability, and its well-established use as neurobiological model.

Oxidative stress has been widely implicated in aging and neurodegeneration. During aging, macromolecules like DNA and proteins can become irreparably damaged by reactive oxygen species. Reactive oxygen species are general byproducts of respiration, but when the body's ability to properly quench excessive free radical production is compromised, this process can eventually lead to pervasive cellular damage that interferes with normal cellular metabolism and causes aging (Harman 1956). Oxidative stress should be a problem for honey bees during the switch to foraging due to the elevation of metabolic rate during flight. In flying insects, the production of reactive oxygen species can be elevated in metabolically active tissues like flight muscle and brain. After all, flying insects do achieve one of the highest mass-specific metabolic rates in the animal kingdom (reviewed by (Harrison and others 2006)). Foraging is also associated with higher levels of flight muscle cytochrome and antioxidants (Harrison and Fewell 2002; Roberts and Elekonich 2005; Schippers and others 2006; Wolschin and Amdam 2007), which presumably mitigate flight-associated oxidative damage. Some studies lend support to the idea that oxidative stress underlies senescence of the brain, but others do not, casting doubt on the role of oxidative stress in learning performance deficits that are associated with age in the honey bee (Farooqui 2008; Seehuus and others 2006a; Tolfsen and others 2011; Williams and others 2008). Collectively, these studies beg the question of whether oxidative stress affects brain functions in honey bee foragers.

The reproductive unit of the honey bee is the colony. The success of a colony depends on the ability of foraging honey bees to learn the spatial and temporal location of floral resources, e.g., nectar and pollen. Most honey bees become foragers toward the end of their lifespans. As free-flying foragers age, their gustatory response score (GRS) — an index of individual sensory acuity to sugar (a proxy for nectar)—remains largely intact (Behrends and others 2007; Scheiner and Amdam 2009). In contrast, associative olfactory learning and tactile learning performance become impaired throughout the aging process. Presumably, gustatory sensitivity and learning ability are both important neurobiological traits for colony fitness and foraging individuals in nature.

In the laboratory, sucrose response predicts the behavioral performance of foraging honey bees in the field and can indicate the amount and composition of stored food resources (honey vs. pollen) in the colony (Page and others 2006). This seemingly similar reliance of a social group on two neurobiological traits raises the question of how gustatory response can be unaffected by senescence whereas learning ability declines during aging.

We contrasted GRS and associative learning performance in honey bees kept in normal oxygen conditions (normoxia; control) and hyperoxia. Hyperoxia is frequently used in insect model systems as a metabolic stressor to prematurely induce pathologies that are often present in aged individuals. In *Drosophila*, hyperoxia reliably produces gradual changes in lifespan and protein oxidative stress (Rascón and Harrison 2010; Sohal and others 1993; Sohal and Dubey 1994), and induces features of premature senescence such as neural system deterioration (Miquel and others 1975), and mitochondrial deformations (Walker and Benzer 2004). Furthermore, 38% of the genes implicated in normal aging are altered in the same direction during hyperoxia (Landis and others 2004). The ability to withstand stress—specifically oxidative stress—has been implicated in contributing to longer lifespans in fruit flies (Lin and others 1998; Orr and Sohal 1992), making hyperoxia an attractive tool for manipulative in-lab experiments.

To test the robustness of GRS and associative learning performance in the face of oxidative stress, and to examine whether a genetic component exists for metabolic stress resistance, we used two distinct genetic honey bee stocks that exhibit consistent differences in gustatory responsiveness, associative learning performance, and lifespan (Amdam and others 2004; Page and others 1998; Pankiw and others 2001; Scheiner and others 2001a; Scheiner and others 2001b). These genotypes were selected from wild-type populations based on the level of pollen-hoarding (referred to as high pollen-hoarding and low pollen-hoarding, or high and low strain), and have been previously used in aging studies. As an extra level of validation, we contrasted wild type or pollen-hoarding honey bee strains of two different

behavioral sub-castes—nurses and foragers. In comparison to nurses, foragers are more susceptible to oxidative stress (Seehuus and others 2006b). This distinct susceptibility to oxidative stress was previously explained by sub-caste-specific expression patterns of vitellogenin, a yolk precursor protein expressed by the functionally sterile workers at high levels as nurses (Seehuus and others 2006a). We thus hypothesized that vitellogenin levels could influence susceptibility to hyperoxia in this study. To test this hypothesis, we reduced *vitellogenin* gene expression via RNAi in worker honey bees of both genotypes and compared survivorship between knockdowns and controls.

2. *Materials and Methods*

2.1 Honey bee stocks

Honey bee colonies with single-drone inseminated queens of high and low pollen-hoarding genotypes (32^{nd} generation) were used in all experiments. We used workers produced by at least three different queens from each strain. The queens represented several (at least three) independent sources (sub-lines) from the selection program. Pollen-hoarding honey bee genotypes were bi-directionally selected for the amount of pollen stored in their colonies (Page and Fondrk 1995). Honey bee pollen-hoarding behavior is genetically linked to a major life-history syndrome that includes reproductive physiology and lifespan. This syndrome is equally present in wild-type honey bee populations (Page and others 2006).

2.2 Hyperoxic stress exposure

Adult worker bees (i.e., essentially sterile female helper bees that make up the majority of individuals in the colony) were kept in isolation to avoid confounding interactions between social influences and genotype. The bees were reared in a HERAcell O_2/CO_2 incubator (Thermo Scientific, Waltham, Massachusetts, USA) that maintained the following controlled conditions: an enriched oxygen atmosphere (75-80% O_2), temperature at 34 °C, and relative humidity at 63±2%. Relative humidity was monitored by Hobo data loggers (Onset Computer Corporation, Framingham, Massachusetts, USA). Bees were individually housed in 1.5 mL Eppendorf tubes, each outfitted with a feeding port, breathing hole, and an opening for waste and defecation, as previously described (Amdam and others 2010). Honey bees were fed 25 µL of a standard diet consisting of 1.5 g of ground pollen per 30 mL of 30% sucrose solution, and were allowed to feed *ad libitum* through an easily accessible food-containing pipette tip. Feeding was verified to prevent starvation and/or caloric restriction, and thereby minimize survivorship effects not associated with oxygen treatment.

2.3 Sensory and learning test preparation

To collect newly emerged bees (0-24 h old), we placed brood combs in an incubator overnight at 34°C in a relative humidity of 65-70%. Upon emergence the following morning, we marked bees on the thorax

with Testors paint (Rockford, Illinois, USA) for identification and placed them into a host colony. Approximately 100 bees per genotype were placed into the incubator for hyperoxic treatment, while all others were introduced into a wild-type, host colony. Honey bees were recaptured as 10-day-old nurses or 20-day-old foragers and placed into either normoxia (21% O_2) or hyperoxia (75-80% O_2), and maintained for 17, 40, or 64 hours. Both nurses and foragers were verified according to their behavior upon collection. Honey bees found dipping their heads into comb cells containing larvae were classified as nurses, while animals that displayed a consistent foraging pattern were considered foragers. Only 10-day-old nurse bees were tested for sensory and learning ability. Both nurses and foragers were tested for survival (methods described below).

Once bees were removed from oxidative stress conditions of 80% O_2, they were restrained in small polyacryl holders using strips of duct tape. To ease handling and placement of bees into individual tubes, they were cooled in 4 °C until immobile. As with all laboratory tests, test subjects were randomized so that experimenters were blind to treatment identity. Thereafter, bees were fed 2 µl of 30% sucrose solution and were placed in an incubator for a 2-hour starvation period. The incubator maintained atmospheric oxygen at normoxic levels.

2.4 Sensory and learning tests

2.4.1 Gustatory response measurements

To measure gustatory responsiveness, we utilized the proboscis extension response (PER). The criterion for a positive PER was complete extension of the proboscis. 10-day-old high and low genotype bees exposed to oxidative stress treatment for 18, 40 and 64 hr were tested. Bees were stimulated over the antennae with water and six subsequent sucrose solutions in the following order: 0.1, 0.3, 1, 3, 10, 30%. We adhered to an inter-stimulus interval of two minutes to prevent sensitization and habituation. An overall index of performance, or gustatory response score (GRS), was calculated for all tested bees by using the sum of all PER responses to seven different stimuli (water and six sucrose solutions). A honey bee with a total score of 7 showed the highest level of sensory responsiveness, while a zero score indicated no responsiveness. Bees that failed to respond to the 30% sucrose stimulus were not included in the olfactory conditioning trials as this sucrose concentration was used as a reward in appetitive (Pavlovian) associative learning.

2.4.2 Olfactory conditioning

To test for stress-induced performance deficits, we measured associative olfactory learning at 18, 40 and 64 hr of hyperoxic exposure in 10-day-old bees that responded to at least 30% sucrose (GRS ≥1). Prior to training, bees were tested for spontaneous PER to carnation (conditioned stimulus, carnation

oil) and cineole. As in previous studies, we only conditioned bees that did not exhibit a spontaneous response to either odor (Amdam and others 2010; Tolfsen and others 2011). For odor preparation, we applied 2 µl of carnation oil to a piece of filter paper that we then placed into a capped 20 ml syringe. During olfactory conditioning, we placed restrained honey bees atop a plastic stand equipped with a vacuum system that constantly neutralized the airstream. Throughout each of the six odor conditioning trials, we applied 5 ml of carnation odor to the antennae of the honey bee for five seconds; during the last three seconds of the odor presentation, we administered a 1 µl sucrose reward (30% sucrose in H_2O) to form a paired stimulus-reward association. The inter-trial interval was 5 min to prevent sensitization and habituation effects. After each conditioning trial, we scored the bee's response as a binary variable via PER (i.e., response or no response). Once all conditioning trials took place, we tested for odor generalization by presenting honey bees with cineole. This allowed us to test the bee's discrimination ability as she should only respond to the conditioned stimulus (carnation). We calculated a learning acquisition score based on conditioned responses. The score, with a numerical value between 0 and 5, was based on 5 conditioning trials and an additional trial that tested reaction spontaneity.

2.5 Survivorship measurements

Survivorship censuses took place twice per day at similar times each day until the last bee was observed dead. During these observation periods, bees were either observed dead or alive, and remaining live bees were transferred to fresh tubes in to prevent bacterial and/or fungal growth. Individuals that appeared to have died due to accident (e.g. killed during routine transfers) were not included in the data analysis. Individual life spans were calculated using the frequency of bees alive at each temporal observation. The mortality dynamics of pilot experiments were utilized to narrow in on three oxygen exposure times (17, 40, 64 hr), which formed the basis of our observations for the gustatory and learning performance assessments. We reasoned that progressive performance deterioration would be detected the longer honey bees spent in hyperoxic treatment.

2.6 Vitellogenin downregulation by RNA interference (RNAi)

2.6.1 dsRNA preparation for vitellogenin gene downregulation

Double-stranded RNA (dsRNA) toward the *vitellogenin* gene was prepared as described previously (Amdam and others 2006; Amdam and others 2003b). Briefly, we used cDNA clone AP4a5 as a template (GenBank accession #: AJ517411). Primers were fused to a T7 promoter sequence (underlined):

Fw:5'-<u>TAATACGACTCACTATAGGGCGA</u>ACGACTCGACCAACGACTT-3'

Re:5'-<u>TAATACGACTCACTATAGGGCGA</u>AACGAAAGGAACGGTCAATTCC-3'

PCR product was purified using the QIAquick PCR purification kit (Qiagen, Valencia, California, USA), and RNA was prepared with the Promega RiboMax T7 system (Promega, Madison, Wisconsin, USA). RNA was extracted by TRIzol LS reagent (GIBCO-BRL, San Diego, California, USA), resuspended in nuclease-free water, heated at 96°C for 2 min in an Eppendorf Thermomixer (Brinkmann Instruments, Westbury, New York, USA), and left to cool at room temperature for 20 min. dsRNA products were diluted with nuclease-free H_2O (Qiagen) to the final concentration of (Amdam and others 2003a; Nelson and others 2007; Seehuus and others 2006b). Nuclease-free water was used as a control, as in a previous study (Ihle and others 2010).

2.6.2 Knockdown verification

Efficacy of this *vitellogenin* RNAi procedure was confirmed previously in honeybees of diverse commercial stocks as well as in the pollen-hoarding genotypes (Amdam and others 2007; Amdam and others 2003b; Marco Antonio and others 2008; Nelson and others 2007), as well as in the present manuscript (Fig. 6). Verification of *vitellogenin* knockdown for this study was performed as described before (eg. (Ihle and others 2010; Nelson and others 2007). We isolated RNA from the abdominal fat bodies (organs responsible for vitellogenin synthesis) for individual workers using TRIzol phenol-chloroform extraction combined with the RNeasy kit (Qiagen). We then used reverse transcriptase real-time PCR (Applied Biosciences, Foster City, CA, USA) to validate knockdown of *vitellogenin* mRNA levels. Relative gene expression levels were obtained against β-actin expression (Nelson and others 2007). β-actin is an effective control gene when measuring gene expression in adult honey bee fat body (Lourenco and others 2008; Scharlaken and others 2008). Primers for *vitellogenin:* 5'-GTTGGAGAGCAACATGCAGA-3' and 5'-TCGATCCATTCCTTGATGGT-3'. Primers for actin: 5'-TGCCAACACTGTCCTTTCTG-3' and 5'-AGAATTGACCCACCAATCCA-3' (Amdam and others 2004).

2.7 Bees

We downregulated *vitellogenin* in newly emerged high and low pollen-hoarding honey bees and raised them in hyperoxia to test whether the divergent response to stress could be attributable to genotype-specific differences in *vitellogenin* expression. The vitellogenin protein can confer oxidative stress protection (Seehuus and others 2006b) and the two genotypes are known to differ in vitellogenin titers (Amdam and others 2004).

Queens from three high and three low genotype source colonies were caged overnight to allow collection of same-aged bees. Frames were pulled from colonies after 20 days, and worker bees emerged in an incubator at 32°C. Newly-emerged workers were randomly assigned to one of two treatments: a control group injected with vehicle (nuclease-free H_2O) and *vg*RNAi, the dsRNA-injected

vitellogenin knockdown group, after experimental designs and protocols established before, e.g. (Guidugli and others 2005; Nelson and others 2007). Treated bees were marked with paint (Testors Enamel; Testor Corporation, Rockford, Illinois, USA) to indicate treatment identity. Injections were performed between the fifth and sixth tergite using Hamilton syringes with G30 disposable needles (BD, Palo Alto, California, USA). Injection volume was 2 µl. Treated workers were introduced into one of two four-frame nuclear colonies where they were allowed to mature for 5 days before collection.

2.8 Statistical analyses

Our gustatory responsiveness and learning performance data were non-normal as determined by normal probability plots. Therefore, we used non-parametric statistics for analysis. We used the Kruskal-Wallis ANOVA to assess overall treatment effects, and the Mann-Whitney U as a *post hoc* test to examine differences between the groups. For analysis of survivorship data, we used Cox's *F*-test and Gehan's Wilcoxon test to verify longevity differences between genotype and social task. The vitellogenin knockdown datasets were also analyzed using Cox's *F*-test and Gehan's Wilcoxon test. The Kaplan–Meier estimator was employed to approximate the survival function for honey bee populations. Quantitative differences were considered statistically significant if alpha values were less than 0.05. All statistical analyses were carried out in *STATISTICA* 7.0 (StatSoft).

3. *Results*

3.1 Gustatory responsiveness

3.1.1 General effects

To test for general effects, we first analyzed our results by pooling both the high and low pollen-hoarding genotypes together. Gustatory responsiveness was measured over 17, 40, 64 hr of treatment exposure (hyperoxia or normoxia) in 10-day-old bees. Overall, the gustatory responsiveness of 10-day-old hyperoxia-exposed bees, compared to normoxic controls, did not change significantly over time (Kruskal-Wallis ANOVA: H=3.803, df=1, n=285, P=0.051).

3.1.2 Genotype-specific effects

To test for the robustness of gustatory and learning performance patterns, we examined the GRS for high and low genotypes. We measured the GRS of high and low strain honey bees in 17, 40, and 64 hours of normoxia or hyperoxia. For low strain genotypes, GRS did not significantly differ between oxygen treatments at any time point (17 hr: Kruskall-Wallis, H(1,79) = 1.0239, P = 0.3116; 40 hr: K-W, H(1,35) = 2.8288, P = 0.0926; 64 hr: K-W, H(1,20) = 3.266, P = 0.0707; Fig. 1). Similarly, the GRS of high strain honey bees did not significantly differ at any time point (17 hr: K-W, H(1,74) = 1.0096, P = 0.3150; 40 hr: K-W, H(1,42) = 2.6145, P = 0.1059; 64 hr: K-W, H(1,35) = 0.5347, P = 0.4646; Fig. 2).

3.2 Associative learning performance

3.2.1 General effects

Associative learning performance was measured over 17, 40, 64 hr of treatment exposure (hyperoxia or normoxia) in 10-day-old bees of high or low pollen-hoarding genotypes that responded to antennal stimulation with a 30% sucrose solution in the preceding gustatory responsiveness trial. Learning performance in 10-day-old nurse honey bees was significantly affected by hyperoxia, compared to normoxic controls (Kruskal-Wallis ANOVA: $H=11.012$, $n=171$, $P<0.001$). The learning ability of hyperoxia-exposed honey bees significantly declined after 64 hr of exposure, indicating that hyperoxic treatment impaired acquisition (Mann-Whitney U post-hoc test: $U=208.500$, $n=28$, $n=23$, $P=0.030$).

3.2.2 Genotype-specific effects

After 17, 40, and 64 hours of treatment exposure, learning did not significantly differ for low strain bees at any time point (17 hr: K-W, $H(1,31) = 0.002$, $P = 0.9641$; 40 hr: K-W, $H(1,26) = 0.3024$, $P = 0.5824$; 64 hr: K-W, $H(1,16) = 1.4815$, $P = 0.2235$; Fig. 3) despite a trend that can be observed at 64 h. In contrast, the learning performance of high strain genotypes was significantly affected at 17 and 64 hr (17 hr: K-W, $H(1,32) = 4.8612$, $P = 0.0275$; 40 hr: K-W, $H(1,31) = 0.0313$, $P = 0.8595$; 64 hr: K-W, $H(1,35) = 4.2072$, $P = 0.0403$; Fig. 4).

3.3 Stress resistance and survivorship

Forager honey bees were more susceptible to hyperoxic stress than their nurse bee counterparts (Cox's F-test: $F(306, 304) = 1.737$, $P<0.001$; Fig. 5) regardless of genotype (Cox's F-test: $F(178, 188) = 1.249760$, $P = 0.066$; Fig. 5). Furthermore, low genotype nurse bees exhibited greater susceptibility to hyperoxia compared to high genotype nurses (Cox's F-Test: $F(306, 304) = 1.736923$, $P <0.001$; Fig. 5).

3.3.1 Survivorship of vitellogenin RNAi knockdowns in hyperoxia

To test whether divergent responses to oxygen stress could be attributable to genotype-specific differences in vitellogenin levels, we used RNA interference to downregulate *vitellogenin* in high and low pollen-hoarding honey bees, and then measured survivorship in hyperoxia. We quantified the effect of treatment (vitellogenin RNAi knockdown or control), colony, and plate on the expression levels of vitellogenin. Treatment had the greatest effect on the protein expression levels of low and high strain honey bees in hyperoxia (FANOVA; low strain: $F=275.832$, $df=1$, $P<0.0001$, high strain: $F=181.818$, $df=1$, $P<0.0001$). Low and high strain honey bees treated with a vitellogenin RNAi knockdown exhibited lower expression levels of vitellogenin compared to control individuals (Student t-test; low strain vgRNAi vs. control: t-value: 5.908, $\mu1=10.487$, $\mu2=4.704$, $N_1=7$, $N_2=8$, F-ratio: 1.075, $P<0.0001$; high strain vgRNA1 vs. control: t-value: 4.471, $\mu1=11.566$, $\mu2=5.371$, $N_1=8$, $N_2=8$, F-ratio: 1.443, $P<0.001$; Fig. 6).

RNAi-mediated knockdown of *vitellogenin*, while resulting in a trend toward lower life span in both genotypes, did not significantly affect the mean lifespan of low (Gehan-Wilcoxon two-sample comparison test; test statistic: -1.57997, P=0.114) or high (Gehan-Wilcoxon two-sample comparison test; test statistic: 1.812602, P=0.070) honey bee strains when the genotypes were examined independently. However, when the genotypes were pooled, the cumulative survival of high and low strains significantly differed between *vitellogenin* knockdown and control populations with *vitellogenin* knockdown populations not living as long as controls (Gehan-Wilcoxon two-sample comparison test; test statistic: -2.28263, P =0.022).

