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Understanding how honey bee flight and senescence are connected through oxidative stress.

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UNDERSTANDING HOW HONEY BEE FLIGHT AND SENESCENCE ARE CONNECTED THROUGH OXIDATIVE STRESS

by

Joseph William Margotta

Bachelor of Science
King's College
2007

Master of Science
Bloomsburg University of Pennsylvania
2010

A dissertation submitted in partial fulfillment
of the requirements for the

Doctor of Philosophy - Biological Sciences

School of Life Sciences
College of Sciences
The Graduate College

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THE GRADUATE COLLEGE

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Joseph W. Margotta

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May 2014

ABSTRACT

Understanding how honey bee flight and senescence are connected through oxidative stress.

By

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The goal of this dissertation was to exploit the tractability of the honey bee (*Apis mellifera*) to understand how the physiological and cellular mechanisms that determine the onset and duration of senescence are shaped by behavioral development and behavioral intensity. These data reveal how behavior can damage cells and consequently limit lifespan. The honey bee represents the ideal model to address these factors because age, behavior, functional senescence, and lifespan are easily manipulated independently of each other while in its natural environment. I determined if there was a cause-effect relationship between honey bee flight and oxidative stress by comparing damage accrued from intense flight bouts to damage accrued from D-galactose treatment, which is a known proxy of oxidative stress in other insects. Previously, we determined a commonly used method to induce oxidative stress, paraquat, did not induce oxidative damage in honey bees as in other animals. I also experimentally manipulated the duration and intensity of honey bee flight across a range of ages to determine their effects on reactive oxygen species (ROS) accumulation, the associated enzymatic antioxidant protective mechanisms, and gene expression. In bees fed D-galactose, lipid peroxidation (measured by MDA) increased when compared to age-matched bees with high flight experience and negligible flight experience. We then found that a marker of oxidative DNA damage (8-

OHdG) increased in flying bees with high amounts of flight experience. These data suggest flight-induced oxidative stress plays a significant role in functional senescence of foraging honey bees. We also observed an imbalance between pro-oxidants (superoxide and H_2O_2) and anti-oxidants (SOD and catalase) in bees with high amounts of flight experience. Our microarray data indicate the transition from behaviors requiring little to no flight (nursing) to those requiring intense flight (foraging), rather than the amount of previous flight *per se*, has a major effect on gene expression. Following behavioral reversion, gene expression partially reverted, but some aspects of forager expression patterns, such as those for genes involved in immune function, remained. Jointly, these data suggest an epigenetic control and energy balance role in honey bee functional senescence along with an imbalance of pro- to antioxidants**.**

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CHAPTER 1

INTRODUCTION

1.1: Purpose

Understanding the physiological and cellular mechanisms that determine the onset and duration of senescence, and how behavioral development and intensity shape these mechanisms is an important goal. Insight into these mechanisms demonstrates how behavior can damage cells and consequently limit lifespan. The molecular mechanisms associated with cellular damage are well-described in model organisms, such as *Drosophila, C. elegans,* and rodents, but the actual behavioral events leading to cellular damage are poorly understood. Based on numerous studies of long-lived transgenic model organisms, and manipulations of behavioral intensity in the laboratory, senescence occurs when a state of increased oxidative stress and decreased stress resistance occurs, which often arises in an age-dependent manner (Martin and Grotewiel, 2006; Sohal, 2002; Sohal and Buchan, 1981; Sohal and Dubey, 1994; Sohal et al., 1995; Sun and Tower, 1999; Sun et al., 2004; Takahashi et al., 2000; Vieira et al., 2000; Yoon et al., 2002; Yu and Chung, 2006). However, to fully understand how behavioral intensity and physical activity affects an organism's lifespan, it is essential to manipulate these behaviors during an organism's natural life history.

Studies linking variation in lifespan limiting, naturally occurring behaviors to molecular mechanisms involved in senescence are rare, because most model organisms demonstrating lifespan limiting behaviors are not easily maintained and manipulated outside of the laboratory. Common laboratory models used in senescence research have simple lifespan dependent behavioral trajectories, and studies in their natural

environment are difficult. However, studies in free-living alpine swifts (birds) suggest oxidative stress plays a significant role in survival in the wild (Bize et al., 2008). In this dissertation, I manipulated the behavioral development of the honey bee (*Apis mellifera*) during its *natural* life history to examine how age, behavior, activity levels, and oxidative stress influence senescence. Understanding these interactions in context provides insight into factors driving an organism to move more quickly or slowly along its life history trajectory, and shows how behavior itself may damage cells and limit lifespan.

1.2: Oxidative stress and senescence

The goal of this dissertation was to determine if predictions proposed by Harman's (1956) theory of oxidative stress and aging support patterns of senescence in honey bees, which have two distinct stages of metabolic expenditure: nurses, which rarely fly, and thus are lowly metabolic, and foragers, which fly often, and thus are highly metabolic. To understand if my data supports these predictions, I determined the effects of honey bee flight on temporal patterns of antioxidant defenses, gene expression, reactive oxygen species (ROS) accumulation, and resulting damage to macromolecules.