4. *Discussion*

4.1 A genetic component for stress resistance

Our results allow us to generalize about the stress resistance of wild-type honey bees. The two pollen-hoarding genotypes are selected from wild-type colonies and are routinely out-crossed to wild-type every 3-4 generations (Page and Fondrk 1995). Phenotypically, the strains represent the opposing tails of life-history trait distributions for the general population. Thus, in some instances, we have pooled data from high and low genotypes in order to obtain a general view of the neurophysiological responses of an aging honey bee population.

4.2 General neurophysiological effects

4.2.1 Divergent responses for gustatory responsiveness and learning performance

We utilized honey bees to examine whether peripheral (gustatory) and central (learning) brain functions exhibit a distinct resilience to a challenging metabolic environment. Based on a previous study, we were able to study whether hyperoxia would elicit physiological responses in the laboratory that were similar to those seen in free-flying honey bees (Amdam and others 2010). Our data, in which we pooled high and low genotypes, reveal that gustatory responsiveness remains constant over time, while learning performance declines during hyperoxic stress. This differential susceptibility of peripheral and central brain functions in response to hyperoxia is consistent with the effects of aging in free-flying honey bees (Behrends and others 2007; Scheiner and Amdam 2009). The agreeability between our laboratory results and those obtained in the field suggest that hyperoxia, or oxidative stress by proxy, can play a role in the aging and neurophysiological decline of the honey bee.

The divergent responses to hyperoxia provide support for the concept of heterogeneity in the honey bee brain. The differential resilience of these neurobiological traits is intriguing because both appear to be essential for colony fitness. This heterogeneity exhibited by the honey bee brain in response to hyperoxic exposure suggests that aging affects a variety of cognitive or information processing functions

in a different manner that cannot be counteracted by colony-level selection. We believe questions of differential tissue susceptibility as well as limits on selection merit further exploration in a variety of aging models.

In honey bees, senescence-related changes in the brain appear to be heterogeneous (Munch and others 2010; Wolschin and others 2009). Structural and proteomic analyses of this insect suggest that the mushroom body calyx—long implicated in learning and memory—is largely unaffected by senescence (Wolschin and others 2009). Interestingly, this same study also showed that proteomic changes did occur on the level of the entire central brain, indicating that regions apart from the calyx could be primarily implicated in the characteristic learning performance decline seen in forager bees. Other neuropiles that may play a role in learning performance decline may include the antennal lobes, where signal integration occurs, and the mushroom body output regions that are critical for the formation of memory (Hammer and Menzel 1998). In general, the patterns of aging heterogeneity in the honey bee brain resemble changes that take place in the primate brain (Fraser and others 2005). The heterogeneity of brain function observed in the present study is consistent with asymmetry reduction models in vertebrates, which propose that aging reduces the lateralization of activity that occurs during cognitive performance in younger adults. Some believe that this reduction in lateralization and increase in heterogeneity, as revealed by neuroimaging studies, may reflect age-related functional compensation (Cabeza and others 2004). Thus, heterogeneity of the honey bee brain may reflect distinct spatial and tissue-specific thresholds for aging. It is possible that these distinct thresholds could ultimately influence behavioral indicators of neurophysiological function such as associative learning performance and gustatory responsiveness.

4.3 Genotype-specific neurophysiological effects

The GRS for both high and low strains did not differ between honey bees maintained in normal oxygen conditions or hyperoxia (Figs. 1, 2). In contrast, the learning performance of high strain honey bees was impaired at 17 and 64 hours of hyperoxic exposure. The lack of a difference at 40 hours may be attributable to an adjustment phase (Fig. 4). Moreover, the fact that learning performance was depressed by hyperoxia in high genotypes, but not in lows, may have its foundations in the physiological divergence between the two genotypes. For instance, these two genotypes exhibit differences in GRS, learning performance, lifespan, and vitellogenin levels (Amdam and others 2004; Page and others 1998; Pankiw and others 2001; Scheiner and others 2001a; Scheiner and others 2001b). High strain honey bees tend to be more sensitive to pollen-foraging stimulus (increasing the presence of larvae to force shift to foraging behavior) and tend to forage earlier (Pankiw and Page 2001). Moreover, high strain

honey bees also possess higher levels of proteins central to neuronal function compared to low strain bees (Humphries and others 2003). Though not all of these differences were noted in our study, it is possible that these factors may help explain why the high strain honey bees exhibited greater sensitivity to oxygen as measured by olfactory learning performance.

4.4 Stress resistance

Our survivorship measurements illustrate that forager honey bees are more susceptible to hyperoxic stress than nurse bees, regardless of genotype (Fig. 5). These results corroborate our previous findings asserting foragers are more vulnerable to functional decline (Behrends and others 2007; Scheiner and Amdam 2009). The survivorship patterns also demonstrate that low genotype nurse bees exhibit a greater sensitivity to hyperoxia than high genotype honey bees of identical life history (Fig. 5).

We reasoned that divergent genotype responses to hyperoxia in nurse bees may be attributable, at least in part, to differences in vitellogenin expression. Vitellogenin is a protein with reproductive function that is implicated in the regulation of honey bee social life history traits (Nelson and others 2007) and can also confer oxidative stress protection (Seehuus and others 2006b). Overall, circulating vitellogenin titers tend to be higher in high genotype honey bees compared to low genotype from emergence up to 10 days (Amdam and others 2004). In the present study, high genotype nurses show greater stress resistance to hyperoxia than low genotype nurse bees (Fig. 5). This finding is consistent with previous experiments which demonstrate high pollen-hoarding nurses have higher vitellogenin levels compared to low strain nurse bees (Amdam and others 2004). Based on this, we expected to see differences in the survival time of vitellogenin RNAi knockdowns compared to controls. Though a trend was present for both strains, we did not detect a significant difference between vitellogenin knockdowns and control individuals for either strain (Fig. 7A). The trend suggests vitellogenin plays a role in the stress resistance of the strains, but the lack of significance indicates that other factors may as well. When both genotypes were pooled (Fig. 7B), vitellogenin-deficient individuals were significantly less stress resistant than control individuals. This result generally supports our hypothesis that vitellogenin levels can influence survivorship in hyperoxia.

Our results demonstrate that hyperoxia can increase mortality in honey bees. Furthermore, gustatory response scores remain unchanged over three successive time intervals of hyperoxia exposure. In contrast, learning ability is affected and declines after prolonged hyperoxia. These laboratory results are consistent with measurements obtained from aged honey bees that developed and lived in ecological settings (Behrends and others 2007; Scheiner and others 2001a; Scheiner and others 2001b).

5. *Conclusions*

5.1 Future Work

Our study suggests that learning dysfunction is a general trait in aging honey bees and that oxidative stress can play a role in this decline. More broadly, our results illustrate that the honey bee model system can add to research on aging and age-related brain deterioration due to its quantifiable, complex brain functions and its manipulability in the laboratory and in the field. This implies that a diversity of labs with interests in neurobiological decline, aging, and oxidative stress would be capable of probing similar questions using this intriguing model system. Furthermore, the decline of learning ability during aging is an important problem in science. Gaining insight into this problem may require that we look beyond lab-reared aging model systems.

Funding

This work was supported by the Research Council of Norway (180504).

Author Contributions

BR and GVA designed the experiments. BR, KI, and GVA wrote the paper. BR and KI conducted the experimental work.

Acknowledgments

We wish to thank Nick Baker and Erin Fennern for their dedicated assistance with experiments, Kirsten Traynor for providing necessary equipment, Brian Smith for providing access to his laboratory for learning tests, and Florian Wolschin for providing comments on this manuscript.

Figure Legends

Figure 1. Gustatory responsiveness of low strain honey bees in 17, 40, and 64 hours of hyperoxic treatment. GRS is not significantly different between oxygen treatments for low strain at any time point (17 hr: KW-H(1,79) = 1.0239, P = 0.3116; 40 hr: KW-H(1,35) = 2.8288, P = 0.0926; 64 hr: KW-H(1,20) = 3.266, P = 0.0707).

Figure 2. Gustatory responsiveness of high strain honey bees in 17, 40, and 64 hours of hyperoxic treatment. GRS is not significantly different in hyperoxia for high strain at any time point (17 hr: KW-H(1,74) = 1.0096, P = 0.3150; 40 hr: KW-H(1,42) = 2.6145, P = 0.1059; 64 hr: KW-H(1,35) = 0.5347, P = 0.4646).

Figure 3. Learning performance of low strain honey bees after 17, 40, and 54 hours of hyperoxic treatment. Learning does not significantly differ in hyperoxia for low strain bees at any time point (17 hr: KW-H(1,31) = 0.002, P = 0.9641; 40 hr: KW-H(1,26) = 0.3024, P = 0.5824; 64 hr: KW-H(1,16) = 1.4815, P = 0.2235).

Figure 4. Learning performance of high strain honey bees after 17, 40, and 64 hours of hyperoxia.The learning performance of high strain honey bees was significantly affected at 17 and 64 hr of hyperoxia exposure (17 hr: KW-H(1,32) = 4.8612, P = 0.0275; 40 hr: KW-H(1,31) = 0.0313, P = 0.8595; 64 hr: KW-H(1,35) = 4.2072, P = 0.0403). Asterisks indicate a significant difference.

Figure 5. Survivorship of low and high strain nurses (NL and NH) and foragers (FL and FH) in hyperoxia. Foragers are more susceptible to hyperoxia than nurse bees (Cox's F-test: F (306, 304) = 1.737, P<0.001). Low genotype nurses show greater vulnerability to stress than their high genotype counterparts (Cox's F-Test: F (306, 304) = 1.736923, P <0.001).

Figure 6. RNAi knockdown reduced vitellogenin mRNA levels in low and high strain honey bees (low: t-test, t-value: 5.908, df=13, P<0.001; high: t-test, t-value: 4.471, df=14, P=0.001). The y-axis, VgRQlog2, represents the log of relative quantities of vitellogenin.

Figure 7. A) Mean survival time in hyperoxia of vitellogenin knockdowns by strain. RNAi-mediated knockdown of vitellogenin did not affect the mean lifespans of low (Gehan-Wilcoxon two-sample comparison test; test statistic: -1.57997, P=0.114) or high (Gehan-Wilcoxon two-sample comparison test; test statistic: 1.812602, P=0.070) honey bee strains. B) The cumulative survival of pooled genotypes. The survival of high and low strains significantly differs between vitellogenin knockdown and control populations (Gehan-Wilcoxon two-sample comparison test; test statistic: -2.28263, P=0.02245).

Figure 1.

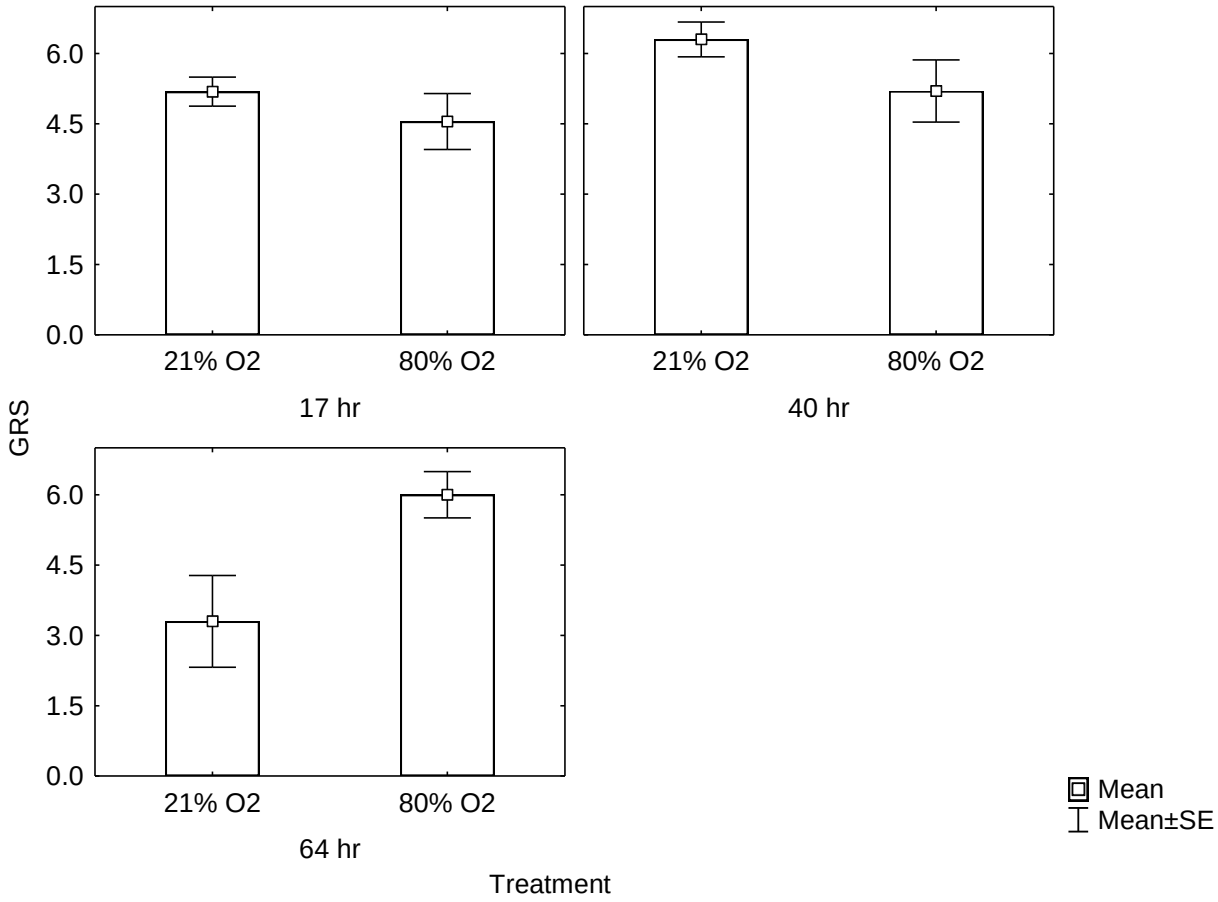

GRS
6.0
4.5
3.0
1.5
0.0
21% O2
80% O2
17 hr
40 hr
64 hr
Treatment
Mean
Mean±SE

Figure 2.

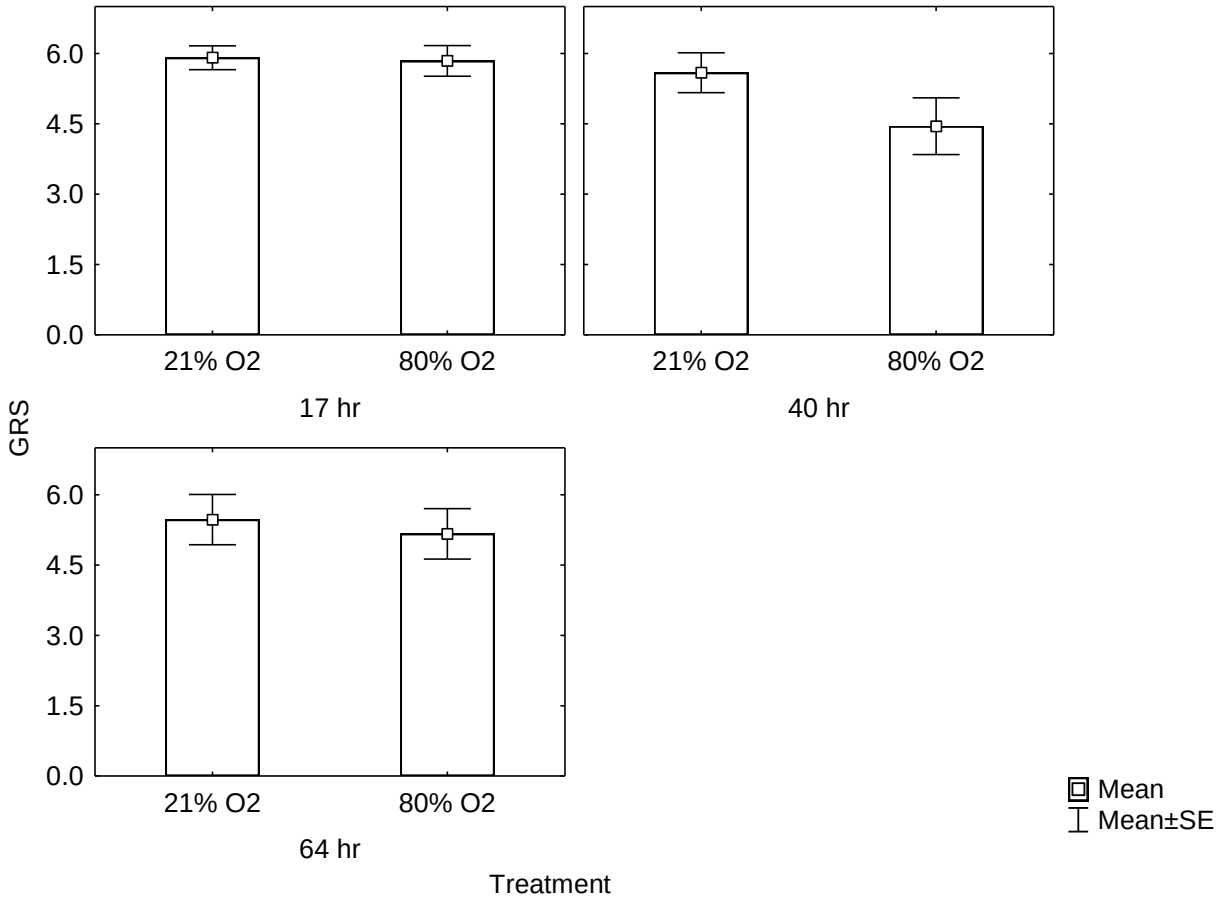

GRS
6.0
4.5
3.0
1.5
0.0
21% O2
80% O2
17 hr
40 hr
64 hr
Treatment
Mean
Mean±SE

Figure 3.

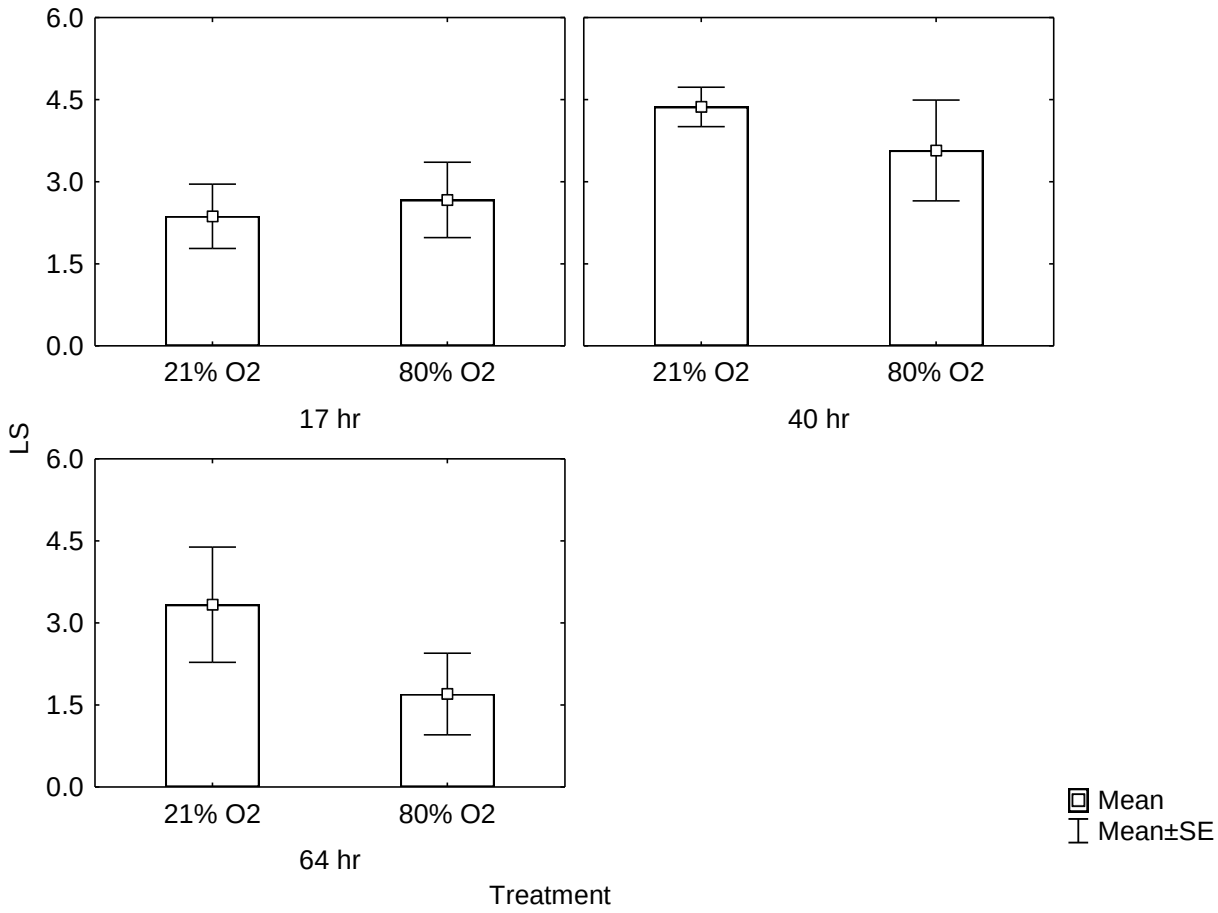

6.0
4.5
3.0
1.5
0.0
LS
21% O2
80% O2
17 hr
40 hr
64 hr
Treatment
Mean
Mean±SE

Figure 4.