The pace and onset of senescence depends on a multitude of factors including genotype, environmental interactions, and ability to mitigate cellular stress (Hughes and Reynolds, 2005). While there are numerous theories of aging, the most dominant mechanistic theories fit into two categories: "programmed" theories of aging and "error" theories of aging (Weinert and Timaras, 2003). According to the programmed theories, aging is dependent on biological clocks regulating lifespan through gene control of metabolism, growth, development, and homeostatic processes. Conversely, the error

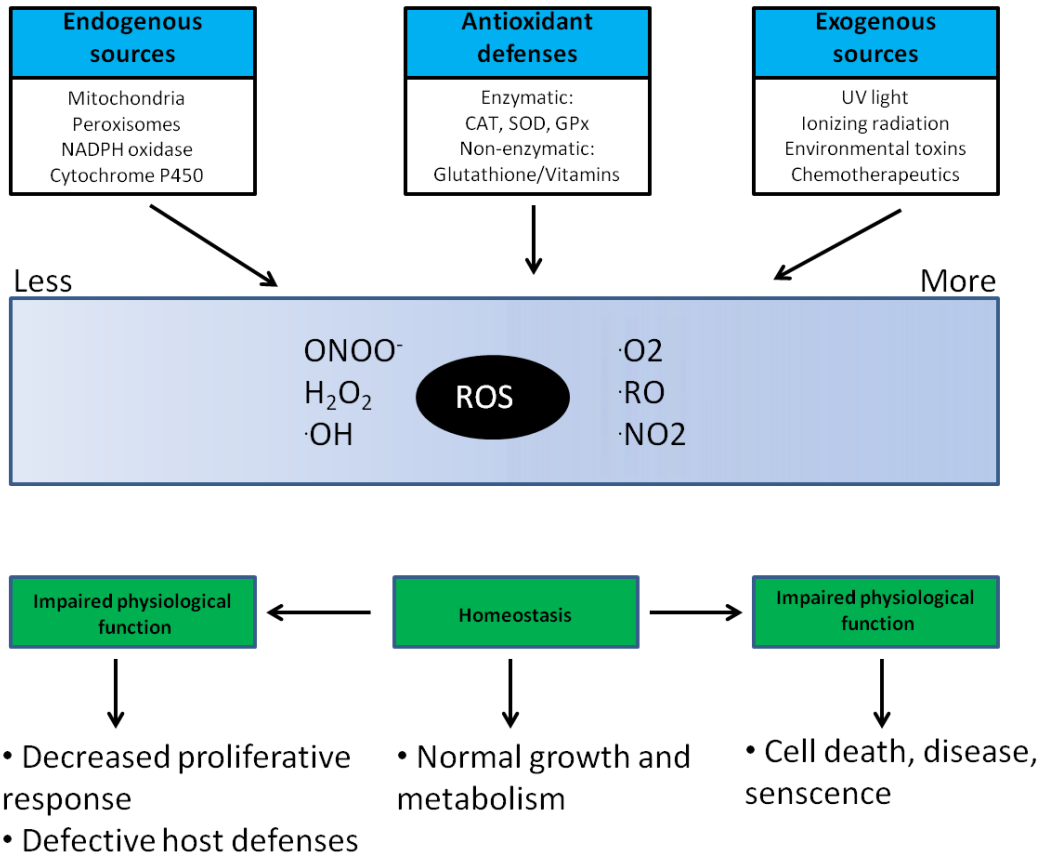
theories suggest aging is the result of accumulation of damage at various levels of biological organization due to environmental interactions (Weinert and Timaras, 2003). Although there is ample evidence for and against the programmed theories of aging, the general consensus is that the error theories play a larger role in aging, (but see Perez et al., 2009). Thus, the focus of this dissertation is on the most widely accepted error theory.

The most prominent of the error theories is the oxidative stress theory of aging, which proposes ROS accumulation through normal cellular metabolism is the foremost cause of aging (Harman, 1956). ROS are produced as normal byproducts of mitochondrial oxygen consumption, and nearly two percent of oxygen reduced by the mitochondria forms reactive oxygen species (Boveris and Chance, 1973). Several studies demonstrate decreased lifespan by knocking out antioxidant genes (Van Raamsdonk and Hekimi, 2009) and increased longevity from overexpression of these genes (Parkes et al., 1998; Sun et al., 2004). Additionally, the ability to overcome cellular damage associated with oxidative stress decreases or occurs more slowly in aged individuals (Butov et al., 2001). Cellular, tissue, or organ senescence thus occurs over time as ROS overwhelm antioxidant protective mechanisms and macromolecule repair systems, leading to cellular damage (Pacifici and Davies, 1991).

Numerous studies demonstrate free radicals are involved in the aging process, thus providing evidence to support Harman's (1956) theory. In rats, superoxide production and lipid peroxidation increase as a function of age (Sawada and Carlson, 1987). Increased production of xanthine oxidase observed with age is likely a source of increased oxidative stress during aging, because xanthine oxidase is a major source of

superoxide (Chung et al., 1999). Superoxide-dependent chemiluminescence is inversely proportionate to lifespan in mice, rats, and pigeons, further suggesting ROS production

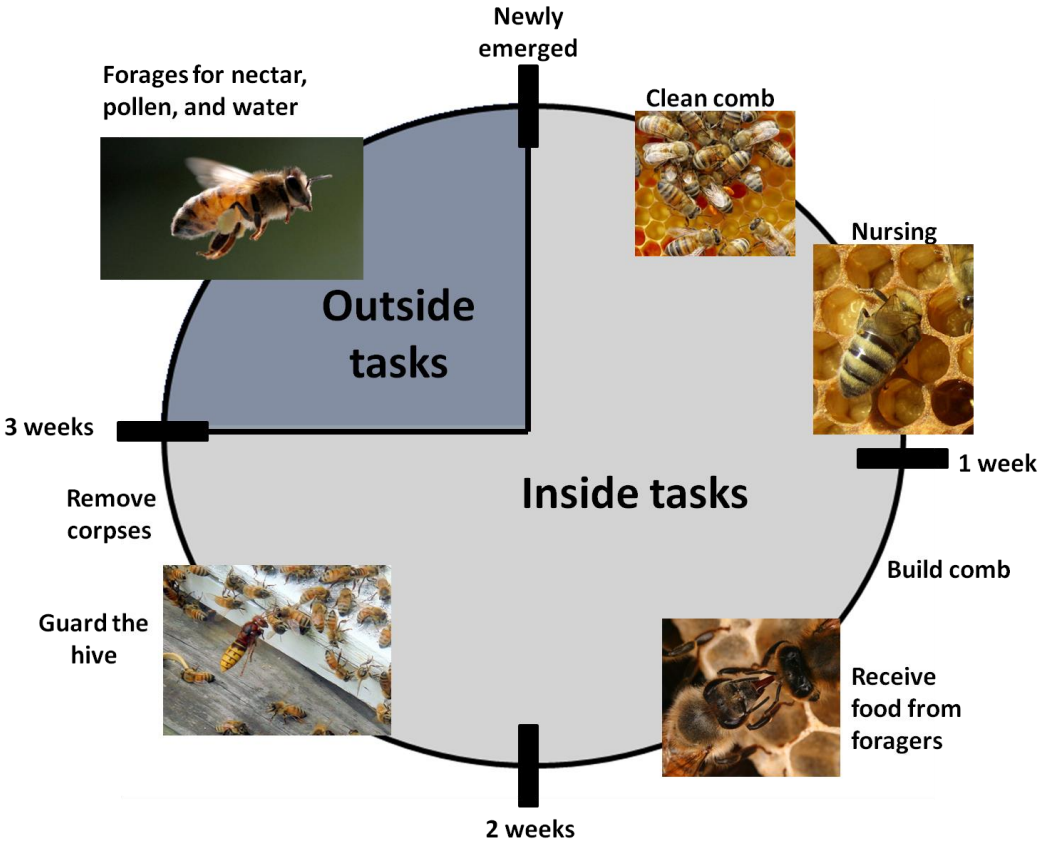
Figure 1.1: Sources and responses to reactive oxygen species. Oxidants are generated as a result of intracellular metabolism, as well as from a variety of cytosolic enzyme systems. Antioxidant systems regulate ROS levels to maintain physiological homeostasis. Lowered ROS levels may impair homeostasis, and increased ROS levels may damage lipids, proteins, or DNA (After Finkel and Holbrook, 2000).



negatively effects lifespan (Sasaki et al., 2008). In *Drosophila*, a multitude of factors including temperature, genotype, and oxidative damage are associated with accelerated aging and senescence (Groteweil et al., 2005; Hughes and Reynolds 2005). Increased resistance to oxidative damage increases longevity in *Drosophila* (Arking et al., 2002; Hughes and Reynolds, 2005; Luckingbell et al., 1984; Patridge and Fowler, 1992), and increased expression of antioxidants such as catalase and superoxide dismutase (Orr and Sohal, 1994). Decreased ROS production also increases lifespan in *Drosophila* (Perez-Campo et al., 1998; Ku and Sohal, 1993). While ample data suggest free radicals cause senescence and aging, most data support a negative correlation between ROS production and lifespan. However, targeted genetic manipulations of antioxidant genes have been less conclusive. For example, in mice, 17 out of 18 genetic mutations of antioxidant defenses had no effect on lifespan, and while SOD and glutathione peroxidase knockout mice had higher levels of oxidative damage and cancer, no effect on lifespan was evident (Jang et al., 2009). In *C. elegans*, single or multiple knockouts of SOD isoforms did not shorten lifespan as expected (Gems and Doonan, 2009; Doonan et al., 2008; Yang and Hekimi, 2007). Knockdown of antioxidant defense systems generally induces greater oxidative damage but unexpectedly had no impact on lifespan. Therefore, the authors of these studies question if Harman's (1956) theory of oxidative stress generalizes into all animals (Perez et al., 2009). Using a highly metabolic and easy-manipulated model system where antioxidant and ROS interactions are likely crucial to lifespan may overcome questions about how ROS accumulation and antioxidant defenses are linked to senescence.