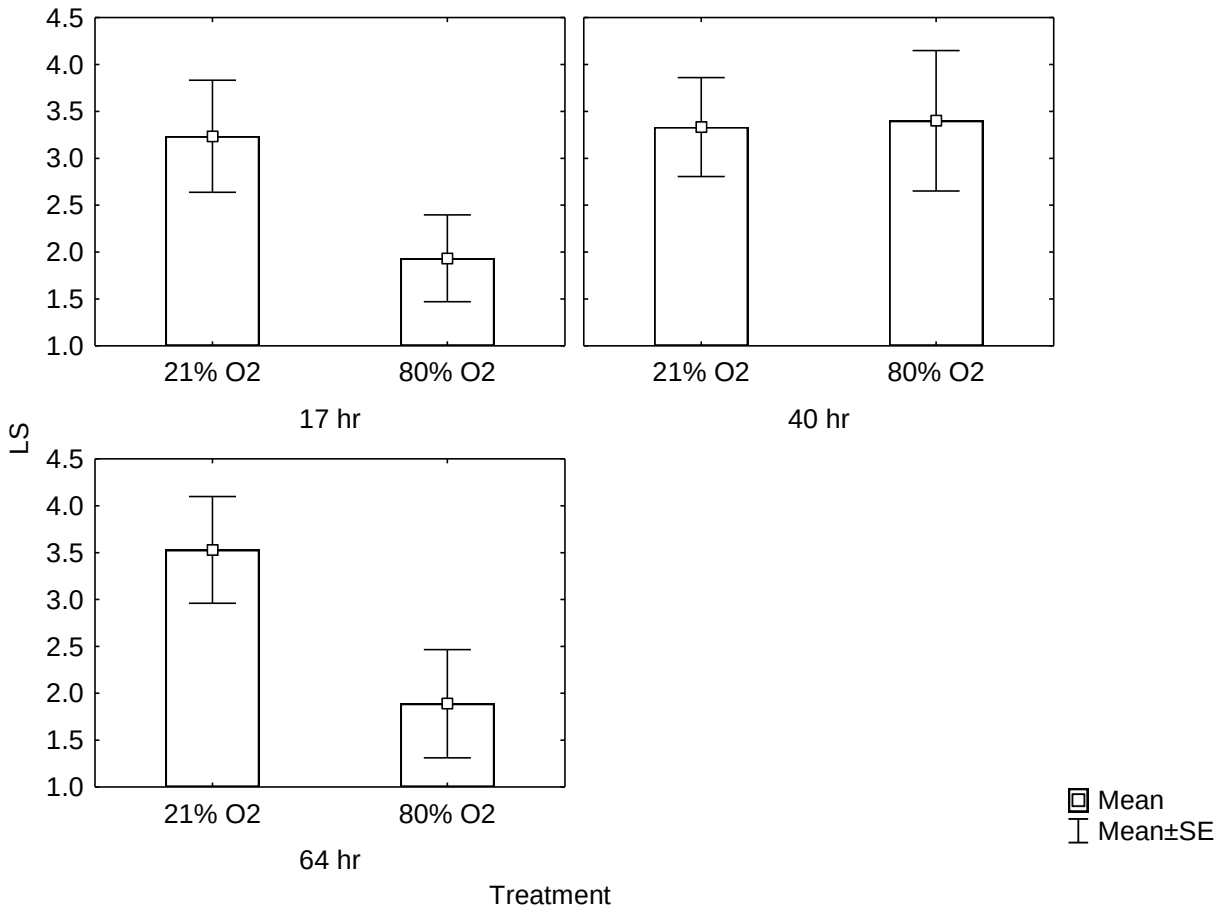

4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
LS
21% O2
80% O2
17 hr
21% O2
80% O2
40 hr
21% O2
80% O2
64 hr
Treatment
Mean
Mean±SE

Figure 5.

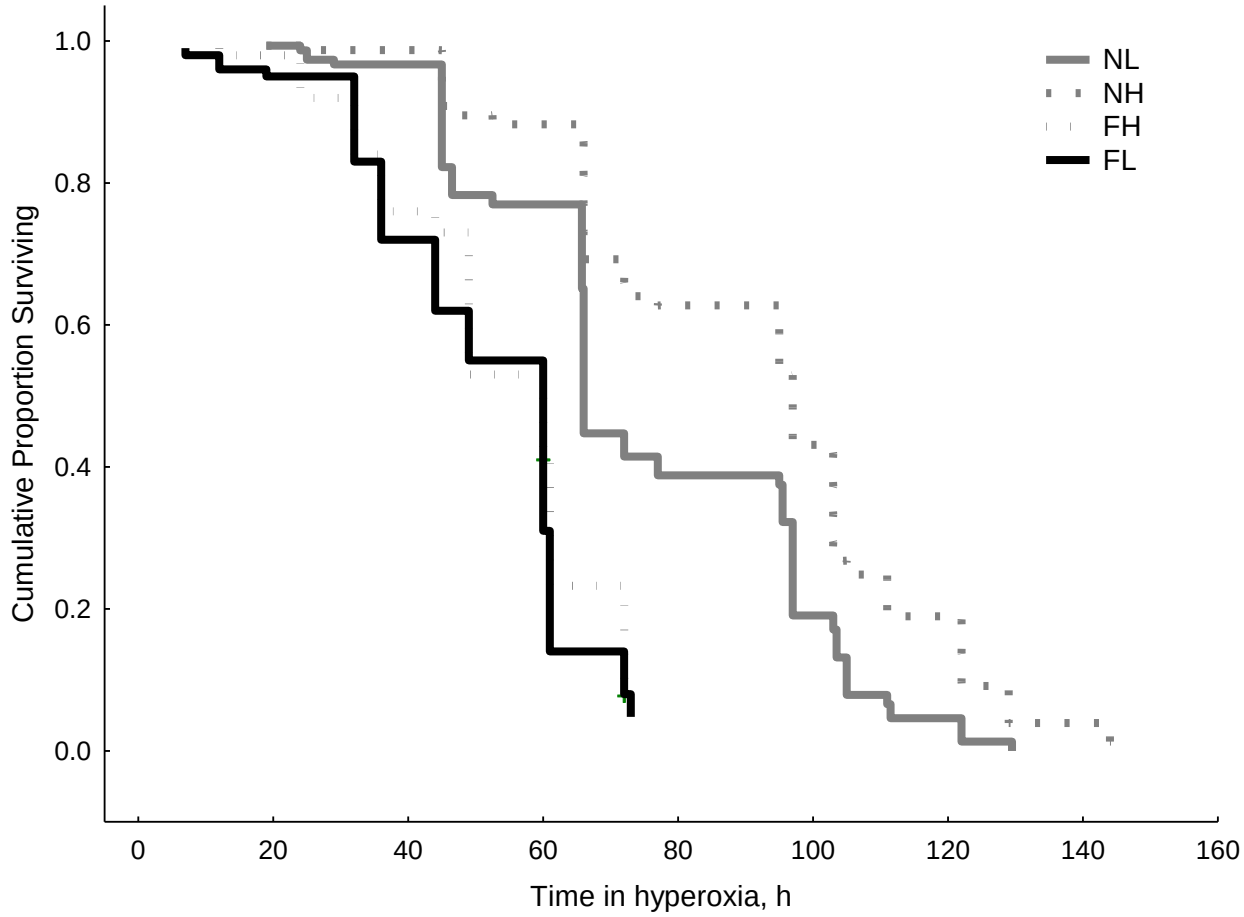

1.0
0.8
0.6
0.4
0.2
0.0
Cumulative Proportion Surviving
0
20
40
60
80
100
120
140
160
Time in hyperoxia, h
NL
NH
FH
FL

Figure 6.

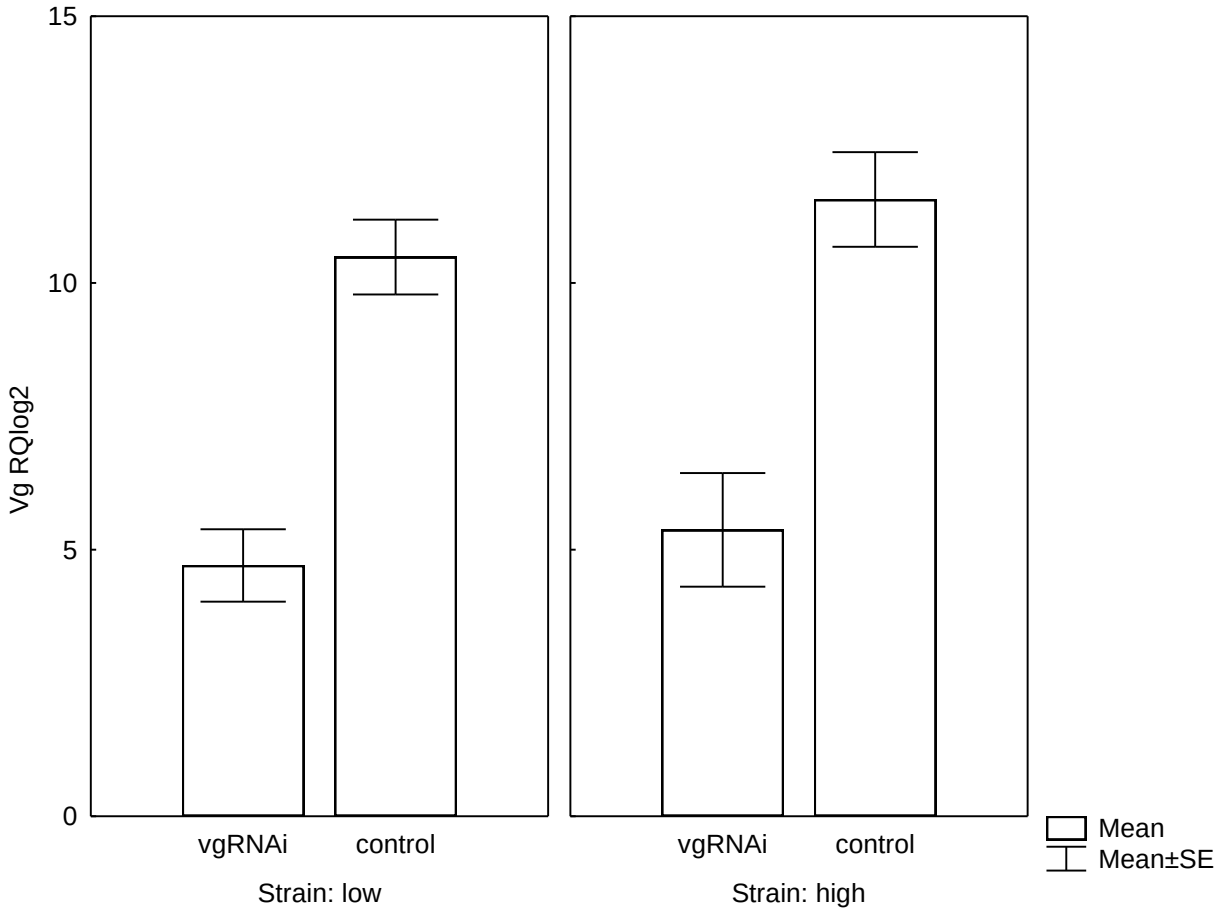

15
10
5
0
Vg RQlog2
vgRNAi
control
vgRNAi
control
Strain: low
Strain: high
Mean
Mean±SE

Figure 7A

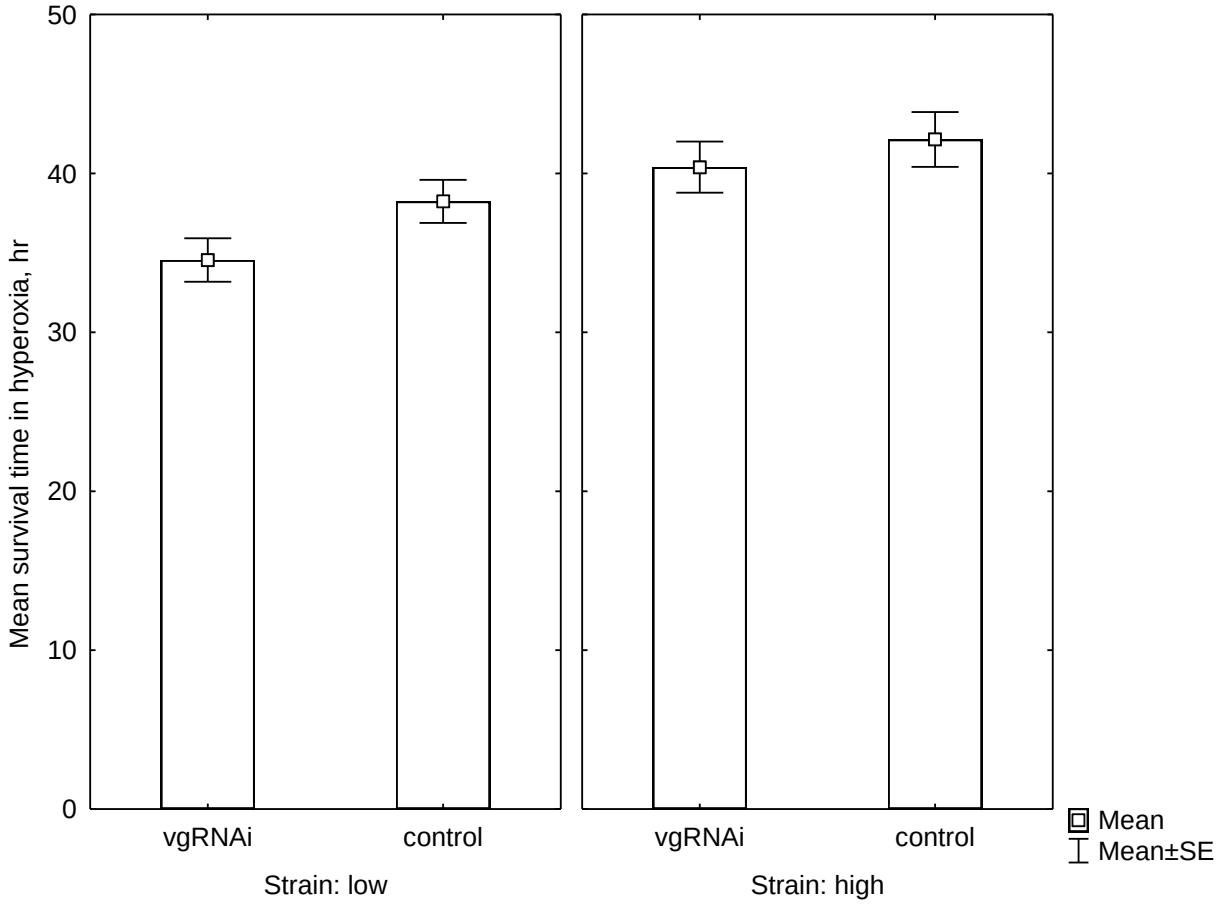
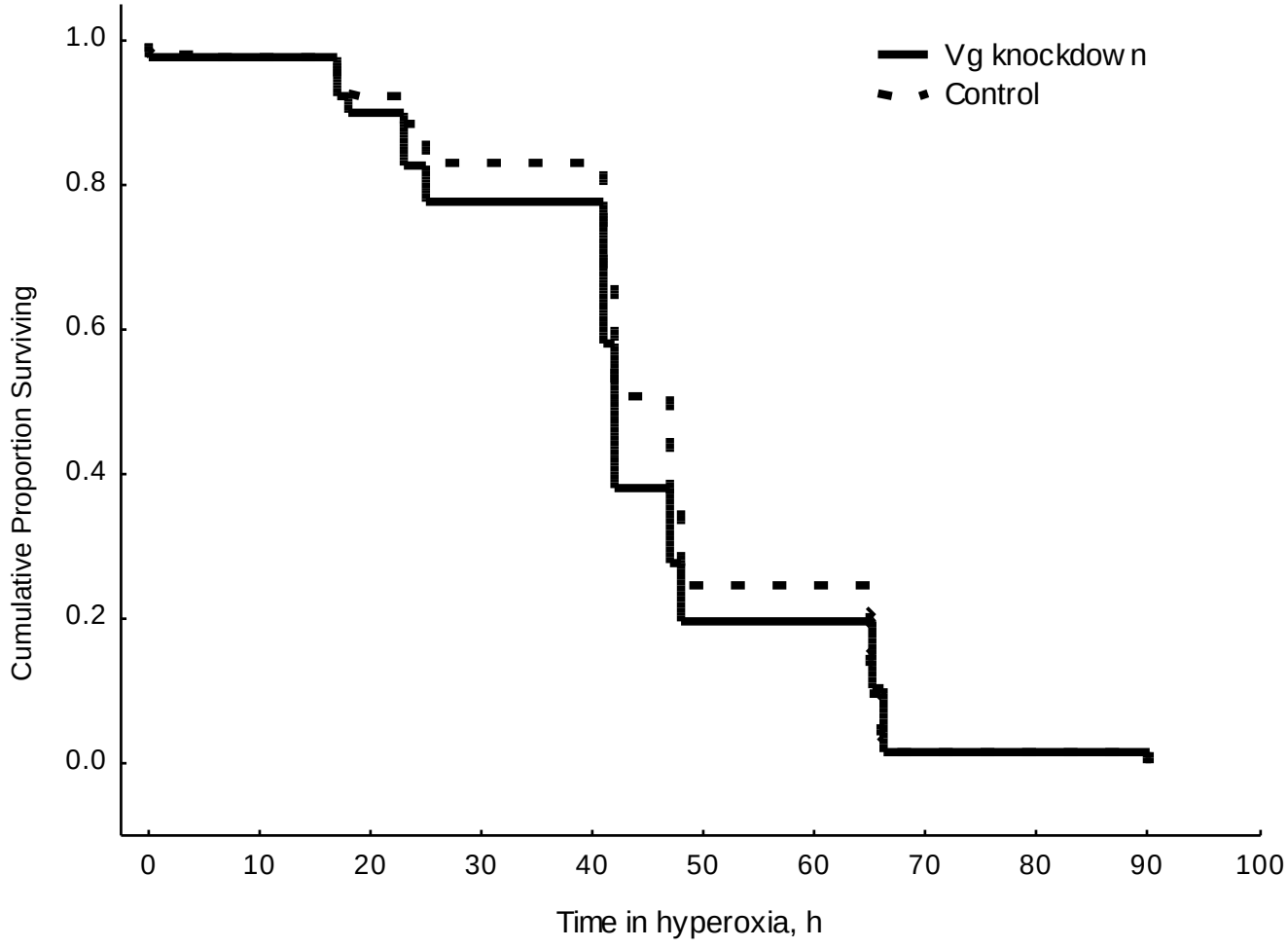

50
40
30
20
10
0
Mean survival time in hyperoxia, hr
vgRNAi
control
vgRNAi
control
Strain: low
Strain: high
Mean
Mean±SE

Figure 7B


Vg knockdown
Control
Cumulative Proportion Surviving
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
90
100
Time in hyperoxia, h

References

Amdam, G.V. Social context, stress, and plasticity of aging. Aging cell. 10:18-27; 2011

Amdam, G.V.; Fennern, E.; Baker, N.; Rascón, B. Honeybee associative learning performance and metabolic stress resilience are positively associated. PloS one. 5:e9740; 2010

Amdam, G.V.; Nilsen, K.A.; Norberg, K.; Fondrk, K.; Hartfelder, K. Variation in Endocrine Signaling Underlies Variation in Social Life History. American Naturalist. 170:37-46; 2007

Amdam, G.V.; Norberg, K.; Fondrk, M.K.; Page, R.E., Jr. Reproductive ground plan may mediate colony-level selection effects on individual foraging behavior in honey bees. Proceedings of the National Academy of Sciences of the United States of America. 101:11350-11355; 2004

Amdam, G.V.; Norberg, K.; Hagen, A.; Omholt, S.W. Social exploitation of vitellogenin. Proceedings of the National Academy of Sciences of the United States of America. 100:1799-1802; 2003a