The honey bee system is highly controllable in its natural environment, and many well-established manipulations are available to control behavioral development, age, and activity levels (Huang and Robinson, 1992; Huang and Robinson, 1996; Schulz et al., 1998; Giray and Robinson, 1994). Only a few of these manipulations succeed in flies, worms, or mice and almost none exist while the organism is in its natural environment, outside of the laboratory. Through colony manipulations life history transitions can be hastened, delayed, or completely reversed, which is not possible is any other model system. Therefore, the honey bee is the ideal model to answer the questions of this dissertation.

Figure 1.2: Worker behavioral development. Worker bees move through a series of behaviorally- defined tasks correlated with age termed a "temporal polyethism." Young adult bees perform tasks inside the hive, while older before perform outdoor foraging tasks.



1.3: The honey bee model system

The honey bee undergoes a behavioral development termed "temporal polyethism" in which bees move through a well-defined series of behaviors correlated with age. After developing for approximately 21 days, workers emerge, or eclose, from waxed-capped cells as fully formed adults. During the first 2 to 3 weeks of life, adult worker bees perform tasks inside of the hive such as tending to the queen and "nursing," or caring for developing brood. At 21 to 25 days of age, workers begin foraging tasks outside the hive, which are risky and result in sharply increased mortality (Winston, 1987). During the nurse to forager transition, numerous physiological changes occur that remodel the bee for foraging, including a 40% drop in body weight, reduced innate immunity, altered gene expression, and altered hormonal patterns (Page et al., 2006; Amdam et al., 2009b; Whitfield et al., 2006). Additionally, mushroom body volume and neural connectivity increase, likely to compensate for increased visual input (Withers et al., 1993). During flight bouts, forager bees gather pollen, nectar, and water, which provide nourishment for the entire hive including other worker bees, drones, developing brood, and the queen. Flying honey bees primarily utilize aerobic respiration. Although anaerobic pathways are present, they are of only used in short-term flight situations, such as take-off and predator avoidance (Harrison and Roberts, 2000).

To fully understand how age and behavior (flight activity) affect senescence, it is essential to decouple these two factors. The core manipulation to decouple age and behavior during honey bee behavioral development is the single-cohort colony (SCC) (Giray and Robinson, 1994, Huang and Robinson, 1992). In a normal colony, nurse bees are the youngest bees in the colony, while forager bees are oldest. (Winston, 1987).

However, only one-day old workers start a SCC, therefore the lack of older workers in the hive causes approximately 10% of the bees in the SCC to start foraging precociously at 7-10 days of age (Giray and Robinson, 1994). As a result of this manipulation, foragers and nurses are the same age in a SCC, allowing same-aged bees with different behaviors to be collected. In the first 10 days of the colony, SCCs yield precocious foragers and typical-aged nurses, and after a few weeks typical-aged foragers and over-aged nurses.

1.4: Life history theory and the honey bee

The classic evolutionary theories of aging, including Medawar's mutation accumulation theory (1952), the antagonistic pleiotrophy theory set forth by Williams (1957), and the disposable soma theory (Kirkwood, 1977), all explain why mortality increases with old age. Based on the concept of extrinsic mortality, natural selection will forgo further investment into the soma when risk of dying is high. Conversely, if extrinsic mortality is low, then natural selection favors somatic maintenance and acts to reduce mortality rates.

These theories focus mainly on reproduction, and are generally thought of as the dominant explanations for the evolution of aging. However, honey bee workers are sterile and act as alloparental caregivers, so these theories may be limited in this model organism. Although a single worker gains some indirect fitness through hive maintenance, one worker bee cannot influence survival of the colony (Amdam and Page, 2005). Consequently, a more recent theory (Lee, 2003) integrates social resource transfers with classical evolutionary thought on aging and centers on parental investment and resource transfer at a multitude of ages. This theory assumes the main factor driving selection on mortality in worker bees at any age is the remaining lifetime transfers to be

made to others (Lee, 2003). Lifetime transfers include behaviors that increase productivity and survival of the hive, and physical transfer of resources between bees. Bees transfer physical resources using trophallaxis, which is the transfer of food from the mouths of individual bees. Trophallaxis transfers two main commodities: 1) floral nectar, which is the basis of honey and provides carbohydrates to the hive, and 2) proteinacious jelly, which workers feed to developing bees and is the main source of protein for honey bees. All bees are able to consume and digest nectar and honey passed to them. Because queens are in a state of constant reproduction and cannot perform other activities, sterile workers conduct intergenerational transfer activities such as hygienic behavior, guarding, foraging, and nursing behavior. Selection then favors survival in individuals making larger investments, intergenerational transfers, or individuals that embody a larger investment of resources (hive bees and queens bees) (Lee, 2003).

Many of the nutrients in developing worker bees are recyclable through cannibalism (Woyke, 1977; Webster et al., 1987), but newly eclosed worker bees are a non-consumable investment. Until 5 days post-eclosion, worker bees exclusively consume nutrients, substantial proteinacious jelly and pollen is before becoming a nurse bee (Winston et al., 1987). During typical hive conditions the remaining lifetime transfers available to others increases until day 12, then declines after day 16 as the nurse bee becomes a net donor of worker jelly to younger bees and developing larvae (Winston, 1987). After the nurse to forager transition, foragers become one of the exclusive providers of nutrients to the hive. Because extrinsic mortality sharply increases with each flight, the remaining lifetime transfers of a forager are small (Amdam and Page, 2005). Therefore, selection for survival is weaker in forager bees and newly emerged bees,

which solely consume nutrients, compared to nurse bees. Additionally, selection for longevity in nurses decreases over time because the likelihood of remaining a nurse (where risk of mortality is low) decreases with age and fewer lifetime resource transfers remain (Amdam and Page, 2005). Thus, stage-dependent senescence of workers is the outcome of selection for transfers (Lee, 2003; Amdam and Page, 2005).

Although Lee's theory (2003) fits particularly well with worker honey bee aging, it was not specially formulated around a eusocial insect species and thus has inherent difficulties explaining all aspects of aging in honey bees. The hypothesis that stage-dependent senescence in honey bees is the outcome of selection on transfers does not provide any information on aging in queen or drone bees. Moreover, this hypothesis does not provide a mechanistic basis for honey bee senescence (Amdam and Page, 2005). Another theory that addresses honey bee aging lies in the conceptual framework of the disposable soma theory, which states resources are optimally divided between somatic maintenance and reproduction (Amdam and Omholt 2002). This theory considers individual worker bees as somatic cells in a super-organism and is based on patterns of resource transfers in the super-organism (Amdam and Omholt, 2002). However, these resource transfers are essentially transfers between individual bees of different stages, so this theory may not be as useful to describe patterns of aging as Lee's (2003) theory, which can be used to directly address inter-individual transfers.

1.5: Senescence in the honey bee

The pace of senescence in honey bees differs from other model organisms, because many traditional model organisms irreversibly age as time progresses. In

contrast, honey bee senescence is largely independent of age and more closely associated with social task (Rueppell et al., 2007). During the nursing period, aging is negligible, but after the transition to foraging, senescence accelerates. After foraging for greater than two weeks, mortality nears 100 percent, likely due to predation and senescence (Visscher and Dukas, 1997; Dukas, 2008). Because foragers bring resources into the hive, the timing of functional senescence is an important component to survival of the colony (Dukas, 2008). Honey bee senescence and colony performance are therefore inversely related to the age at which nurse-to-foraging transitions occur (Reuppell et al., 2007; Amdam et al., 2009b).

One of the mechanisms possibly responsible for senescence in honey bee workers is oxidative stress. Forager bees fly several hours per day over long distances (up to 8 km) (Winston, 1987), and to meet the energetic needs of intense flight metabolic rates of forager bees reach 100-120 mL O_2 g^{-1} h^{-1}, which is 10-100 times higher than infrequently flying nurse bees (Suarez et al., 1996). Senescence continually increases in foragers, and after approximately 14 days of foraging, oxidatively damaged proteins accumulate in the brain (Seehuus et al., 2006) and cognitive ability declines (Behrends et al., 2007). The flight capacity of foraging bees decreases with age, which may be partially due to oxidative damage to flight muscles (Vance et al., 2009). In the flight muscle of nurse and forager bees, total antioxidant activity decreases as age increases, but brain total antioxidant activity is independent of age (Williams et al., 2008). Although these data collectively suggest honey bee flight causes oxidative stress, it is unclear whether physiological differences between nurses and foragers and/or time spent flying causes oxidative stress. It is also unknown if the increased metabolic rate associated with foraging is the true source of oxidative stress in foraging honey bees.

The time of the nurse to forager transition, which is a main determinant of lifespan and senescence, varies depending on several factors, including the season. When forced to forgo foraging behaviors, nurse bees can live for more than 130 days (Reuppell et al., 2007), and bees that eclose just before winter, in the absence of nursing activity, survive more than 280 days (Seehuus et al., 2006a). Another aspect of honey bee plasticity is their ability to revert from foraging tasks to in-hive nursing duties (Robinson et al 1992; Huang and Robinson 1996), with an accompanying reversion of many physiological characteristics. Behavioral reversion occurs when colonies are deficient in younger worker bees, which may be due to predation, disease, colony reproduction, or seasonal differences in birth rates (Robinson et al., 1992). Previous studies demonstrate juvenile hormone mediates the behavioral plasticity in worker bees, and worker-worker interactions influence juvenile hormone levels, which are low in nurse bees and high in forager bees (Robinson et al., 1987). The presence or absence of foragers in the hive controls behavioral reversion as well as the initial transition from nurse to forager, demonstrating social interactions control the division of labor in honey bees (Robinson et al., 1992; Huang and Robinson, 1996, Le Conte et al., 2001).