Amdam, G.V.; Norberg, K.; Page, R.E., Jr.; Erber, J.; Scheiner, R. Downregulation of vitellogenin gene activity increases the gustatory responsiveness of honey bee workers (Apis mellifera). Behavioural brain research. 169:201-205; 2006

Amdam, G.V.; Simoes, Z.L.; Guidugli, K.R.; Norberg, K.; Omholt, S.W. Disruption of vitellogenin gene function in adult honeybees by intra-abdominal injection of double-stranded RNA. BMC biotechnology. 3:1; 2003b

Behrends, A.; Scheiner, R.; Baker, N.; Amdam, G.V. Cognitive aging is linked to social role in honey bees (Apis mellifera). Experimental gerontology. 42:1146-1153; 2007

Brown, S.; Strausfeld, N. The effect of age on a visual learning task in the American cockroach. Learning & memory (Cold Spring Harbor, NY. 16:210-223; 2009

Cabeza, R.; Daselaar, S.M.; Dolcos, F.; Prince, S.E.; Budde, M.; Nyberg, L. Task-independent and task-specific age effects on brain activity during working memory, visual attention and episodic retrieval. Cereb Cortex. 14:364-375; 2004

Farooqui, T. Iron-induced oxidative stress modulates olfactory learning and memory in honeybees. Behavioral neuroscience. 122:433-447; 2008

Flood, J.F.; Morley, J.E. Learning and memory in the SAMP8 mouse. Neuroscience and biobehavioral reviews. 22:1-20; 1998

Fraser, H.B.; Khaitovich, P.; Plotkin, J.B.; Paabo, S.; Eisen, M.B. Aging and gene expression in the primate brain. PLoS biology. 3:e274; 2005

Grotewiel, M.S.; Martin, I.; Bhandari, P.; Cook-Wiens, E. Functional senescence in Drosophila melanogaster. Ageing research reviews. 4:372-397; 2005

Guidugli, K.R.; Nascimento, A.M.; Amdam, G.V.; Barchuk, A.R.; Omholt, S.; Simoes, Z.L.; Hartfelder, K. Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect. FEBS letters. 579:4961-4965; 2005

Hammer, M.; Menzel, R. Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. Learning & memory (Cold Spring Harbor, NY. 5:146-156; 1998

Harman, D. Aging: a theory based on free radical and radiation chemistry. Journal of gerontology. 11:298-300; 1956

Harrison, J.; Frazier, M.R.; Henry, J.R.; Kaiser, A.; Klok, C.J.; Rascon, B. Responses of terrestrial insects to hypoxia or hyperoxia. Respiratory physiology & neurobiology. 154:4-17; 2006

Harrison, J.F.; Fewell, J.H. Environmental and genetic influences on flight metabolic rate in the honey bee, Apis mellifera. Comparative biochemistry and physiology. 133:323-333; 2002

Humphries, M.A.; Muller, U.; Fondrk, M.K.; Page, R.E., Jr. PKA and PKC content in the honey bee central brain differs in genotypic strains with distinct foraging behavior. J Comp Physiol A Neuroethol Sens Neural Behav Physiol. 189:555-562; 2003

Ihle, K.E.; Page, R.E.; Frederick, K.; Fondrk, M.K.; Amdam, G.V. Genotype effect on regulation of behaviour by vitellogenin supports reproductive origin of honeybee foraging bias. Animal behaviour. 79:1001-1006; 2010

Landis, G.N.; Abdueva, D.; Skvortsov, D.; Yang, J.; Rabin, B.E.; Carrick, J.; Tavare, S.; Tower, J. Similar gene expression patterns characterize aging and oxidative stress in Drosophila melanogaster. Proceedings of the National Academy of Sciences of the United States of America. 101:7663-7668; 2004

Lin, Y.J.; Seroude, L.; Benzer, S. Extended life-span and stress resistance in the Drosophila mutant methuselah. Science (New York, NY. 282:943-946; 1998

Lourenco, A.P.; Mackert, A.; Cristino, A.D.; Simoes, Z.L.P. Validation of reference genes for gene expression studies in the honey bee, Apis mellifera, by quantitative real-time RT-PCR. Apidologie. 39:372-U333; 2008

Marco Antonio, D.; Guidugli-Lazzarini, K.; do Nascimento, A.; Simões, Z.; Hartfelder, K. RNAi-mediated silencing of vitellogenin gene function turns honeybee (Apis mellifera) workers into extremely precocious foragers. Die Naturwissenschaften. 95:953-961; 2008

Mery, F. Aging and its differential effects on consolidated memory forms in Drosophila. Experimental gerontology. 42:99-101; 2007

Miquel, J.; Lundgren, P.R.; Bensch, K.G. Effects of exygen-nitrogen (1:1) at 760 Torr on the life span and fine structure of Drosophila melanogaster. Mechanisms of ageing and development. 4:41-57; 1975

Munch, D.; Amdam, G.V. The curious case of aging plasticity in honey bees. FEBS letters. 584:2496-2503; 2010

Munch, D.; Amdam, G.V.; Wolschin, F. Ageing in a eusocial insect: molecular and physiological characteristics of life span plasticity in the honey bee. Functional ecology. 22:407-421; 2008

Munch, D.; Baker, N.; Kreibich, C.D.; Braten, A.T.; Amdam, G.V. In the laboratory and during free-flight: old honey bees reveal learning and extinction deficits that mirror mammalian functional decline. PloS one. 5:e13504; 2010

Nelson, C.M.; Ihle, K.E.; Fondrk, M.K.; Page, R.E.; Amdam, G.V. The gene vitellogenin has multiple coordinating effects on social organization. PLoS biology. 5:e62; 2007

Orr, W.C.; Sohal, R.S. The effects of catalase gene overexpression on life span and resistance to oxidative stress in transgenic Drosophila melanogaster. Archives of biochemistry and biophysics. 297:35-41; 1992

Page, R.E., Jr.; Erber, J.; Fondrk, M.K. The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (Apis mellifera L.). Journal of comparative physiology. 182:489-500; 1998

Page, R.E., Jr.; Fondrk, M.K. The Effects of Colony-Level Selection on the Social Organization of Honey Bee (Apis mellifera L.) Colonies: Colony-Level Components of Pollen Hoarding. Behavioral Ecology and Sociobiology. 36:135-144; 1995

Page, R.E., Jr.; Peng, C.Y. Aging and development in social insects with emphasis on the honey bee, Apis mellifera L. Experimental gerontology. 36:695-711; 2001

Page, R.E., Jr.; Scheiner, R.; Erber, J.; Amdam, G.V. 8. The development and evolution of division of labor and foraging specialization in a social insect (Apis mellifera L.). Current topics in developmental biology. 74:253-286; 2006

Pankiw, T.; Page, R.E., Jr. Genotype and Colony Environment Affect Honeybee (Apis mellifera L.) Development and Foraging Behavior. Behavioral Ecology and Sociobiology. 51:87-94; 2001

Pankiw, T.; Waddington, K.D.; Page, R.E., Jr. Modulation of sucrose response thresholds in honey bees (Apis mellifera L.): influence of genotype, feeding, and foraging experience. Journal of comparative physiology. 187:293-301; 2001

Rascón, B.; Harrison, J.F. Lifespan and oxidative stress show a non-linear response to atmospheric oxygen in Drosophila. The Journal of experimental biology. 213:3441-3448; 2010
Roberts, S.P.; Elekonich, M.M. Muscle biochemistry and the ontogeny of flight capacity during behavioral development in the honey bee, Apis mellifera. The Journal of experimental biology. 208:4193-4198; 2005
Scharlaken, B.; de Graaf, D.C.; Goossens, K.; Brunain, M.; Peelman, L.J.; Jacobs, F.J. Reference gene selection for insect expression studies using quantitative real-time PCR: The head of the honeybee, Apis mellifera, after a bacterial challenge. Journal of Insect Science. 8; 2008
Scheiner, R.; Amdam, G.V. Impaired tactile learning is related to social role in honeybees. The Journal of experimental biology. 212:994-1002; 2009
Scheiner, R.; Page, R.E., Jr.; Erber, J. The effects of genotype, foraging role, and sucrose responsiveness on the tactile learning performance of honey bees (Apis mellifera L.). Neurobiology of learning and memory. 76:138-150; 2001a
Scheiner, R.; Page, R.E., Jr.; Erber, J. Responsiveness to sucrose affects tactile and olfactory learning in preforaging honey bees of two genetic strains. Behavioural brain research. 120:67-73; 2001b
Schippers, M.P.; Dukas, R.; Smith, R.W.; Wang, J.; Smolen, K.; McClelland, G.B. Lifetime performance in foraging honeybees: behaviour and physiology. The Journal of experimental biology. 209:3828-3836; 2006
Seehuus, S.C.; Krekling, T.; Amdam, G.V. Cellular senescence in honey bee brain is largely independent of chronological age. Experimental gerontology. 41:1117-1125; 2006a
Seehuus, S.C.; Norberg, K.; Gimsa, U.; Krekling, T.; Amdam, G.V. Reproductive protein protects functionally sterile honey bee workers from oxidative stress. Proceedings of the National Academy of Sciences of the United States of America. 103:962-967; 2006b
Sohal, R.S.; Agarwal, S.; Dubey, A.; Orr, W.C. Protein oxidative damage is associated with life expectancy of houseflies. Proceedings of the National Academy of Sciences of the United States of America. 90:7255-7259; 1993
Sohal, R.S.; Dubey, A. Mitochondrial oxidative damage, hydrogen peroxide release, and aging. Free radical biology & medicine. 16:621-626; 1994
Tamura, T.; Chiang, A.S.; Ito, N.; Liu, H.P.; Horiuchi, J.; Tully, T.; Saitoe, M. Aging specifically impairs amnesiac-dependent memory in Drosophila. Neuron. 40:1003-1011; 2003
Tolfsen, C.C.; Baker, N.; Kreibich, C.; Amdam, G.V. Flight restriction prevents associative learning deficits but not changes in brain protein-adduct formation during honeybee ageing. The Journal of experimental biology. 214:1322-1332; 2011
Walker, D.W.; Benzer, S. Mitochondrial "swirls" induced by oxygen stress and in the Drosophila mutant hyperswirl. Proceedings of the National Academy of Sciences of the United States of America. 101:10290-10295; 2004
Williams, J.B.; Roberts, S.P.; Elekonich, M.M. Age and natural metabolically-intensive behavior affect oxidative stress and antioxidant mechanisms. Experimental gerontology. 43:538-549; 2008
Wolschin, F.; Amdam, G.V. Comparative proteomics reveal characteristics of life-history transitions in a social insect. Proteome science. 5:10; 2007
Wolschin, F.; Munch, D.; Amdam, G.V. Structural and proteomic analyses reveal regional brain differences during honeybee aging. The Journal of experimental biology. 212:4027-4032; 2009

Paper III

Honey bee associative learning performance and metabolic stress resilience are positively associated

Gro V. Amdam, Erin Fennern, Nicholas Baker, and Brenda Rascón

Abstract

Social-environmental influences can affect animal cognition and health. Also, human socio-economic status is a covariate factor connecting psychometric test-performance (a measure of cognitive ability), educational achievement, lifetime health, and survival. The complimentary hypothesis, that mechanisms in physiology can explain some covariance between the same traits, is disputed. Possible mechanisms involve metabolic biology affecting integrity and stability of physiological systems during development and ageing. Knowledge of these relationships is incomplete, and underlying processes are challenging to reveal in people. Model animals, however, can provide insights into connections between metabolic biology and physiological stability that may aid efforts to reduce human health and longevity disparities.

OPEN ACCESS Freely available online

PLoS one

Honeybee Associative Learning Performance and Metabolic Stress Resilience Are Positively Associated

Gro V. Amdam[1,2]*, Erin Fennern[1], Nicholas Baker[1], Brenda Rascón[2]

1 School of Life Sciences, Arizona State University, Tempe, Arizona, United States of America, **2** Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas, Norway

Abstract

Background: Social-environmental influences can affect animal cognition and health. Also, human socio-economic status is a covariate factor connecting psychometric test-performance (a measure of cognitive ability), educational achievement, lifetime health, and survival. The complimentary hypothesis, that mechanisms in physiology can explain some covariance between the same traits, is disputed. Possible mechanisms involve metabolic biology affecting integrity and stability of physiological systems during development and ageing. Knowledge of these relationships is incomplete, and underlying processes are challenging to reveal in people. Model animals, however, can provide insights into connections between metabolic biology and physiological stability that may aid efforts to reduce human health and longevity disparities.

Results: We document a positive correlation between a measure of associative learning performance and the metabolic stress resilience of honeybees. This relationship is independent of social factors, and may provide basic insights into how central nervous system (CNS) function and metabolic biology can be associated. Controlling for social environment, age, and learning motivation in each bee, we establish that learning in Pavlovian conditioning to an odour is positively correlated with individual survival time in hyperoxia. Hyperoxia induces oxidative metabolic damage, and provides a measure of metabolic stress resistance that is often related to overall lifespan in laboratory animals. The positive relationship between Pavlovian learning ability and stress resilience in the bee is not equally established in other model organisms so far, and contrasts with a genetic cost of improved associative learning found in *Drosophila melanogaster*.

Conclusions: Similarities in the performances of different animals need not reflect common functional principles. A correlation of honeybee Pavlovian learning and metabolic stress resilience, thereby, is not evidence of a shared biology that will give insight about systems integrity in people. Yet, the means to resolve difficult research questions often come from findings in distant areas of science while the model systems that turn out to be valuable are sometimes the least predictable. Our results add to recent findings indicating that honeybees can become instrumental to understanding how metabolic biology influences life outcomes.

Citation: Amdam GV, Fennern E, Baker N, Rascón B (2010) Honeybee Associative Learning Performance and Metabolic Stress Resilience Are Positively Associated. PLoS ONE 5(3): e9740. doi:10.1371/journal.pone.0009740

Editor: Georges Chapouthier, L'université Pierre et Marie Curie, France

Received January 8, 2010; **Accepted** February 22, 2010; **Published** March 17, 2010

Funding: This work was supported by the Research Council of Norway (#175413, 180504, 185306, and 191699), the National Institute on Aging (NIA P01 AG22500), The PEW Charitable Trust, and the Wissenschaftskolleg zu Berlin. 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.

* E-mail: Gro.Amdam@asu.edu

Introduction

Childhood psychometric (IQ) scores correlate with age at death [1–3] and can, statistically, predict mortality with a strength similar to that of smoking [4]. Covariance of psychometric scores and longevity is explained by complex inter-related factors, such as socio-economic status, education, health behaviour, disease factors and illnesses, as well as pre- and postnatal privations [2,3,5,6]. Yet, IQ-longevity relationships can remain largely intact when markers of fetal development (birth weight) and early-life conditions (parental social status) are taken into account during statistical processing of data [2,4]. Such patterns of persistence led to the debated claim (e.g. [3,7,8]) that a fraction of covariance in cognition-survival correlations is explained by physiological 'systems integrity', a poorly understood factor [2,5,9].

Systems integrity encompasses functional reserve capacity and metabolic robustness [4,9,10]. The former refers to the capacity to maintain brain function during degenerative processes. The latter to the ability to maintain metabolic stability despite induced oxidative damage. Mechanisms of longevity, and the physiology of central nervous system (CNS) function, ageing, and frailty, are much-studied in genetic workhorses *Caenorhabditis elegans*, *Drosophila*, and mice, where some mutants maintain youthful levels of CNS function at advanced ages [11–13]. However, positive correlations between early-life performance of CNS computational processes, such as learning, and physiological stability or survival are generally not measured in prior studies (reviewed by Burger and coworkers [13], see also citations [14–16]). In *Drosophila*, furthermore, the strongest correlated response to artificial selection for improved associative learning is shorter lifespan — revealing a negative genetic link between learning ability and survival [13].

Research on poorly understood factors that potentially influence lifespan may ultimately benefit efforts to reduce health and longevity disparities between people [17–19]. However, studies motivated by IQ-longevity relationships are debated and difficult to justify. At the same time, it is uncertain whether variables related to early-life CNS computational task performance, such as learning, are positively correlated with survival in the laboratory, and whether these connections can be generalized to model animals. Here, we directly address the latter questions by studying a relationship between a measure of associative learning performance and metabolic stress resilience in the honeybee (*Apis mellifera*).

Social effects have strong influences on honeybee life outcomes [20–24]. Individuals that are largely identical genetically can be very different phenotypically, as exemplified by the reproductive division of labour between sister queens (primary egg-layers) and workers (essentially sterile female helpers), and in the social division of labour between workers that move between behavioural roles: nursing, nest building, guarding, colony defence, and foraging [20]. CNS function differs between workers, as measured in laboratory learning and memory retention tests (see citations [25,26] for recent reviews). In such tests, the individual bee learns to respond to stimuli (olfactory, tactile, visual), and shows different memory forms [27–30]. Worker longevity also varies greatly, from weeks to months, and is partly contingent on social role as nurse bees can generally outlive foragers—in the colony as well as in laboratory confinement (reviewed by Amdam and co-workers [31,32]). Such differences in worker survival correlate with the bees' resistance to laboratory-induced oxidative stress, a test of metabolic stress resilience that nurse bees can endure longer than foragers [32–34].

The opportunity to quantify these variables in honeybees led us to examine whether Pavlovian learning ability can be positively correlated with survival during oxidative insult.

Results and Discussion

We obtained adult worker bees from single-cohort colonies (N = 4), a method that provides animals of known (same) age and social role (see Materials and Methods). To control for social role, we chose a single well-defined behavioural group — nurse bees (young caregivers)—and quantified individual associative learning performance using a well-established procedure for Pavlovian olfactory learning [35]. Nurse bees were trained to a conditioned stimulus (CS) — an odour — which was associated with a sucrose reward (unconditioned stimulus, US). Gustatory responsiveness was determined prior to training as a control for individual motivational state; this responsiveness conveyed the subjective value each animal placed on the US, the sucrose reward [29]. Learning ability was scored on an integer scale from 0 (poorest score) to 5 (best score). Thereafter, individual metabolic stress resilience was measured as survival time in 80% O_2 (hyperoxia). Hyperoxia induces oxidative stress, metabolic damage, and features of premature senescence in model animals [36–38]. This reproducible approach gives a measure of metabolic stress resistance, a variable that often is related to lifespan of model organisms [36,39], as shown in honeybees [32–34].

Pavlovian learning ability and metabolic stress resilience

By comparing all animals with data on learning ability (learning categories 0–5) and subsequent survival time in hyperoxia (between 4–100 h, N = 390), we found a modest but significant positive correlation between individual associative learning performance and longevity (Pearson's correlation; R = 0.11, P = 0.036, N = 390). This pattern was consistent throughout the experiment, and repeatable between independent replicate setups (visualized as mean plots of survival times, Figure 1A). Accordingly, poor learning ability would be a predictor of short survival time in hyperoxia, while good learning performance would be associated with higher resilience and extended survival. We tested the robustness of this connection by excluding bees with mid performance scores in learning (N = 49, learning categories 2–3), thereby strictly comparing workers with the poorest and best

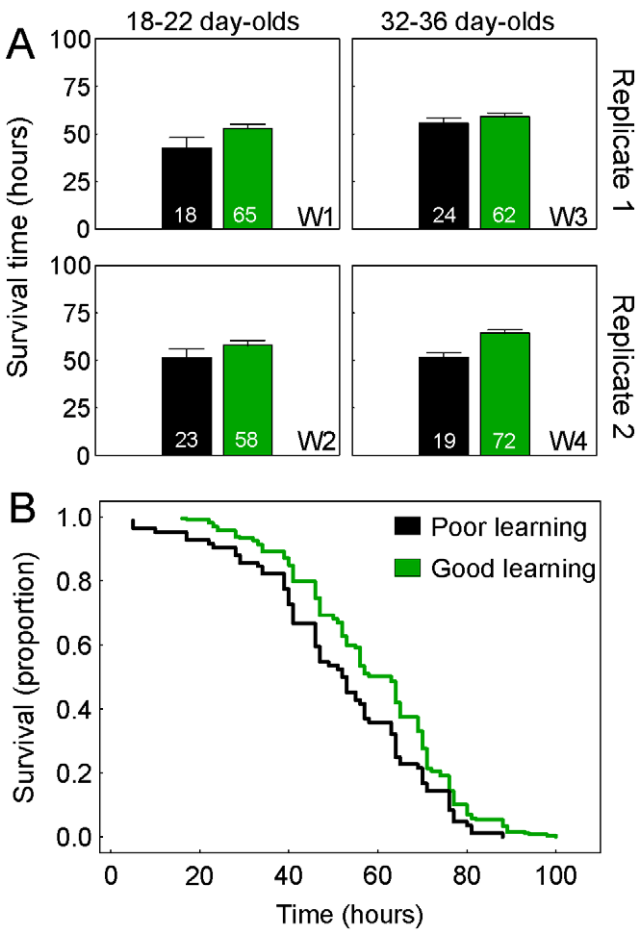


Figure 1. A positive association between Pavlovian learning ability and survival time in worker honeybees. (**A**) Average + S.E. survival time (h) in hyperoxia (80% O_2) of honeybees with poor (black bars) vs. good (green bars) associative learning ability. Bees were collected in equal numbers from four single-cohort colonies assembled from <24 h old bees (see Materials and Methods). The four colonies were prepared as two pairs, independent Replicate 1 and 2, which were set up one week apart. During the course of the experiment, each replicate pair was tested twice; when bees were 18–22 day-olds (from Replicate 1 during sample week 1 (W1) and from Replicate 2 during W2), and when bees were 32–36 day-olds (from Replicate 1 during W3 and from Replicate 2 during W4). In hyperoxia, the survival time of workers with poor performance (learning score 0–1) was shortened compared with the bees that had performed better in Pavlovian learning (scores 4–5). Sample sizes inside bars. (**B**) Proportional survival probability during the time course of metabolic insult in hyperoxia, summing over the workers shown in panel A (N = 341). Learning ability and metabolic stress resistance are positively connected. Compared to the individuals with poor learning scores (N = 84), bees that did well in associative learning (N = 257) showed significantly higher proportional survival (greater metabolic stress resistance) throughout the experiment.
doi:10.1371/journal.pone.0009740.g001

performance scores (learning categories 0, 1, 4, and 5). The correlation remained significant (R = 0.15, P = 0.007, N = 341). Next, we used proportional hazard statistics to contrast the survival data from the poorest learners (scores 0–1) toward the bees with the best performance (scores 4–5). This analysis confirmed that associative learning ability was a significant predictor of longevity during laboratory-induced metabolic stress in hyperoxia (Cox's Regression; $\chi^2 = 7.259$, P = 0.007, N = 341; Fig. 1B).