During behavioral reversion, hypopharyngeal glands (which produce the food that young larvae consume and atrophy in foraging bees) redevelop (Amdam et al., 2005; Milojevic, 1940), juvenile hormone levels drop (Robinson et al 1992; Huang and Robinson 1996), vitellogenin levels increase (Amdam et al., 2004b), immunosenescence reverses (Amdam et al., 2005), and age-related learning deficits cease (Baker et al., 2012). However, despite exhibiting many of the physiological traits of typical nurse bees, reverted nurse bees are not identical to typical nurse bees, because reverted nurse bees

have a mixed proteome that is similar to both nurses and foragers (Wolschin and Amdam, 2007b). Additionally, some foragers appear to reach a threshold where they are unable to revert and continue to progress towards functional senescence (Wolschin and Amdam, 2007b). Although much is known about the physiology of reverted nurses, it remains unclear if reverted nurse bees revert at the genomic level or if the effects of extended flight bouts on gene expression are permanent.

1.5: Summary and objectives

The fundamental goal of this dissertation is to exploit tractability in the honey bee model system to decouple age, behavior, and activity levels to understand specific factors that may limit the lifespan of an organism living in its natural environment. Furthermore, because honey bees have the highest mass-specific metabolic rates measured thus far, oxidative stress and the corresponding resistance mechanisms likely play a critical role in functional senescence and longevity. The main objectives of this work are to (a) determine if there is a cause-effect relationship between honey bee flight and oxidative damage, by comparing damage accrued from intense flight bouts to damage accrued from pharmacologic treatment, and (b) experimentally manipulate the duration and intensity of honey bee flight and age to determine their effects on ROS accumulation and the associated enzymatic antioxidant protective mechanisms. These experiments provide a deeper mechanistic understanding of how honey bee flight leads to oxidative stress. Finally, (c) examine how performance of behaviors with low *vs.* high metabolic cost affects gene expression as bees transition to foraging and during reversion from forager to nurse. These data will show how flight affects the transciptome and if these changes are permanent.

In chapter 2, I tested D-galactose to determine if it causes oxidative damage as in other insects. In chapter 3 I demonstrated lipid peroxidation increases with D-galactose treatment indicating a cause and effect relationship between an oxidative insult and oxidative damage in honey bees. I then showed DNA oxidation increases in bees with high amounts of flight experience. Because DNA oxidation increased with high amounts of flight, which is the point flight-induced senescence is likely to occur, these data indicated flight-induced oxidative stress plays a significant role in functional senescence of foraging honey bees. I also demonstrated an imbalance between pro-oxidants and anti-oxidants in bees with high amounts of flight experience. Together these data indicated flight is causative in oxidative damage, and likely contributes to whole-organism senescence.

In chapter 3, to test possible pharmacologic treatments to induce oxidative stress, I determined that abdominal injections of paraquat, an herbicide classically used to induced oxidative damage, produce heterogeneous effects in ROS levels, antioxidant activity, and oxidative damage in both foragers and nurses, and my data showed injections induced a response similar to the paraquat antioxidant response. Based on my data, I concluded paraquat injections are not an effective oxidative stressor in honey bees because of the confounding effect of the injection and possible cytotoxic effects.

The data presented in chapter 4 show that transitioning from behaviors requiring little to no flight (nursing) to those requiring prolonged flight bouts (foraging), rather than the amount of previous flight *per se*, has a major effect on gene expression. Because many of the biological processes and pathways affected by flight were involved in the response to oxidative stress, this provided further evidence that oxidative stress and

functional senescence are intimately connected. Additionally, I demonstrated that following behavioral reversion from forager to nurse, there was a partial reversion in gene expression, but some aspects of forager expression patterns remained.

CHAPTER 2

EFFECTS OF FLIGHT ACTIVITY AND AGE ON OXIDATIVE STRESS IN THE HONEY BEE, APIS MELLIFERA

2.1: Introduction

The exact role of oxidative stress in aging is the subject of great debate (Lapointe and Hekimi, 2010; Sanz and Stefanatos, 2008; Salmon et al., 2010; Constantini, 2008; see Parker, 2010 for social insects). Understanding how naturally-occurring behaviors are linked to lifespan and oxidative stress is an important goal. Reaching this goal may reveal how activity levels are linked to senescence (Metcalf and Alonoso-Alvarez, 2010). Because honey bees (*Apis mellifera*) have the highest mass-specific metabolic rate measured thus far (Suarez et al., 1996), accumulation of reactive oxygen species (ROS) resulting from highly-aerobic flying behavior and the associated antioxidant defenses are likely critically important to honey bee lifespan. Yet, the mechanisms linking life history and behavioral traits to oxidative stress and functional senescence remain unclear (Metcalf and Alonoso-Alvarez., 2010). Understanding the relationship between age, behavioral intensity, senescence, and the accrual of cellular damage may provide mechanistic insight into how behavior can actually damage a cell. Manipulations of behavioral intensity and studies of longevity mutants clearly demonstrate that senescence is due, in part, to age-related increases oxidative stress and decreases in associated protective mechanisms (Golden et al., 2002; Martin and Grotwiel. 2006; Sohal, 2002, Sohal and Buchan, 1981; Sohal and Dubey, 1994; Sohal et al., 1995; Sun and Tower, 1999; Sun et al., 2004; Takahashi et al., 2000; Vieira et al., 2000; Yoon et al., 2002; Yu and Chung, 2006). However, few studies link lifespan-limiting behaviors that occur in an