By using poor vs. good learning in Pavlovian conditioning to an odour (learning categories 0–1 vs. 4–5) as the predictor of survival time in hyperoxia, we could establish that the relationship between honeybee learning ability and metabolic stress resistance persisted when variance from social environment (colony) and age at testing were controlled for (MANOVA; F = 7.03, P = 0.008, N = 341). This analysis showed that the social environment did not influence the bees' longevity in hyperoxia (F = 2.09, P = 0.102), while their age at testing had a positive effect on survival that was independent of learning performance (F = 13.00, P = 0.0004, see also Figure 1A). A comparable response was identified by Seehuus and co-workers [33], who measured increased oxidative stress resistance in mid-aged nurse bees compared with younger bees. Similarly, we used nurse bees in our experiment (Materials and Methods). Seehuus and co-workers attributed the effect of nurse bees' age to vitellogenin, a multifunctional antioxidant protein that can accumulate over time in nurse bees [23,31,40,41]. This physiological factor may also explain the effect of age in our study.

Finally, we went back to the full dataset (N = 390) to test whether the positive association between Pavlovian learning ability and subsequent survival time in hyperoxia also influenced the olfactory acquisition (learning) curves of the worker bees. We contrasted the workers that died during the first half of the survival experiment (≤50 h in hyperoxia, N = 135) to bees that died during the second half (>50 h in hyperoxia, N = 255). Plotting the two curves revealed that the increase in conditioned responses was steepest after the initial conditioning trial and then gradually levelled out for both groups (Figure 2). After the second trial, however, the learning curve increased significantly more steeply for workers that survived >50 h in hyperoxia (ANCOVA, one sided test; F = 4.41, P = 0.038, N = 390); and this group also reached higher plateau levels of acquisition (89% in the 6th and final trial) in comparison to those surviving ≤50 h in hyperoxia (77%, Figure 2). These results suggest that faster learning after the initial conditioning trial and a higher level of final memory acquisition characterised the workers with the highest resistance to metabolic stress.

Gustatory responsiveness and metabolic stress resilience

Our control data on individual responsiveness to sucrose identified a positive correlation between the gustatory responsiveness score and learning score of the bees. This association was significant in the full dataset (Pearson's correlation; R = 0.33, P<0.001, N = 390) as well as when the workers with the mid performance scores (learning categories 2–3) were excluded (Pearson's correlation; R = 0.36, P<0.001, N = 341). This result corroborated a general finding: bees that place a high subjective value on sucrose rewards often perform better in reward learning [29,42]. The same result was conveyed by plotting the learning curves of bees with lower gustatory responsiveness (did not respond to sucrose at ≤0.1% in H_2O, N = 63) toward those with higher gustatory responsiveness (did respond to sucrose at ≤0.1 in H_2O, N = 327). From the first conditioning trial, the acquisition curve increased significantly more steeply for bees with higher gustatory responsiveness (ANCOVA, one sided test; F = 56.14, P<0.001, N = 390, Figure 3). Thus, a larger percentage of these workers (91%) showed the conditioned response in the final trial compared to the group with lower gustatory responsiveness (0.65%, Figure 3). Faster learning and a higher level of final memory acquisition, accordingly, characterised the bees with higher gustatory responsiveness.

Although responsiveness to sucrose was a predictor of learning performance, and learning performance was a predictor of survival during induced metabolic damage, the bees' appraisal of sucrose

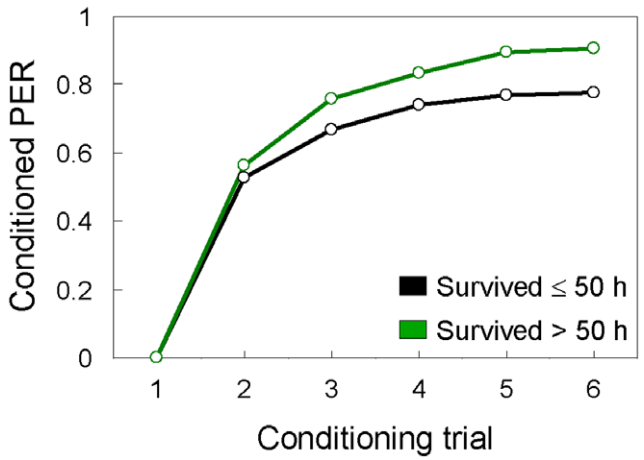


Figure 2. Olfactory learning in worker honeybees with different metabolic stress resilience. Acquisition (learning) curves for the proportion of worker bees that showed conditioned responses to an odour (CS) in each of six conditioning trials. Learning was quantified by the bees' proboscis extension response (PER), which was monitored during every presentation of the odour. Bees that after the conditioning experiment survived ≤50 h (black line, N = 135) vs. >50 h (green line, N = 255) in hyperoxia (80% O_2) are graphed separately. See text for details on statistics.
doi:10.1371/journal.pone.0009740.g002

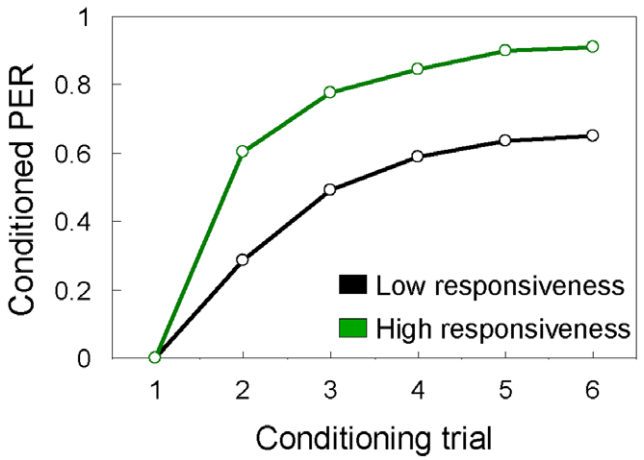


Figure 3. Olfactory learning in worker honeybees with different gustatory responsiveness. Acquisition (learning) curves for the proportion of bees that showed conditioned proboscis extension response (PER) to an odour in six conditioning trials. Bees with different responsiveness to sucrose are graphed separately. Low responsiveness (black line, N = 63) refers to worker bees that did not respond to sucrose at ≤0.1% in H_2O; High responsiveness (green line, N = 327) refers to bees that did respond to sucrose at ≤0.1% in H_2O. See text for details on statistics.
doi:10.1371/journal.pone.0009740.g003

rewards did not similarly explain longevity in hyperoxia. This lack of association was seen in the full dataset (R = 0.070, P = 0.167, N = 390) as well as when the workers with mid performance in learning (scores 2–3) were excluded (R = 0.058, P = 0.285, N = 341). The pattern was consistent between our replicate setups (Figure 4A). We also used proportional hazard statistics to contrast bees with lower vs. higher gustatory responsiveness (did not vs. did respond to sucrose at ≤0.1% in H_2O, respectively). The analysis confirmed that sucrose responsiveness did not predict survival time in hyperoxia (Cox's Regression; $\chi^2 = 1.001$, P = 0.464, N = 341; Figure 4B).

From these results, we inferred that the variance in learning ability that correlates with metabolic stress resistance in worker bees is independent of the variance that is explained by the bees' subjective motivation to learn. In other words, only a fraction of variation in learning is explained by gustatory responsiveness [29]. Here, this proportion of explained variance, R^2, was 10.89% (R = 0.33; N = 390, above), which leaves much variation in learning to be explained by factors other than sucrose responsiveness. Our results suggest that one or more of these latent factors, which affect learning but not motivation, can influence metabolic stress resilience — causing learning scores and survival times to correlate independent of the gustatory responsiveness of the bees.

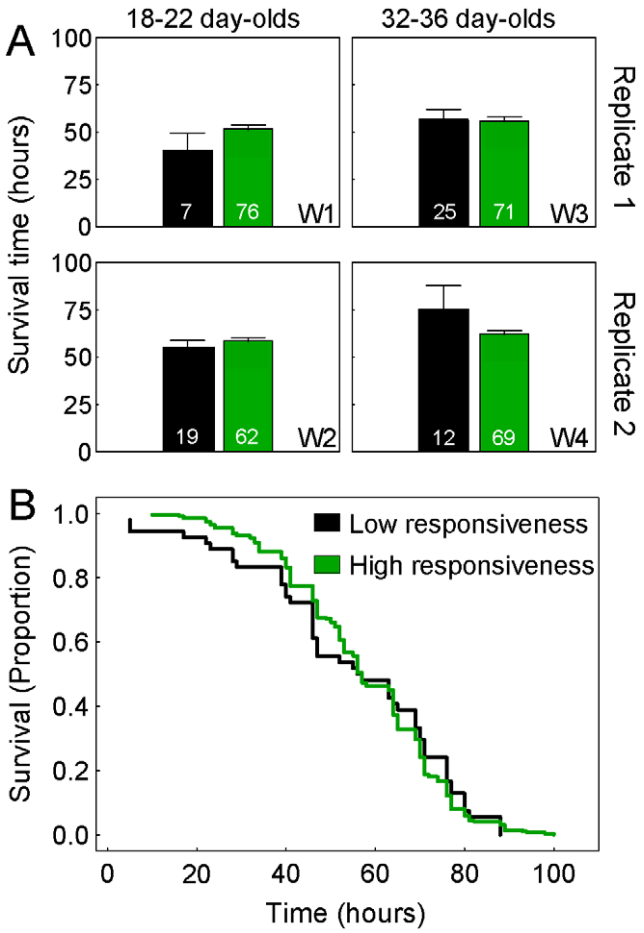


Figure 4. The gustatory responsiveness of honeybee workers is not associated with metabolic stress resistance. (**A**) Average + S.E. survival time (h) in hyperoxia (80% O_2) of bees with low (black bars) vs. high (green bars) gustatory responsiveness (not responding vs. responding to sucrose at ≤0.1% in H_2O, respectively). Bees were collected from four single-cohort colonies that were prepared as two pairs one week apart (Replicate 1 and 2). Each replicate pair was tested when bees were 18–22 day-olds (from Replicate 1 during sample week 1 (W1) and from Replicate 2 during W2), and when bees were 32–36 day-olds (from Replicate 1 during W3 and from Replicate 2 during W4). Gustatory responsiveness failed to show a consistent relationship to the bees' subsequent survival time in hyperoxia. Sample sizes inside bars. (**B**) Proportional survival probability during the time course of metabolic insult in hyperoxia, summing over the workers shown in panel A (N = 341). Gustatory responsiveness and metabolic stress resistance are not associated. See text for statistics.
doi:10.1371/journal.pone.0009740.g004

Conclusions

Our work establishes that in young caregiver (nurse) honeybees, individual performance in Pavlovian olfactory learning is positively associated with metabolic stress resistance measured in hyperoxia. This finding exemplifies that a positive correlation between early-life CNS function and a variable related to organismal survival can be detected in, and perhaps generalized to, a laboratory animal.

While the correlation between learning in Pavlovian conditioning to an odour and subsequent survival time in hyperoxia was modest in our worker bees (Pearson's correlation: R = 11 for the full dataset; R = 15 with mid performance values excluded, above), a Pearson's analysis of correlation between childhood IQ and age at death, similarly, gave only R = 0.18 for 722 human subjects [2]. Thus, our results are statistically significant and in line with the interpretation that positive associations between variables related to CNS computational task-performance (in our case associative olfactory learning) and longevity are moderate.

Bees have rich and quantifiable learning and memory repertoires [25,26], are amenable to functional genomic research, and provide the best-studied social invertebrate system to date [32,43]. In this model, genotype, social environment, social history, behaviour, workload, nutrition, physiology, and health can be controlled [32,43–45]—helping us identify and understand mechanisms that affect life-history. Such experiments already propose that life outcomes in social insects can be strongly influenced by metabolic biology [46,47].

Similarities in patterns of test performance between different organisms need not reflect common functional principles [48], yet it is also difficult to predict which models will become the most valuable for addressing and understanding unresolved challenges in research [49]. Many more studies will need to be conducted before we fully grasp how honeybees can best contribute toward research efforts to reduce health and longevity disparities between people.

Materials and Methods

Bees

The experiments were performed in Spring 2009 at Arizona State University in Tempe AZ, USA, and utilized four single-cohort colonies [50,51]. Each single-cohort colony was assembled with one egg-laying queen and several thousand workers. Within every colony, all workers belonged to one age-cohort. This demography was achieved by collecting honeybee combs with mature brood from a set of nine donor colonies. The combs were placed in an incubator overnight at 33°C in a relative humidity (RH) of 65–70%. The next morning, newly emerged bees (0–24 h old) were collected from the incubator and marked on the thorax with paint (Testors™) for identification.

Two genetic sources were donors of newly emerged bees: *i)* genetically diverse wild type stocks from four colonies headed by openly mated queens of Californian commercial origin, and *ii)* a standard research stock maintained by instrumental insemination, using five colonies headed by queens inseminated with 1–2 drones

(males) each. The wild type provided a background population for the single-cohort colonies, but all sampled bees came from the standard research stock, which has a well-documented and broad distribution of learning behaviour [52,53].

The four colonies were prepared as two separate pairs for independent replication of our experiment. The first paired colonies (Replicate 1) each contained 2,700 wild type workers plus 2,300 bees of standard stock. The second paired colonies (Replicate 2) were assembled with 3,400 wild type workers plus 3,000 bees of standard stock.

Sampling and handling

For experimental Replicate 1, collections were performed in calendar week 20 (bees aged 18–22 days old) and 22 (bees aged 32–36 days old). Sampling for Replicate 2 took place during calendar weeks 21 (18–22 day-olds) and 23 (32–36 day-olds). These staggered collections provided two replicates of age-matched bees. Collections started at 7 AM, and only marked bees of the standard stock that demonstrated typical nursing behaviour (inserting their heads into cells containing larvae) were retrieved from the colonies. The nurse bees were placed into $7.0\times3.5\times3.5$ cm plastic tubes containing a moist paper towel and brought to the laboratory (<5 min transit time). There, bees were incubated at 4°C until movement was reduced. Next, they were mounted onto individual plastic holders, and affixed with removable tape behind the head and across the thorax (Supporting Figure S1A). After restraining, the bees were fed 2 µl of 30% sucrose solution before being starved for 2 h at 37°C, 65–70% RH.

Quantification of gustatory responsiveness

After the 2 h starvation period, gustatory responsiveness [54,55] was measured by the proboscis extension response (PER). Bees were observed for PER while being stimulated with H_2O, followed by six sucrose solutions (sucrose in H_2O) in an ascending order (0.1%, 0.3%, 1%, 3%, 10%, 30%) at a minimum interval of 2 min between trials. Thereafter, bees were assigned a gustatory response score (GRS) that totalled the number of times PER was observed throughout the seven trials. The maximum GRS of 7 indicated that bees responded to all sucrose concentrations and H_2O (high gustatory responsiveness). In contrast, bees with a GRS of 0 did not respond to any of the seven stimuli. GRS provides a measure for the subjective value that the bee places on sucrose solutions, which are later used as rewards in the associative learning paradigm (see below). Thus, via GRS quantification, we ensured that only bees that responded to a reward (and thus could be rewarded) were trained [42,56].

Quantification of associative learning ability

Because we used 30% sucrose solution as reward [42,56], only bees that showed a PER response to a solution of at least 30% sucrose were allowed to participate in the associative learning assay. Over the course of the study, 48 bees did not respond to 30% sucrose and were thus not trained. As olfactory stimuli [35], 2 µl of each of two odours (carnation and cineole) were applied to separate pieces of filter paper, which were then placed into two different 10 ml syringes (BD Luer-Lock™ Tip). Each bee was initially stimulated for 6 sec directly to the antennae with approximately 6 ml of the carnation odour, which served as the conditioned stimulus (CS) during associative conditioning (see below). Thereafter, the alternative odour (cineole) was administered in the same manner. Bees that responded spontaneously to either odour were omitted (N = 57), as we could not validate learning for individuals whose response to the CS was spontaneous prior to conditioning [42].

During conditioning, each bee was subjected to six CS reward pairings with an approximate inter-trial interval of 15 min. During every trial, bees were stimulated with 6 ml of carnation odour applied directly to their antennae for 6 sec. Using a Gilmont® syringe, the final 3 ml of the CS was paired with 1 µl of 30% sucrose reward for 3 sec in order to form an association between the two [42]. For each trial, those bees who displayed PER to the odour stimulus prior to the introduction of the reward were recorded as positive, while the bees that did not respond prior to reward were noted as negative for PER.

Following the six conditioning trials, we performed a retention test where the specific CS memory of the worker bees was evaluated. The bees were first presented with cineole (the alternative odour), and then CS without reward. The outcome was not associated with survival time in hyperoxia: Longevity was the same whether bees demonstrated specific CS memory (did not respond to alternative odour, N = 304) or not (responded to alternative odour, N = 86, Student t-test; $t = -0.022$, $P = 0.983$, Supporting Figure S2).

Bees that responded to the final CS without reward were given a learning score ranging from 1 to 5, reflecting the total number of conditioning trials in which PER was observed minus the number of responses to the alternative odour (this number was 0 for 304 bees and 1 for 86 bees, above). The learning score, thereby, took into account how precise the learning was. Bees that did not respond to the final CS without reward and had not responded to any of the prior six conditioning trials were given a learning score of 0. Finally, the few bees that responded in all or some conditioning trials but did not respond to the final CS presentation without reward were omitted (N = 14), as we could not validate the learned association in them (details in [42]).

For all trials, bees were placed in front of a neutral air stream approximately 8 sec before and after odour stimulation. A minimum of 5 min passed between trials to prevent habituation effects [42]. The general activity of each bee was also monitored in every trial to ensure that all animals remained healthy.

Survival in hyperoxia

Bees that completed the olfactory conditioning test and received a measure of learning ability (learning scores 0–5) were placed in hyperoxia to monitor survival capability. Hyperoxia induces features of premature senescence in many laboratory systems, and can provide a reproducible test of metabolic stress resistance that often, but not without exception [39,57], is relevant to lifespan in a general way [36–38]. Bees were housed in 1.5 ml Eppendorf tubes that had two holes on top and an opening at the bottom for animal waste (Supporting Fig. S1B). The experimental bees were kept in an incubator (HERAcell O_2/CO_2, Thermo Scientific) with a constant 80% O_2 concentration; incubator temperature was 34°C and RH averaged 63±2%. A standard diet of 1.5 g of ground pollen per 30 ml of 30% sucrose solution was administered twice per day into a pipette tip that rested in one of the holes atop the Eppendorf tube (Supporting Fig. S1B). The other hole was left unobstructed for breathing.

Survivorship censuses took place four times daily: 7–8 AM; 1–2 PM; 6–7 PM; 11 PM–12 AM until the last bee was observed dead. As needed, alive bees were transferred to fresh tubes in order to prevent bacterial and/or fungal growth. Individuals that were likely harmed during routine transfers were excluded. Individual lifespans were calculated as the number of hours spent in hyperoxia prior to observed death.

Statistics

The datasets on associative learning, gustatory responsiveness and survival time in hyperoxia conformed to Levene's and Bartlett's tests of equal variance and parametric statistics were used [58]. The relationships between learning ability, gustatory responsiveness, and survival were tested with Pearson's correlations, and investigated further with the Proportional hazard (Cox) regression, which we have applied to bee survival data in previous studies [23,59]. One-sided analysis of covariance (ANCOVA) for comparison of regression curve slopes was used to test differences between olfactory learning [58]. Log-linear transformation was tested but the outcome was similar to raw data (for bees surviving $\leq$50 h vs. >50 h in hyperoxia: $F = 3.79$, $P = 0.046$; for bees not responding vs. responding to sucrose at $\leq$0.1% in H_2O: $F = 21.18$, $P = 0.002$). Thus, results from the untransformed dataset were reported. To control for variance linked to social environment (different single-cohort colonies) and age at testing (18–22 vs. 32–36 days old), we utilized Main effect ANOVA (MANOVA) with learning ability, colony, and age-group as categorical predictors of survival. This reporting was preferred over the Cox regression with colony or age-group as stratifying variables, because the Cox regression model does not allow the input of three predictors. Yet, the significant effect of learning performance on survival capability persisted even in the stratified Cox regression analyzes in which colony or sampling age were controlled for separately ($\chi^2 = 4.678$, $P = 0.031$; $\chi^2 = 4.738$, $P = 0.030$, respectively, $N = 341$). The relationship between the binary outcome of the retention test (bees demonstrating specific CS memory, or not) and the survival time in hyperoxia was tested with a Student t-test using survival time as the dependent variable. All analyses performed in Statistica 6.0 (StatSoft).

Supporting Information

Figure S1 (A) Worker honeybee prepared for testing of Pavlovian learning ability. The restraint holder is custom-made from Plexiglas, and the bee was affixed with straps of tape. After quantification of gustatory responsiveness and learning, the straps were removed and the bee was released unharmed. (B) Worker bee in the modified Eppendorf tube design used in our assay of survival capability in hyperoxia. The lid has holes for feeding and air-exchange. The end of the tube is cut open and sealed with cotton to absorb animal waste.
Found at: doi:10.1371/journal.pone.0009740.s001 (10.12 MB TIF)

Figure S2 Relationship between the outcome of the retention test, where bees were monitored for specific CS memory, and survival time in hyperoxia (80% O2). Bars are averages + S.E. for survival time (hours). Specific CS memory for the conditioned stimulus (carnation) was measured after olfactory conditioning by presenting the bees with an alternative odour (cineole) one time. No (zero) proboscis extension response (PER) to the alternative odour demonstrated specific CS memory. The performance in the retention test was not associated with survival time in hyperoxia (Student t-test; $t = -0.022$, $P = 0.983$). Sample sizes inside bars.
Found at: doi:10.1371/journal.pone.0009740.s002 (0.01 MB TIF)

Acknowledgments

We thank C. Ozturk and O. Kaftanoglu for assistance in the apiary, and L. Harshman, T. Flatt, B. Smith and D. Münch for helpful comments.

Author Contributions

Conceived and designed the experiments: GVA. Performed the experiments: EF NB. Analyzed the data: GVA. Contributed reagents/materials/analysis tools: GVA BR. Wrote the paper: GVA BR.

References

1. Jokela M, Elovainio M, Singh-Manoux A, Kivimaki M (2009) IQ, socioeconomic status, and early death: The US National Longitudinal Survey of Youth. Psychosom Med 71: 322–328.
2. Whalley LJ, Deary IJ (2001) Longitudinal cohort study of childhood IQ and survival up to age 76. Bmj 322: e819.
3. Hemmingsson T, Melin B, Allebeck P, Lundberg I (2009) Cognitive ability in adolescence and mortality in middle age: a prospective life course study. J Epidemiol Community Health 63: 697–702.
4. Deary I (2008) Why do intelligent people live longer? Nature 456: 175–176.
5. Batty GD, Deary IJ, Gottfredson LS (2007) Premorbid (early life) IQ and later mortality risk: systematic review. Ann Epidemiol 17: 278–288.
6. Kanazawa S (2008) IQ and the health of states. Biodemography Soc Biol 54: 200–213.
7. Ramsden E (2007) A differential paradox: the controversy surrounding the Scottish mental surveys of intelligence and family size. J Hist Behav Sci 43: 109–134.
8. Jokela M, Batty GD, Deary IJ, Gale CR, Kivimaki M (2009) Low childhood IQ and early adult mortality: The role of explanatory factors in the 1958 British birth cohort. Pediatrics 124: e380–e388.
9. Hall PA, Dubin JA, Crossley M, Holmqvist ME, D'Arcy C (2009) Does executive function explain the IQ-mortality association? Evidence from the Canadian study on health and aging. Psychosom Med 71: 196–204.
10. Batty GD, Gale CR, Mortensen LH, Langenberg C, Shipley MJ, et al. (2008) Pre-morbid intelligence, the metabolic syndrome and mortality: the Vietnam Experience Study. Diabetologia 51: 436–443.
11. Murakami H, Bessinger K, Hellmann J, Murakami S (2005) Aging-dependent and -independent modulation of associative learning behavior by insulin/insulin-like growth factor-1 signal in *Caenorhabditis elegans*. J Neurosci 25: 10894–10904.
12. Bartke A (2005) Minireview: role of the growth hormone/insulin-like growth factor system in mammalian aging. Endocrinology 146: 3718–3723.
13. Burger JM, Kolss M, Pont J, Kawecki TJ (2008) Learning ability and longevity: a symmetrical evolutionary trade-off in *Drosophila*. Evolution 62: 1294–12304.
14. Mery F, Kawecki TJ (2005) A cost of long-term memory in *Drosophila*. Science 308: 1148.
15. Mery F, Kawecki TJ (2004) An operating cost of learning in *Drosophila melanogaster*. Anim Behav 68: 589–598.
16. Mery F, Kawecki TJ (2003) A fitness cost of learning ability in *Drosophila melanogaster*. Proc Royal Soc B 270: 2465–2469.
17. Finch CE (1990) Longevity, senescence and the genome. Chicago: University of Chicago Press. pp 67–72.
18. Sloan FA, Ayyagari P, Salm M, Grossman D (2009) The longevity gap between black and white men in the United States at the beginning and end of the 20th century. Am J Public Health 100: 357–363.
19. Espinoza SE, Hazuda HP (2008) Frailty in older Mexican-American and European-American adults: is there an ethnic disparity? J Am Geriatr Soc 56: 1744–1749.
20. Winston ML (1987) The Biology of the Honey Bee. Cambridge MA: Harvard University Press. pp 296.
21. Huang Z-Y, Robinson GE (1996) Regulation of honey bee division of labor by colony age demography. Behav Ecol Sociobiol 39: 147–158.
22. Huang Z-Y, Robinson GE (1992) Honeybee colony integration: Worker-worker interactions mediate hormonally regulated plasticity in division of labor. Proc Natl Acad Sci USA 89: 11726–11729.
23. Amdam GV, Rueppell O, Fondrk MK, Page RE, Nelson CM (2009) The nurse's load: early-life exposure to brood-rearing affects behavior and lifespan in honey bees (*Apis mellifera*). Exp Gerontol 44: 467–471.
24. Smedal B, Brynem M, Kreibich CD, Amdam GV (2009) Brood pheromone suppresses physiology of extreme longevity in honeybees (*Apis mellifera*). J Exp Biol 212: 3795–3801.
25. Giurfa M (2007) Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. J Comp Physiol A 193: 801–824.
26. Menzel R, Leboulle G, Eisenhardt D (2006) Small brains, bright minds. Cell 124: 237–239.
27. Roussel E, Carcaud J, Sandoz JC, Giurfa M (2009) Reappraising social insect behavior through aversive responsiveness and learning. PLoS ONE 4: e4197.
28. Scheiner R, Arnold G (2010) Effects of patriline on gustatory responsiveness and olfactory learning in honey bees. Apidologie 41: 29–37.
29. Scheiner R, Kuritz-Kaiser A, Menzel R, Erber J (2005) Sensory responsiveness and the effects of equal subjective rewards on tactile learning and memory of honeybees. Learn Mem 12: 626–635.
30. Scheiner R, Barnert M, Erber J (2003) Variation in water and sucrose responsiveness during the foraging season affects proboscis extension learning in honey bees. Apidologie 34: 67–72.

31. Amdam GV, Omholt SW (2002) The regulatory anatomy of honeybee lifespan. J Theor Biol 216: 209–228.
32. Muench D, Amdam GV, Wolschin F (2008) Physiological and cognitive aging in honey bees: robustness, plasticity and molecular regulation. Func Ecol 22: 407–421.
33. Seehuus SC, Norberg K, Gimsa U, Krekling T, Amdam GV (2006) Reproductive protein protects sterile honey bee workers from oxidative stress. Proc Natl Acad Sci USA 103: 962–967.
34. Corona M, Velarde RA, Remolina S, Moran-Lauter A, Wang Y, et al. (2007) Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. Proc Natl Acad Sci USA 104: 7128–7133.
35. Bitterman ME, Menzel R, Fietz A, Schäfer S (1983) Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). J Comp Physiol 97: 107–119.
36. Douglas RM, Haddad GG (2008) Can O_2 dysregulation induce premature aging? Physiology 23: 333–349.
37. Klimova TA, Bell EL, Shroff EH, Weinberg FD, Snyder CM, et al. (2009) Hyperoxia-induced premature senescence requires p53 and pRb, but not mitochondrial matrix ROS. FASEB J 23: 783–794.
38. Darr D, Fridovich I (1995) Adaptation to oxidative stress in young, but not in mature or old, *Caenorhabditis elegans*. Free Radic Biol Med 18: 195–201.
39. Mockett RJ, Bayne AC, Kwong LK, Orr WC, Sohal RS (2003) Ectopic expression of catalase in *Drosophila* mitochondria increases stress resistance but not longevity. Free Radic Biol Med 34: 207–217.
40. Amdam GV, Norberg K, Hagen A, Omholt SW (2003) Social exploitation of vitellogenin. Proc Natl Acad Sci USA 100: 1799–1802.
41. Amdam GV, Ihle KE, Page RE (2009) Regulation of honey bee (*Apis mellifera*) life histories by vitellogenin. In: Pfaff D, Arnold A, Etgen A, Fahrbach S, Rubin R, eds. Hormones, Brain and Behavior. 2 ed. San Diego, CA: Elsevier Academic Press.
42. Behrends A, Scheiner R, Baker N, Amdam GV (2007) Cognitive aging is linked to social role in honey bees (*Apis mellifera*). Exp Gerontol 42: 1146–1153.
43. Robinson GE, Grozinger CM, Whitfield CW (2005) Sociogenomics: Social life in molecular terms. Nat Rev Genet 6: 257–270.
44. Toth AL, Robinson GE (2005) Worker nutrition and division of labour in honeybees. Anim Behav 69: 427–435.
45. Weinstock GM, Robinson GE, Gibbs RA, Weinstock GM, Weinstock GM, et al. (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443: 931–949.
46. Alaux C, Sinha S, Hasadsri L, Hunt GJ, Guzman-Novoa E, et al. (2009) Honey bee aggression supports a link between gene regulation and behavioral evolution. Proc Natl Acad Sci USA 106: 15400–15405.
47. Wolschin F, Amdam GV (2007) Plasticity and robustness of protein patterns during reversible development in the honey bee (*Apis mellifera*). Anal Bioanal Chem 389: 1095–1100.
48. Bitterman ME (1975) The comparative analysis of learning. Science 188: 699–709.
49. Golstein P, Aubry L, Levraun JP (2003) Cell-death alternative model organisms: why and which? Mol Cell Biol 4: 1–10.
50. Nelson FC (1927) Adaptability of young bees under adverse conditions. Am Bee J 67: 242–243.
51. Rothenbuhler WC, Page RE (1989) Genetic variability for temporal polyethism in colonies consisting of similarly-aged worker honey bees. Apidologie 29: 433–437.
52. Scheiner R, Erber J, Page RE (1999) Tactile learning and the individual evaluation of the reward in honey bees (*Apis mellifera*). J Comp Physiol A 185: 1–10.
53. Scheiner R, Page RE, Erber J (2001) The effects of genotype, foraging role, and sucrose responsiveness on the tactile learning performance of honey bees (*Apis mellifera* L.). Neurobiol Learn Mem 76: 138–150.
54. Scheiner R, Page RE, Erber J (2001) Responsiveness to sucrose affects tactile and olfactory learning in preforaging honey bees of two genetic strains. Behav Brain Res 120: 67–73.
55. Scheiner R, Page RE, Erber J (2004) Sucrose responsiveness and behavioral plasticity in honey bee (*Apis mellifera*). Apidologie 35: 133–142.
56. Scheiner R, Amdam GV (2009) Impaired tactile learning is related to social role in honeybees. J Exp Biol 212: 994–1002.
57. Salmon AB, Perez VI, Bokov A, Jernigan A, Kim G, et al. (2009) Lack of methionine sulfoxide reductase A in mice increases sensitivity to oxidative stress but does not diminish life span. FASEB J 23: 3601–3608.
58. Montgomery DC (1997) Design and Analysis of Experiments, 4th ed. New York: John Wiley & Sons. 672 p.
59. Nelson CM, Ihle K, Amdam GV, Fondrk MK, Page RE (2007) The gene *vitellogenin* has multiple coordinating effects on social organization. PLoS Biol 5: 673–677.

Paper IV

The lifespan extension effects of resveratrol are conserved in the honey bee and may be driven by a mechanism related to caloric restriction

Brenda Rascón, Basil P. Hubbard, David A. Sinclair, and Gro V. Amdam

Abstract

Our interest in healthy aging and in evolutionarily conserved mechanisms of lifespan extension prompted us to investigate whether features of age-related decline in the honey bee could be attenuated with resveratrol. Resveratrol is regarded as a caloric restriction mimetic known to extend lifespan in some but not all model species. The current, prevailing view is that resveratrol works largely by activating signaling pathways. It has also been suggested that resveratrol may act as an antioxidant and confer protection against nervous system impairment and oxidative stress. To test whether honey bee lifespan, learning performance, and food perception could be altered by resveratrol, we supplemented the diets of honey bees and measured lifespan, olfactory learning, and gustatory responsiveness to sucrose. Furthermore, to test the effects of resveratrol under metabolic challenge, we used hyperoxic environments to generate oxidative stress. Under normal oxygen conditions, two resveratrol treatments—30 and 130 μM—lengthened average lifespan in wild-type honey bees by 38% and 33%, respectively. Both resveratrol treatments also lengthened maximum and median lifespan. In contrast, hyperoxic stress abolished the resveratrol life-extension response. Furthermore, resveratrol did not affect learning performance, but did alter gustation. Honey bees that were not fed resveratrol exhibited greater responsiveness to sugar, while those supplemented with resveratrol were less responsive to sugar. We also discovered that individuals fed a high dose of resveratrol—compared to controls—ingested fewer quantities of food under *ad libitum* feeding conditions.

Research paper

The lifespan extension effects of resveratrol are conserved in the honey bee and may be driven by a mechanism related to caloric restriction

Brenda Rascón[1], Basil P. Hubbard[2], David A. Sinclair[2], and Gro V. Amdam[1, 3]

[1] *Department of Chemistry, Biotechnology and Food Science (IKBM), Norwegian University of Life Sciences (UMB), Ås N-1432, Norway*
[2] *Departments of Pathology and Genetics, Harvard Medical School, Boston, MA, 02115, USA*
[3] *School of Life Science, Arizona State University, Tempe, AZ 85287-4501, USA*

Key words: *hyperoxia, learning performance, aging, lifespan, Apis mellifera, resveratrol*
Received: *7/17/12; Accepted: 7/30/12; Published: 7/31/12*
Correspondence to: *Brenda Rascón, PhD;* ***E-mail:*** *brenda.rascon@asu.edu*

Abstract: Our interest in healthy aging and in evolutionarily conserved mechanisms of lifespan extension prompted us to investigate whether features of age-related decline in the honey bee could be attenuated with resveratrol. Resveratrol is regarded as a caloric restriction mimetic known to extend lifespan in some but not all model species. The current, prevailing view is that resveratrol works largely by activating signaling pathways. It has also been suggested that resveratrol may act as an antioxidant and confer protection against nervous system impairment and oxidative stress. To test whether honey bee lifespan, learning performance, and food perception could be altered by resveratrol, we supplemented the diets of honey bees and measured lifespan, olfactory learning, and gustatory responsiveness to sucrose. Furthermore, to test the effects of resveratrol under metabolic challenge, we used hyperoxic environments to generate oxidative stress. Under normal oxygen conditions, two resveratrol treatments—30 and 130 µM—lengthened average lifespan in wild-type honey bees by 38% and 33%, respectively. Both resveratrol treatments also lengthened maximum and median lifespan. In contrast, hyperoxic stress abolished the resveratrol life-extension response. Furthermore, resveratrol did not affect learning performance, but did alter gustation. Honey bees that were not fed resveratrol exhibited greater responsiveness to sugar, while those supplemented with resveratrol were less responsive to sugar. We also discovered that individuals fed a high dose of resveratrol—compared to controls—ingested fewer quantities of food under *ad libitum* feeding conditions.

INTRODUCTION

The decline in brain function that accompanies senescence in diverse organisms and the mechanisms that underlie this dysfunction are of great interest. Aging intervention strategies, such as genetic manipulation and dietary restriction, have shown that lifespan extension is possible e.g. [1]. However, enhanced longevity coupled to brain deterioration is an undesirable combination that warrants further research into the prolongation of health span. Our previous experiments in the honey bee have revealed that hyperoxic environments can mimic normal patterns of aging dysfunction, which include increased mortality and learning performance decline (Rascon et al., submitted). In the current study, we tested whether we could promote healthy aging in the honey bee via the administration of resveratrol.

Resveratrol is a plant polyphenol with reported lifespan extension effects in some [2-4], but not all studies [5, 6]. This particular polyphenol is a well-known activator of Silent Information Regulator 1 (SIRT1), which is thought to mediate the beneficial effects of caloric restriction [7, 8]. Resveratrol also elicits neuroprotective effects in vertebrate cell lines [9-12] and prevents the

decline of locomotory function in fish [3]. In the brains of healthy rats, resveratrol increases the activity of antioxidants such as superoxide dismutase and catalase, and decreases the level of oxidative stress [13]. The purported, beneficial effects of resveratrol also extend to cognitive performance. For instance, resveratrol can reverse cognitive deficits and maintain memory in aged rats [14]. Also, rats suffering from traumatic brain injury [15] or demonstrating Parkinsonian characteristics [16], benefit from resveratrol treatment. However, it is unclear whether resveratrol can improve learning performance in healthy animals.

With some exceptions, most of the studies that demonstrate a resveratrol-dependent lifespan extension rely on solitary model species such as yeast, worms, and fruit flies [2-4] . To date, only one study of social animals— mice fed either a high fat diet or fed every other day—has showed improved survival in response to resveratrol [17]. In the present study, we investigated the effects of resveratrol on lifespan and learning performance in a social animal with a well-established neurobiological pedigree: the honey bee.

Briefly, the characteristic signatures of honey bee social life are the caste differences between the functionally sterile worker and highly fecund queen, and the ontogenetic specialization of tasks undertaken by worker bees. These complexities often prevent the application of many well-known evolutionary theories of aging to the honey bee [18]. For instance, explanatory paradigms such as life history theory, which postulates that the pressure of natural selection on survival falls after reproductive capacity has been reached, cannot adequately explain the aging of sterile worker honey bees. Furthermore, the disposable soma theory, which states that natural selection will not favor investment into the soma if extrinsic mortality rates are high [19], is applicable to worker honey bees only for part of their lives. For example, during nursing tasks, workers experience low hazard and low mortality, but the switch to foraging tasks outside of the nest increases hazard and mortality. However, if honey bee society is viewed as a superorganism, the worker honey bee can be considered the disposable social caste due to their high numbers, short lives, and inability to reproduce. From this standpoint, there should be evolutionary constraints on worker lifespan. But is this so? Is worker lifespan fixed or can its limits be extended?

At present, there is no published work on the effects of resveratrol in the honey bee. To achieve insight, we combine resveratrol administration with the application of metabolic stress and the classic neurobiological paradigm of olfactory conditioning to examine whether resveratrol can: 1) lengthen lifespan in the honey bee, 2) affect brain function, and 3) affect food perception (gustation) and consumption. In this study, we demonstrate that resveratrol elicits life extension in wild-type honey bees, alters gustation, and food consumption.

RESULTS

Learning and gustatory performance tests

All sensory and gustatory tests were evaluated in normoxic conditions after honey bees had spent five days in the laboratory under resveratrol treatment(refer to materials and methods).