organism's natural environment to accumulated cellular damage. In this study, we manipulated the onset of high intensity flight behavior and its duration to test the role of behavioral development and behavioral intensity on the accrual of oxidative damage and the balance of pro-oxidants to antioxidants.

Increased metabolic rate is positively associated with production of several highly reactive chemical species including superoxide anions (SO^-), hydroxyl radicals ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2), which react with and damage lipids, proteins and nucleic acids (Boveris et al., 1972; Hulbert et al., 2007). However, increased availability of antioxidants such as superoxide dismutase (SOD) and catalase neutralizes the negative effects of ROS (Orr and Sohal, 1994; Cui et al., 2012). In worms, flies, and mice, increased oxidative stress resistance or decreased ROS production leads to increased longevity (Arking et al., 2002; Hughes and Reynolds, 2005; Luckingbell et al., 1984; Patridge and Fowler, 1992). Overexpression of SOD1 and catalase increases lifespan in *Drosophila*. But, increased antioxidant capacity does not universally extend longevity because ROS may still overwhelm defense systems, especially in organisms that normally have extremely high metabolic rates (Monaghan et al., 2008). Oxidative damage typically occurs when the balance between pro-oxidants and antioxidants is perturbed. However, how this phenomenon is connected to age, behavioral intensity, and senescence in nature remains uncertain (Metcalf and Alonoso-Alvarez, 2010).

Senescence in most organisms tends to increase with age, while honey bee senescence is related to social task and behavioral development (Reuppell et al., 2008), which are determined by the social environment in the hive. During the first 2-3 weeks of life honey bee workers infrequently fly and perform tasks inside the hive, such as hive

maintenance and brood care or "nursing" (Winston, 1987). During the nursing period, senescence is negligible. If the transition to outside foraging tasks is delayed, then longevity increases up to 8-fold (Reuppell, 2007). After transitioning to outside foraging tasks, which occurs near the third week of life, foraging bees leave the hive in search of pollen and nectar flying several hours per day over long distances (up to 8 km) (Winston 1987). To meet intense power requirements of hovering flight, the metabolic rate of forager bees is 100-120 mL O_2 g^{-1} h^{-1}, which is 10-100 times higher than during non-flying behaviors (Suarez et al., 1996). After the transition to foraging, senescence accelerates and combined with increased predation leads to nearly 100% mortality within 14 days (Visscher and Dukas, 1997). Honey bee lifespan is therefore inversely related to the age at which the nurse-to-foraging transition occurs (Reuppell et al., 2008).

Because the performance of forager bees is crucial in the health and survival of the honey bee colony, accelerated senescence of forager bees can decrease the overall performance of the entire colony (Vissher and Dukas, 1997; Dukas, 2008; Vance et al., 2009). Current data suggests oxidative stress plays a role in functional senescence of foraging honey bees, but the precise series of cellular events leading to this phenomenon are not fully understood (Williams et al., 2009). After foragers reach the point of senescence, which occurs after approximately 14 days of foraging, oxidatively damaged proteins accumulate in the brain (Seehuus et al., 2006) and cognitive ability declines (Behrends et al., 2007). The flight capacity of foraging bees decreases with age, which may be partially due to oxidative damage to flight muscle (Vance et al., 2009). In the flight muscle of nurse and forager bees, total antioxidant activity decreases as age increases. In contrast, brain total antioxidant activity is independent of age (Williams et

al., 2008), and the brain appears to be able to mitigate damage more effectively than flight muscle (Margotta et al., 2013). Although data suggest honey bee flight produces high levels of ROS, it is unclear whether differences in age, the physiology of nurses and foragers, and/or amount of flight lead to ROS accumulation and oxidative damage.

In this study, we investigated how honey bee age, flight behavior and senescence are connected by testing the effects of age, behavioral development, and behavioral intensity on the accumulation of oxidative damage to lipids and DNA along with levels of ROS activity and antioxidant activity. We measured markers of oxidative stress in nurses of increasing age, foragers with low, medium, and high amounts of flight experience, and foragers restricted from flight activity. Because previous experiments suggest brain tissue and flight muscle respond differently to cellular stress, we examined both tissues in this study. We found that oxidative damage, ROS accumulation, and antioxidant activity are significantly influenced by the presence of absence of flight activity as well as increased age.