Learning performance

Learning performance was not affected by resveratrol supplementation (Figure 1; Kruskal-Wallis ANOVA: H (2, N= 350) =1.529602 P=0.465).

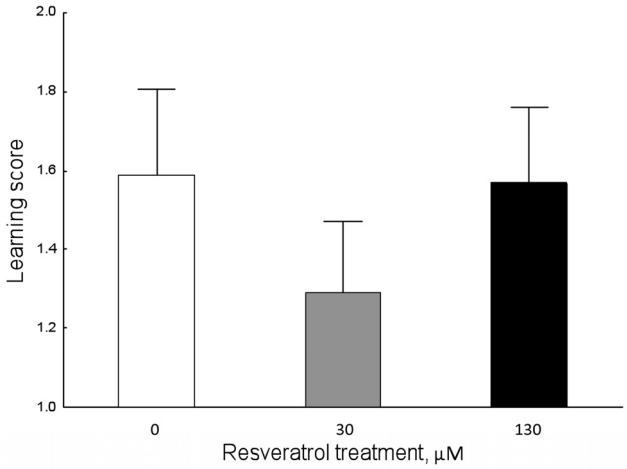


Figure 1. Resveratrol does not affect learning performance in 9-day-old honey bees in normoxic conditions (Kruskal-Wallis ANOVA: H (2, N= 350) =1.529602 P =0.465). Data shown as mean±SE.

Gustatory responsiveness

Resveratrol significantly influenced gustatory responsiveness (Figure 2; Kruskall-Wallis: H (2, 566)=11.363, N=578, P=0.003). The gustatory responsiveness of 30 and 130 μM resveratrol-fed individuals significantly differed from controls (Mann-Whitney U test; 0 vs 30 μM: [U=14117, N=168, N=193, P=0.017] 0 vs 130: [U=14061, N=168, N=205, P<0.001]). Resveratrol-supplemented honey bees showed lower gustatory responsiveness scores, while

unsupplemented control animals showed higher scores (Figure 2). There were no significant differences in gustatory responsiveness between 30 and 130 µM resveratrol-fed honey bees (Mann-Whitney U test: U=18847.50, N=193, N=205, P=0.38).

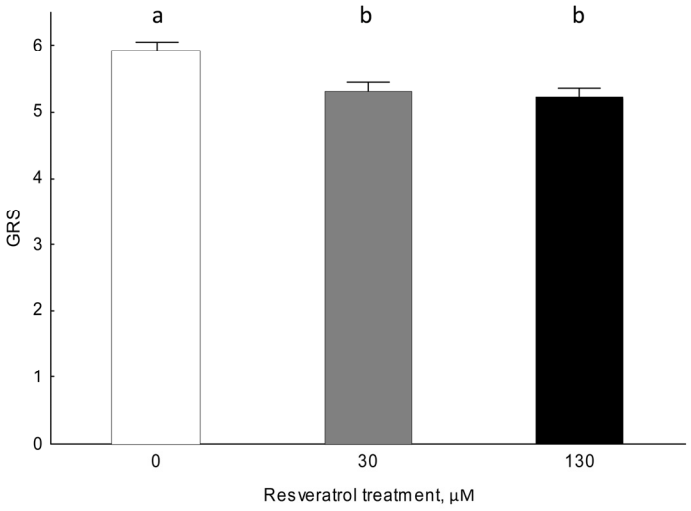


Figure 2. Gustatory responsiveness is altered by resveratrol in 9-day-old honey bees in normoxic conditions (Kruskall-Wallis ANOVA: H (2, 566)=11.363, N=578, P=0.003). Data shown as mean±SE. Identical letters indicate that groups are not significantly different from one another at an alpha significance level of 5% for the Mann-Whitney U test.

Effect of resveratrol on honey bee lifespan and food consumption

Honey bees reared in hyperoxia compared to normoxic controls showed decreased survival rates regardless of drug dosage (Figure 3; Cox's F-test: F(328, 238)=2.69, P<0.01). However, under normal oxygen conditions, resveratrol significantly lengthened lifespan in honey bees (Figure 4; Multi-group survival test: $\chi 2$ =11.72, df=2, N=130, P=0.003). The two resveratrol treatments of 30 and 130 µM increased average lifespan by 38% and 33%, respectively (Figure 4). Both resveratrol treatments also lengthened maximum and median lifespan. Hyperoxia abolished the life extension effect of resveratrol previously seen in normoxic controls (Figure 5; Multi-group survival test: $\chi 2$ =4.08, df=2, N=60, P=0.130).

Food consumption

Resveratrol affected food consumption compared to controls (Figure 6; Mann-Whitney: U=775, N1=49, N2=46, P=0.008). Resveratrol-supplemented honey bees significantly reduced their food consumption, compared to unsupplemented individuals.

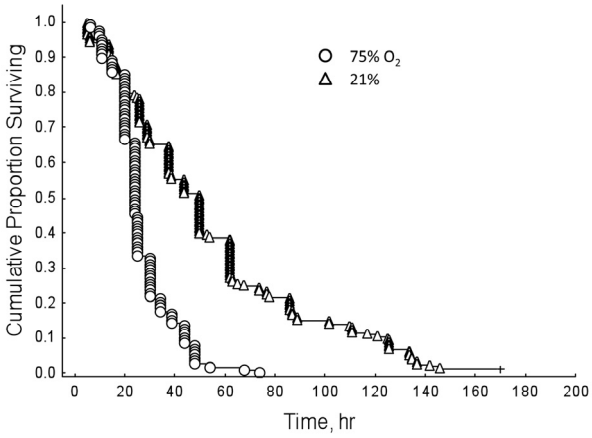


Figure 3. Honey bees show decreased survival rates in hyperoxia regardless of resveratrol dosage, compared to normoxic controls (Cox's F-test: F(328, 238)=2.69, P<0.01).

CONCLUSIONS & DISCUSSION

Resveratrol and lifespan

Prior studies on yeast, worms, and fruit flies [2, 4] (Figure 2) have demonstrated a life-prolonging effect of resveratrol. However, the resveratrol-dependent lifespan effects in yeast, flies, and worms have been called into question by the experimental results of some studies [5, 6]. Nevertheless, several other inquires have provided support for the original findings of Howitz et al and Wood et al [20-23]. Our results in the honey bee reveal that resveratrol enhances lifespan under normal oxygen conditions and are thus consistent with the original reports of Howitz et al. and Wood et al.

Resveratrol is purported to function as a signaling molecule [24]. Various studies also show resveratrol may act as an antioxidant [13, 25, 26] or a pro-oxidant DNA mutagen [25, 27, 28]. In the present study, hyperoxia abolished the lifespan extension effect of resveratrol seen under normoxic conditions and failed to act as a compensatory agent in restoring lifespan to previous lengths seen in ambient oxygen (Figure 3). That said, the tension of hyperoxia (75% O2) we used was near pure oxygen, which is known to induce an array of deleterious physiological responses in

Drosophila, including increased levels of protein carbonyl formation [29], nervous system destruction [30, 31], and neurodegeneration [32]. Furthermore, resveratrol is susceptible to *in vitro* oxidation under normal oxygen conditions [33], so it is possible that the hyperoxygenated environment used in these experiments altered the functionality of resveratrol enough to prevent lifespan extension. Lastly, the possibility that stress may account for the lack of a resveratrol-dependent lifespan extension in other studies is worth mentioning. However, more replicates in animal models would be necessary to confirm this speculation.

Neurophysiological effects of resveratrol

Several studies in neurons, cells, and a short-lived fish species have demonstrated that resveratrol and some of its derivatives can elicit neuroprotective effects [3, 9-12] (Figure 4). In the fish, *Nothobranchius furzeri*, resveratrol delayed the onset of locomotory and learning performance decline during aging [3]. Furthermore, resveratrol increased the activity of antioxidants and decreased oxidative stress in rat brains [13]. Resveratrol can also preserve brain function in neurologically impaired rats [15, 16]. But it is unknown whether resveratrol can improve learning performance in healthy animals. As a result, our aim was to examine this question in wild-type worker honey bees. We hypothesized that resveratrol would enhance learning performance in the honey bee.

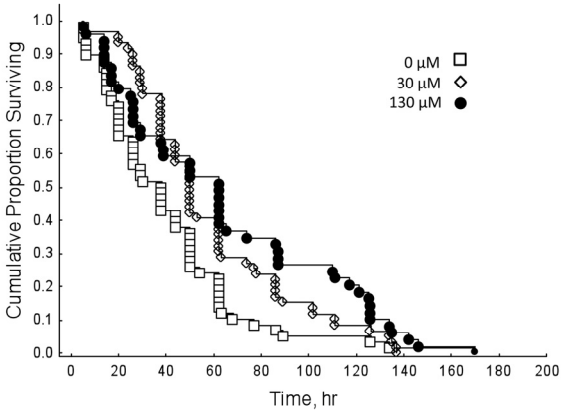


Figure 4. Under normal oxygen conditions, resveratrol extends lifespan in 9-day-old honey bees (Multi-group survival test: $\chi 2 = 11.72$, df=2, N=130, P<0.01). Both 30 and 130 µM resveratrol treatments increased average lifespan in wild-type honey bees by 38% and 33%, respectively. Resveratrol treatments also lengthened maximum and median lifespan. Differences considered significant if $P < 0.05$.

In contrast to our hypothesis, resveratrol did not improve the learning performance of honey bees in a significant way. However, resveratrol treatment did significantly change the gustatory responsiveness score (Figure 5). Unsupplemented honey bees exhibited greater responsiveness to sugar during this test, while animals supplemented with resveratrol were less responsive to sugar. This finding is particularly interesting because observations in natural settings reveal that gustatory responsiveness remains intact throughout aging in the honey bee, a response we previously replicated in the laboratory using hyperoxia (Rascón et al., in progress).

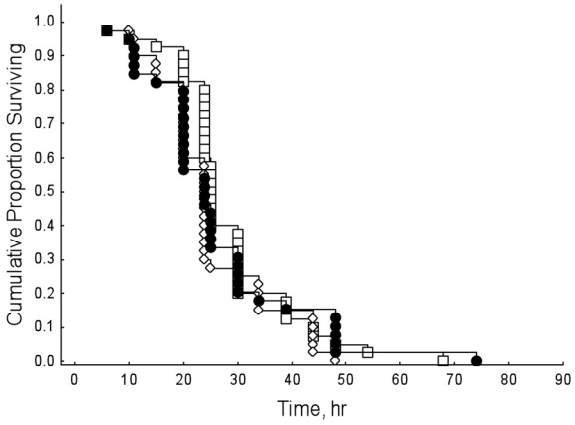


Figure 5. The life-lengthening effect of resveratrol, previously seen in normoxia, is abolished under sub-optimal conditions of hyperoxia (Multi-group survival test: $\chi 2 = 4.08$, df=2, N=60, P=0.130).

We hypothesized that an altered gustatory response score could indicate that resveratrol was eliciting a satiety effect on honey bees. This prompted us to measure individual food consumption in resveratrol-supplemented and unsupplemented subjects.

Effects of resveratrol on food consumption

Resveratrol is thought to slow aging by mechanisms that may be related to caloric restriction. Caloric restriction is widely known to increase lifespan across organisms, as well as prevent the onset of diseases associated with old age [1]. Several studies in diverse organisms indicate that sirtuins may facilitate the effects of caloric restriction [34-36]. Added to this, the lifespan extension effect of resveratrol appears to depend on the

activation of sirtuins [37], which play known roles in energy metabolism [38-40]. Most [6, 35, 41, 42], but not all studies [43], demonstrate that the overexpression of Sir2 homologs extends lifespan. Notably, the overexpression of SIRT1 in mice produces phenotypes reminiscent of caloric restriction [44]. A recent bioinformatics study that compared the gene expression profiles of species subjected to caloric restriction, Sir2 overexpression, and resveratrol administration discovered that 23 genes involved in stress, metabolism, and growth were conserved in response to caloric restriction and resveratrol [45]. This suggests that resveratrol supplementation and caloric restriction share some common molecular responses.

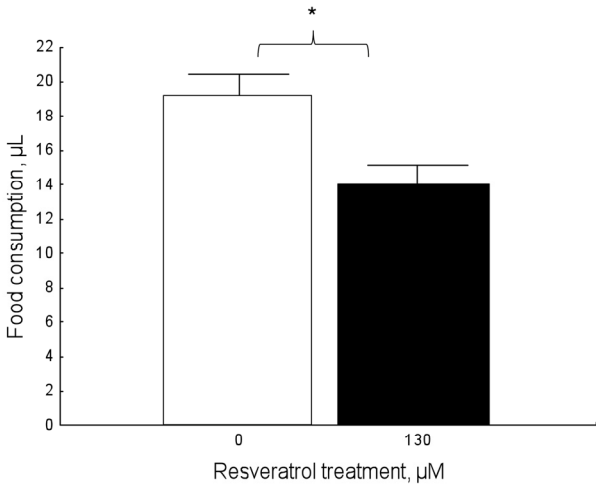


Figure 6. Resveratrol affects food consumption in a dose-dependent fashion in 9-day-old honey bees in normoxic conditions (Mann-Whitney: U=775, N1=49, N2=46, P=0.008). Data shown as mean±SE. The star denotes significant differences between the groups.

In the present study, resveratrol-supplemented individuals showed a significant reduction in food consumption under *ad libitum* feeding conditions, compared to unsupplemented controls, which consumed more food (Figure 6). To the best of our knowledge, this has never been demonstrated for the honey bee, but our results are consistent with a study on mouse lemurs in which resveratrol decreased food intake by increasing satiety [46]. Furthermore, mice with an additional copy of SIRT1 exhibit decreased food intake [47] and hypothalamic SIRT1 seems to regulate food consumption in rats [48]. Resveratrol is a well-known activator of SIRT1, which is thought to mediate the beneficial effects of caloric restriction [7, 8, 49], so it is possible that resveratrol may be activating SIRT1 in the honey bee and influencing food consumption. Lastly, it should be noted that the decrease in food consumption in honey bees is not likely related to factors of palatability since honey bees showed no adverse behavior to resveratrol in prior trials.

Resveratrol, sirtuins, and the epigenome

The environmental conditions of early life, particularly nutrition, leave consequential signatures on the epigenome that can span across generations [50-52]. In the honey bee, differences in nutrition during development lead to the formation of distinct castes in genetically identical individuals. These differences in nutritional input in the honey bee can be traced to changes in DNA methylation [53]. Resveratrol is a nutritional supplement and an activator of SIRT1, a histone deacetylase [2]. SIRT1 can influence DNA methylation in mammalian cells [54]. In mammals, aging is associated with a loss of DNA methylation and declining transcriptional control of methyltransferases [55, 56]. This loss of DNA methylation during aging may contribute to the signature genomic instability and shortened telomeres that characterize aging cells [57, 58]. Recently, a study demonstrated that SIRT1 represses a large set of genes in the mouse genome and promotes repair of DNA strand breaks [59]. This particular study also determined that increased SIRT1 promotes survival in the mouse and reduces transcriptional abnormalities associated with aging. Thus, it is possible that resveratrol and its subsequent activation of SIRT1 may promote genomic stability and a delay in aging via similar mechanisms.

Resveratrol, sirtuins, and the TOR network

In addition to activating sirtuins, resveratrol has been shown to inhibit several members of the Target of Rapamycin (TOR) network in mammals, e.g. AMP-activated protein kinase (AMPK), Phosphatidylinositol 3-kinase (PI3K), and Mitogen-activated protein kinase (MAPK) [17, 60-62]. Suppression of TOR activity is known to slow aging in yeast, worms, and flies [6, 63-65]. Notably, rapamycin also extends the lifespan of fruit flies and mice [66, 67]. A recent study of mammalian cells demonstrated that resveratrol preserved their proliferative capacity and inhibited S6 kinase phosphorylation, thereby eliciting an indirect repression of mTOR activity [68]. This result is similar to the effect of rapamycin on mTOR in cells [69]. A separate cellular study revealed that resveratrol blocks autophagy via the inhibition of S6 kinase under nutrient-limited conditions [70]. In contrast, cells grown in nutrient-rich media supplemented with resveratrol

showed an increase in autophagy [70]. Furthermore, the negative regulation of S6 kinase homologs produces anti-aging effects in yeast [71-73] and fruit flies [64, 74]. It is possible that sirtuins and TOR may be part of the same caloric restriction pathway in mammals since both caloric restriction and TOR inhibition lead to increased expression of a key sirtuin regulator [75].

The molecular connections between resveratrol, sirtuins, the epigenome, and TOR remain unexplored in the honey bee. However, if some of the aforementioned links are conserved in this species, they may explain some of the organismal-level changes observed in this study.

Conclusion

In summary, we demonstrated that resveratrol significantly affected gustatory responsiveness and prolonged lifespan in wild-type honey bees under normal oxygen conditions. However, the enhanced lifespan effect of resveratrol was abolished under hyperoxic conditions. Moreover, resveratrol had a satiety effect on honey bees and reduced their food consumption. These findings support the hypothesis that the lifespan extension effects of resveratrol are evolutionarily conserved.

Future work

Our subsequent projects in honey bees will focus on using pharmacological agents to explore whether there is a SIRT1-dependence for the lifespan and neuro-physiological effects noted here.

MATERIALS AND METHODS

Sample collection scheme and honey bees. Experiments were performed at Arizona State University in Tempe, AZ, USA. We utilized four genetically diverse wild type stock colonies (*Apis mellifera*) headed by queens of Californian commercial origin that were mated with multiple males. The colonies each had a single queen and several thousand workers. Each queen was caged onto a comb and allowed to lay eggs over a period of one day, with an additional day for proper acclimatization. This procedure made brood easy to track temporally and spatially.

To ensure robust experimental replication and manageable workloads, we employed a staggered honey bee collection scheme, which was repeated every week for a total of five weeks. This experimental design allowed for a predictable supply of age-matched newly emerged bees three times per week every Thursday, Friday, and Saturday beginning calendar week 44 and ending week 49. To collect newly emerged bees (0-24 h old), brood combs were placed in an incubator overnight at 34°C in a relative humidity of 65-70%. Upon emergence the following morning, bees were marked on the thorax with a designated paint color (Testors, Rockford, Illinois, USA) for identification and placed in a host colony. After four days, marked honey bees were recaptured and taken into the laboratory. We reasoned that newly emerged bees— which cannot feed themselves—should remain in the colony for the first days of life before transfer to a laboratory setting so that they could procure essential social provisions [76, 77].

In-lab processing. We captured four-day-old honey bees at 9 AM every Monday, Tuesday, and Wednesday and then placed individuals into 7.0 x 3.5 x 3.5 cm plastic tubes. Then, we brought the honey bees to the laboratory (< 5 min transit time) where they were incubated at 4°C until movement was reduced. Next, we placed bees into wire mesh cages in groups of 30 and randomly assigned them to 0, 30, or 130 μM resveratrol treatment. Honey bees were fed and maintained in cages for five days until they reached the age of nine days. In the cages, honey bees had *ad libitum* access to water and a pollen-sucrose diet. Subsequently, we placed the cages containing treatment animals in an incubator that maintained optimal environmental conditions of 34°C and 65-75% relative humidity. Diets were freshly prepared each day for all treatment groups. After spending five days in cages, we tested each nine-day-old honey bee cohort (3 cohorts per week) for gustatory responsiveness and olfactory learning performance every Saturday, Sunday, and Monday for five weeks. All sensory and gustatory tests were performed on honey bees that had only experienced normoxia. After the completion of sensory and learning tests, we placed honey bees in either a hyperoxygenated (75% O2) or a normoxic (21% O2) environment, and then measured survivorship.

Diet preparation. We prepared a liquefied diet of protein and carbohydrates consisting of 1.5 g of freshly ground pollen per 30 mL of 30% sucrose solution. Batches of this mixture were stored in frozen aliquots of 25 ml and thawed daily upon use. We solubilized resveratrol— which was kept in the dark at -20 ˚C— in molecular grade ethanol and added it to the pollen-sucrose diet (1:1000 dilution of resveratrol to liquid diet) daily. The daily addition of fresh resveratrol to the diet ensured optimal activity of the drug since it is known that resveratrol degrades in food medium after 24 h in 37˚ C [33]. Three concentrations of resveratrol (0, 30, 130 μM) were chosen as the basis for our treatment groups based on in vitro activation levels for *Drosophila*. The

resveratrol we utilized is routinely tested for activity. We began administering resveratrol when honey bees were four-days-old because resveratrol works best when fed during early adulthood [4].

Sensory and learning test preparation. Following a five-day resveratrol treatment in cages, approximately 30 bees per resveratrol group/day (3 days per week, 5 weeks total) aged nine days were prepared for sensory and learning performance tests. To ease handling and placement of bees into individual tubes, they were cooled in 4°C until reduced movement was detected. Honey bees were then restrained in small polyacryl holders using strips of duct tape. As with all laboratory tests, test bees were randomized so that experimenters were blind to treatment identity. Thereafter, bees were fed 2 µl of 30% sucrose solution and were placed in an incubator for a starvation period of two hours. The incubator maintained atmospheric oxygen at normoxic levels.

Gustatory response measurements. To measure gustatory responsiveness, we utilized the proboscis extension response (PER). The criterion for a positive PER was complete extension of the proboscis. Nine-day-old honey bees under resveratrol or control treatment were stimulated over the antennae with water and six subsequent sucrose solutions in the following order: 0.1, 0.3, 1, 3, 10, 30%. We adhered to an inter-stimulus interval of two minutes to prevent sensitization and habituation. An overall index of performance, or gustatory response score (GRS), was calculated for all tested bees by using the sum of all PER to seven different stimuli (water and six sucrose solutions). A honey bee with a total score of 7 showed the highest level of sensory responsiveness, while a zero score indicated no responsiveness. Bees that failed to respond to the 30% sucrose stimulus were not included in the olfactory conditioning trials as this sucrose concentration was used as a reward in subsequent learning trials.

Olfactory conditioning. In an effort to examine the whether resveratrol could enhance or improve brain function, we tested learning performance using a reward-based olfactory conditioning paradigm which we have previously published (Rascón & Amdam, in progress). Briefly, this method involves pairing an odor with a sucrose reward over six trials of conditioning to test associative learning performance. After each conditioning trial, we scored PER as binary variable via PER (i.e., response or no response). Once all conditioning trials took place, we tested the honey bees for odor generalization by presenting them with the unconditioned stimulus, cineole. This allowed us to test the bee's discrimination ability. Subsequently, we calculated a learning acquisition score based on conditioned responses. The score, with a numerical value between 0 and 5, was based on 5 conditioning trials and an additional trial that tested reaction spontaneity.

Hyperoxic stress exposure. Oxygen exposure has been shown to impair honey bees faster and in a more controlled fashion than free-flight recapture setups. On their ninth day of life following gustatory and learning performance tests, honey bees were maintained in an incubator (HERAcell O_2/CO_2, Thermo Scientific) under an enriched oxygen atmosphere (75% O_2), 34°C, and a relative humidity of 63±2%. Relative humidity was monitored by Hobo data loggers (Onset, MA, USA). Bees were individually housed in 1.5 mL Eppendorf tubes, each outfitted with a feeding port, breathing hole, and an opening for waste and defecation, as previously described [78]. Honey bees were fed 25 µL of the aforementioned protein-carbohydrate-resveratrol diet and were allowed to feed *ad libitum* through an easily accessible food-containing pipette tip. Feeding was verified to prevent starvation and/or caloric restriction, and thereby minimize survivorship effects not associated with treatment. Honey bees were continually fed their respective resveratrol diet mixtures until death.

Survivorship measurements. The survivorship assay began when honey bees were nine-days-old and while maintained in either hyperoxia or normoxia. Survivorship was scored at four to five times per day until all subjects were dead. During these monitoring periods, bees were either observed dead or alive, and remaining live bees were transferred to fresh tubes in order to prevent bacterial and/or fungal growth. Individuals that appeared to have died due to accident (e.g. killed during routine transfers) were not included in the data analysis. Individual life spans were calculated using the frequency of bees alive at each temporal observation.

Food consumption measurements. We measured individual food consumption in honey bees that were reared on ad libitum diets of protein-carbohydrate-resveratrol. Following an administration of 0 or 130 µM resveratrol treatment for five days, we prepared 50 nine-day-old honey bees per group for food consumption measurements, exactly as noted above for sensory and learning tests. After a starvation period of two hours, bees were allowed to feed until satiation. Diets were administered one microliter at a time and in this way we were able to quantify food consumption on an individual-level basis.

Statistical analyses. Our gustatory responsiveness and learning performance data were non-normal as determined by normal probability plots, so we used non-parametric statistics for analysis. We used the Kruskal-Wallis ANOVA to assess overall treatment effects, and the Mann-Whitney U test as a *post hoc* test to examine differences between the groups. For analysis of survivorship data, we used Cox's F-test and the multi-sample survival test to verify longevity differences between treatment groups. The Kaplan–Meier estimator was employed to approximate the survival function for honey bee populations. Quantitative differences were considered statistically significant if alpha values were less than 0.05. All statistical analyses were carried out using *STATISTICA* 7.0 (Statsoft) and graphs were generated using R statistical software, version 2.12.1 (www.r-project.org).

ACKNOWLEDGEMENTS

We thank Erin Fennern and Nick Baker for their dedicated assistance; Osman Kaftanoglu for help with bees, Brian Smith for providing access to his lab, and Florian Wolschin for his helpful comments. BR and GVA were supported by the Research Council of Norway (180504), BPH by a NSERC PGS-D fellowship, and DAS by grants from the National Institute on Aging (RO1AG19719, AG028730, and AG027916) and The Glenn Foundation for Medical Research.

CONFLICT OF INTERESTS STATEMENT

The authors of this manuscript have no conflict of interest to declare.

REFERENCES

1. Arking R. Altering Aging: Interventions That Modify Longevity and Aging. In: Arking R, ed. The Biology of Aging: Observations and Principles. (New York: Oxford University Press), 2006; pp. 202-236.

2. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B and Sinclair DA. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature. 2003; 425:191-196.

3. Valenzano DR, Terzibasi E, Genade T, Cattaneo A, Domenici L and Cellerino A. Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. Curr Biol. 2006; 16:296-300.

4. Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M and Sinclair D. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature. 2004; 430:686-689.

5. Bass TM, Weinkove D, Houthoofd K, Gems D and Partridge L. Effects of resveratrol on lifespan in Drosophila melanogaster and Caenorhabditis elegans. Mechanisms of ageing and development. 2007; 128:546-552.

6. Kaeberlein M, McDonagh T, Heltweg B, Hixon J, Westman EA, Caldwell SD, Napper A, Curtis R, DiStefano PS, Fields S, Bedalov A and Kennedy BK. Substrate-specific activation of sirtuins by resveratrol. The Journal of biological chemistry. 2005; 280:17038-17045.

7. Feige JN, Lagouge M, Canto C, Strehle A, Houten SM, Milne JC, Lambert PD, Mataki C, Elliott PJ and Auwerx J. Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation. Cell metabolism. 2008; 8:347-358.

8. Smith JJ, Kenney RD, Gagne DJ, Frushour BP, Ladd W, Galonek HL, Israelian K, Song J, Razvadauskaite G, Lynch AV, Carney DP, Johnson RJ, Lavu S, et al. Small molecule activators of SIRT1 replicate signaling pathways triggered by calorie restriction in vivo. BMC systems biology. 2009; 3:31.

9. Bastianetto S, Zheng WH and Quirion R. Neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide-related toxicity in cultured hippocampal neurons. British journal of pharmacology. 2000; 131:711-720.

10. Han YS, Zheng WH, Bastianetto S, Chabot JG and Quirion R. Neuroprotective effects of resveratrol against beta-amyloid-induced neurotoxicity in rat hippocampal neurons: involvement of protein kinase C. British journal of pharmacology. 2004; 141:997-1005.

11. Jang JH and Surh YJ. Protective effect of resveratrol on beta-amyloid-induced oxidative PC12 cell death. Free radical biology & medicine. 2003; 34:1100-1110.

12. Marambaud P, Zhao H and Davies P. Resveratrol promotes clearance of Alzheimer's disease amyloid-beta peptides. The Journal of biological chemistry. 2005; 280:37377-37382.

13. Mokni M, Elkahoui S, Limam F, Amri M and Aouani E. Effect of Resveratrol on Antioxidant Enzyme Activities in the Brain of Healthy Rat. Neurochemical Research. 2007; 32:981-987.

14. Joseph JA, Fisher DR, Cheng V, Rimando AM and Shukitt-Hale B. Cellular and behavioral effects of stilbene resveratrol analogues: implications for reducing the deleterious effects of aging. Journal of agricultural and food chemistry. 2008; 56:10544-10551.

15. Sonmez U, Sonmez A, Erbil G, Tekmen I and Baykara B. Neuroprotective effects of resveratrol against traumatic brain injury in immature rats. Neuroscience letters. 2007; 420:133-137.

16. Jin F, Wu Q, Lu YF, Gong QH and Shi JS. Neuroprotective effect of resveratrol on 6-OHDA-induced Parkinson's disease in rats. European journal of pharmacology. 2008; 600:78-82.

17. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, et al. Resveratrol improves health and survival of mice on a high-calorie diet. Nature. 2006; 444:337-342.

18. Amdam GV and Page RE, Jr. Intergenerational transfers may have decoupled physiological and chronological age in a eusocial insect. Ageing research reviews. 2005; 4:398-408.

19. Kirkwood TB. Evolution of ageing. Nature. 1977; 270:301-304.

20. Bauer JH, Goupil S, Garber GB and Helfand SL. An accelerated assay for the identification of lifespan-extending interventions in Drosophila melanogaster. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101:12980-12985.

21. Gruber J, Tang SY and Halliwell B. Evidence for a trade-off between survival and fitness caused by resveratrol treatment of

Caenorhabditis elegans. Annals of the New York Academy of Sciences. 2007; 1100:530-542.
22. Jarolim S, Millen J, Heeren G, Laun P, Goldfarb DS and Breitenbach M. A novel assay for replicative lifespan in Saccharomyces cerevisiae. FEMS yeast research. 2004; 5:169-177.
23. Viswanathan M, Kim SK, Berdichevsky A and Guarente L. A role for SIR-2.1 regulation of ER stress response genes in determining C. elegans life span. Developmental cell. 2005; 9:605-615.
24. Howitz KT and Sinclair DA. Xenohormesis: sensing the chemical cues of other species. Cell. 2008; 133:387-391.
25. de la Lastra CA and Villegas I. Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. Biochemical Society transactions. 2007; 35:1156-1160.
26. Mahal H and Mukherjee T. Scavenging of reactive oxygen radicals by resveratrol: antioxidant effect. Research on Chemical Intermediates. 2006; 32:59-71.
27. Ahmad A, Syed FA, Singh S and Hadi SM. Prooxidant activity of resveratrol in the presence of copper ions: Mutagenicity in plasmid DNA. Toxicology Letters. 2005; 159:1-12.
28. Zheng L-F, Wei Q-Y, Cai Y-J, Fang J-G, Zhou B, Yang L and Liu Z-L. DNA damage induced by resveratrol and its synthetic analogues in the presence of Cu (II) ions: Mechanism and structure-activity relationship. Free Radical Biology and Medicine. 2006; 41:1807-1816.
29. Rascon B and Harrison JF. Lifespan and oxidative stress show a non-linear response to atmospheric oxygen in Drosophila. The Journal of experimental biology. 2011; 213:3441-3448.
30. Kloek GP. Survivorship of drosophila in nitrogen reduced hypobaric atmospheres. Aviation, space, and environmental medicine. 1978; 49:720-721.
31. Miquel J, Lundgren PR and Bensch KG. Effects of exygen-nitrogen (1:1) at 760 Torr on the life span and fine structure of Drosophila melanogaster. Mechanisms of ageing and development. 1975; 4:41-57.
32. Gruenewald C, Botella JA, Bayersdorfer F, Navarro JA and Schneuwly S. Hyperoxia-induced neurodegeneration as a tool to identify neuroprotective genes in Drosophila melanogaster. Free radical biology & medicine. 2009; 46:1668-1676.
33. Yang NC, Lee CH and Song TY. Evaluation of resveratrol oxidation in vitro and the crucial role of bicarbonate ions. Bioscience, biotechnology, and biochemistry. 2010; 74:63-68.
34. Lin SJ, Defossez PA and Guarente L. Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. Science New York, NY. 2000; 2892126-2128.
35. Rogina B and Helfand SL. Sir2 mediates longevity in the fly through a pathway related to calorie restriction. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101:15998-16003.
36. Wang C, Chen L, Hou X, Li Z, Kabra N, Ma Y, Nemoto S, Finkel T, Gu W, Cress WD and Chen J. Interactions between E2F1 and SirT1 regulate apoptotic response to DNA damage. Nature cell biology. 2006; 8:1025-1031.
37. Frye RA. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochemical and biophysical research communications. 2000; 273:793-798.
38. Boily G, Seifert EL, Bevilacqua L, He XH, Sabourin G, Estey C, Moffat C, Crawford S, Saliba S, Jardine K, Xuan J, Evans M, Harper ME, et al. SirT1 regulates energy metabolism and response to caloric restriction in mice. PloS one. 2008; 3:e1759.
39. Imai S, Armstrong CM, Kaeberlein M and Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature. 2000; 403:795-800.
40. Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L and Weinberg RA. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell. 2001; 107:149-159.
41. Rizki G, Iwata TN, Li J, Riedel CG, Picard CL, Jan M, Murphy CT and Lee SS. The evolutionarily conserved longevity determinants HCF-1 and SIR-2.1/SIRT1 collaborate to regulate DAF-16/FOXO. PLoS genetics. 2011; 7:e1002235.
42. Tissenbaum HA and Guarente L. Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. Nature. 2001; 410:227-230.
43. Burnett C, Valentini S, Cabreiro F, Goss M, Somogyvari M, Piper MD, Hoddinott M, Sutphin GL, Leko V, McElwee JJ, Vazquez-Manrique RP, Orfila AM, Ackerman D, et al. Absence of effects of Sir2 overexpression on lifespan in C. elegans and Drosophila. Nature. 2011; 477:482-485.
44. Bordone L, Cohen D, Robinson A, Motta MC, van Veen E, Czopik A, Steele AD, Crowe H, Marmor S, Luo J, Gu W and Guarente L. SIRT1 transgenic mice show phenotypes resembling calorie restriction. Aging cell. 2007; 6:759-767.
45. Antosh M, Fox D, Helfand SL, Cooper LN and Neretti N. New comparative genomics approach reveals a conserved health span signature across species. Aging. 2011; 3:576-583.
46. Dal-Pan A, Blanc S and Aujard F. Resveratrol suppresses body mass gain in a seasonal non-human primate model of obesity. BMC physiology. 2010; 10:11.
47. Banks AS, Kon N, Knight C, Matsumoto M, Gutierrez-Juarez R, Rossetti L, Gu W and Accili D. SirT1 gain of function increases energy efficiency and prevents diabetes in mice. Cell metabolism. 2008; 8:333-341.
48. Cakir I, Perello M, Lansari O, Messier NJ, Vaslet CA and Nillni EA. Hypothalamic Sirt1 regulates food intake in a rodent model system. PloS one. 2009; 4:e8322.
49. Price NL, Gomes AP, Ling AJ, Duarte FV, Martin-Montalvo A, North BJ, Agarwal B, Ye L, Ramadori G, Teodoro JS, Hubbard BP, Varela AT, Davis JG, et al. SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. Cell metabolism. 2012; 15:675-690.
50. Dolinoy DC, Huang D and Jirtle RL. Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:13056-13061.
51. Dolinoy DC, Weidman JR, Waterland RA and Jirtle RL. Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. Environmental health perspectives. 2006; 114:567-572.
52. Kaati G, Bygren LO, Pembrey M and Sjostrom M. Transgenerational response to nutrition, early life circumstances and longevity. Eur J Hum Genet. 2007; 15:784-790.
53. Kucharski R, Maleszka J, Foret S and Maleszka R. Nutritional control of reproductive status in honeybees via DNA methylation. Science New York, NY. 2008; 319:1827-1830.
54. O'Hagan HM, Mohammad HP and Baylin SB. Double strand breaks can initiate gene silencing and SIRT1-dependent onset of DNA methylation in an exogenous promoter CpG island. PLoS genetics. 2008; 4:e1000155.
55. Lopatina N, Haskell JF, Andrews LG, Poole JC, Saldanha S and Tollefsbol T. Differential maintenance and de novo methylating

activity by three DNA methyltransferases in aging and immortalized fibroblasts. Journal of cellular biochemistry. 2002; 84:324-334.
56. Casillas MA, Jr., Lopatina N, Andrews LG and Tollefsbol TO. Transcriptional control of the DNA methyltransferases is altered in aging and neoplastically-transformed human fibroblasts. Molecular and cellular biochemistry. 2003; 252:33-43.
57. Eden A, Gaudet F, Waghmare A and Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. Science New York, NY. 2003; 300:455.
58. Gonzalo S, Jaco I, Fraga MF, Chen T, Li E, Esteller M and Blasco MA. DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nature cell biology. 2006; 8:416-424.
59. Oberdoerffer P, Michan S, McVay M, Mostoslavsky R, Vann J, Park SK, Hartlerode A, Stegmuller J, Hafner A, Loerch P, Wright SM, Mills KD, Bonni A, et al. SIRT1 redistribution on chromatin promotes genomic stability but alters gene expression during aging. Cell. 2008; 135:907-918.
60. Cao Z, Fang J, Xia C, Shi X and Jiang BH. trans-3,4,5'-Trihydroxystibene inhibits hypoxia-inducible factor 1alpha and vascular endothelial growth factor expression in human ovarian cancer cells. Clin Cancer Res. 2004; 10:5253-5263.
61. Dasgupta B and Milbrandt J. Resveratrol stimulates AMP kinase activity in neurons. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:7217-7222.
62. Frojdo S, Cozzone D, Vidal H and Pirola L. Resveratrol is a class IA phosphoinositide 3-kinase inhibitor. The Biochemical journal. 2007; 406:511-518.
63. Jia K, Chen D and Riddle DL. The TOR pathway interacts with the insulin signaling pathway to regulate C. elegans larval development, metabolism and life span. Development (Cambridge, England). 2004; 131:3897-3906.
64. Kapahi P, Zid BM, Harper T, Koslover D, Sapin V and Benzer S. Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Curr Biol. 2004; 14:885-890.
65. Powers RW, 3rd, Kaeberlein M, Caldwell SD, Kennedy BK and Fields S. Extension of chronological life span in yeast by decreased TOR pathway signaling. Genes & development. 2006; 20:174-184.
66. Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A and Partridge L. Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. Cell metabolism. 2010; 11:35-46.
67. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009; 460:392-395.
68. Demidenko ZN and Blagosklonny MV. At concentrations that inhibit mTOR, resveratrol suppresses cellular senescence. Cell cycle (Georgetown, Tex. 2009; 8:1901-1904.
69. Demidenko ZN and Blagosklonny MV. Quantifying pharmacologic suppression of cellular senescence: prevention of cellular hypertrophy versus preservation of proliferative potential. Aging. 2009; 1:1008-1016.
70. Armour SM, Baur JA, Hsieh SN, Land-Bracha A, Thomas SM and Sinclair DA. Inhibition of mammalian S6 kinase by resveratrol suppresses autophagy. Aging. 2009; 1:515-528.
71. Longo VD. The Ras and Sch9 pathways regulate stress resistance and longevity. Experimental gerontology. 2003; 38:807-811.
72. Madia F, Gattazzo C, Wei M, Fabrizio P, Burhans WC, Weinberger M, Galbani A, Smith JR, Nguyen C, Huey S, Comai L and Longo VD. Longevity mutation in SCH9 prevents recombination errors and premature genomic instability in a Werner/Bloom model system. The Journal of cell biology. 2008; 180:67-81.
73. Wei M, Fabrizio P, Hu J, Ge H, Cheng C, Li L and Longo VD. Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9. PLoS genetics. 2008; 4:e13.
74. Patel PH and Tamanoi F. Increased Rheb-TOR signaling enhances sensitivity of the whole organism to oxidative stress. Journal of cell science. 2006; 119:4285-4292.
75. Medvedik O, Lamming DW, Kim KD and Sinclair DA. MSN2 and MSN4 link calorie restriction and TOR to sirtuin-mediated lifespan extension in Saccharomyces cerevisiae. PLoS biology. 2007; 5:e261.
76. Crailsheim K. The protein balance of the honey bee worker. Apidologie. 1990; 21:417-429.
77. Naiem E-S, Hrassnigg N and Crailsheim K. Nurse bees support the physiological development of young bees (Apis mellifera L.). J Comp Physiol B 1999; 169:271-279.
78. Amdam GV, Fennern E, Baker N and Rascón B. Honeybee associative learning performance and metabolic stress resilience are positively associated. PloS one. 2010; 5:e9740.

Amendment to Doctoral Dissertation
(Last revised 9 June 2013)

The following errors were found in the completed work.

Paper IV
The article published in the journal *Aging* entitled, “The lifespan extension effects of resveratrol are conserved in the honey bee and may be driven by a mechanism related to caloric restriction,” contains an editorial mistake in the “Correspondence” section. The manuscript erroneously states that I, Brenda Rascón, have a PhD. Here, I declare that on the publishing date of 31 July 2012, I, Brenda Rascón, did not hold a PhD.