2.2: Methods

2.2.1: Single-cohort colonies

Honey bees for this study were reared at the University of Nevada, Las Vegas apiary. Forager bees with varying amounts of flight experience and age-matched nurse bees were obtained using single-cohort colonies (SCCs), which induce precious foraging by altering colony demography. The SCC decouples the effects of age and behavior, allowing comparison of same-aged bees with drastically different flight histories and bees

of different ages with the same behavioral activity. In this study, 10 SCCs were created using bees from multiple source colonies headed by naturally-mated queens.

Frames of wax comb containing metamorphosing bees from source colonies were placed in an incubator (35°C, 75% relative humidity, 24 h dark cycle), and newly emerged adult bees were removed from the frames every 24 h. SCCs were formed by housing approximately 2000 day-old workers, which emerged over 2 consecutive days, with a naturally-mated queen. These bees were placed in a nucleus colony containing one frame each of pollen and honey and 3 empty frames to allow the queen to lay eggs. The dorsal thorax of each bee was marked with a single dot of paint (Testors, Rockford, IL) to indicate age prior to placing them in their SCC. The SCC was kept in the laboratory for 5 days post-adult emergence to allow for adult maturation and queen egg laying before being moved to the outdoor apiary on the UNLV campus.

2.2.2: Flight experience

Once a SCC was placed outdoors, the colony was observed until the onset of first foraging, which occurred between 8 to 11 days of age in this study. Once a bee was identified as a forager an additional dot of paint was placed on the posterior end of the abdomen. Foragers were marked continuously throughout the day until all foragers returning were consistently marked. This process was continued each day with a new color paint mark until the 14^{th} and 15^{th} days after emergence. Only marked foragers were collected in this study. Table 1 shows which flight groups were used in these analyses.

2.2.3: Flight restriction

We collected 25-35 marked 10 day old foragers, which by this time had <3 days of flight experience, from each colony and restricted them from taking additional flights by placing them in a wire mesh cage pushed into the frame of wax comb, which was then returned to the hive. Care was taken to place foragers in an area of the frame that contained no eggs, larvae, or pupae to prevent the possibility of them reverting to nursing behaviors. To ensure any effects seen were not due to starvation, these foragers were placed in areas of a frame that contained ample food. Moreover, trophallaxis was observed between caged bees and non-caged bees suggesting in the event of their food stores being depleted nest mates will feed caged foragers.

2.2.4: Collections and dissections

Forager bees and nurse bees used for all assays were identified based on behavioral observations (Robinson, 1987). Nurses were identified as bees repeatedly placing their head into cells containing larvae. Foragers were identified as bees returning to the colony with pollen in their corbiculae or a distended abdomen from nectar. To determine production of ROS and MDA levels intact heads and thoraces were assayed immediately after bees were collected off the comb (nurses) or at the entrance of the colony (foragers). Bees used for the DNA damage and enzymatic activity assays were collected and flash-frozen in liquid nitrogen to preserve enzyme integrity and later dissected on dry ice. Brains were carefully dissected from the head capsule to include the optic lobes, antennal lobes and mushroom bodies, but exclude the hypopharyngeal and subesophogeal glands. Thoraces were carefully dissected to remove only flight muscle

and avoid the esophagus and wings. Dissected tissues were kept frozen until the time of assay. Foragers of an unknown age used for the D-galactose lifespan analysis were collected from a typical honey bee colony maintained using standard beekeeping practices. Because the thorax is composed of 95 percent flight muscle (Marden, 1987), in some assays whole thoraces were used instead of solely flight muscles to hasten assays requiring fresh tissue.

2.2.5: D-galactose treatment

To investigate if D-galactose treatment induces oxidative damage and mimics the effects of increased flight activity, the sucrose solution (50% w/v) used to maintain honey bees in the laboratory was supplemented with 10% D-galactose (w/v). Bees were collected from colonies and placed in Lebenfelder cages inside the laboratory (37°C and 75% RH). One set of bees were fed *ad libitum* for 5 days then collected and immediately assayed for MDA content, while another set was used for the lifespan analysis. Due to the dry environment of the Mojave Desert region, water was also given *ad libitum.*

2.2.6: Lifespan analysis

D-galactose treatment decreases lifespan in mice, rats, fruit flies and house flies through oxidative damage resulting from unknown mechanisms (Cui et al., 2004; Xu et al., 1985; Zhang et al., 1990). To determine if D-galactose treatment attenuated forager lifespan, a total of 60 foragers (carrying pollen) were randomly collected (unknown age) at the entrance of a typical honey bee colony. These bees were transferred to Lebenfelder cages and given the sucrose solution (30 bees, 3 cages) or D-galactose solution (30 bees,

3 cages), *ad libitum*. Dead honey bees were counted daily until all D-galactose-fed bees had died, at which point the experiment was discontinued.

2.2.7: Malondialdehyde measurement

Lipid peroxidation was estimated by measuring MDA levels in D-galactose/sucrose fed bees and age-matched nurses, foragers and flight-restricted foragers using a commercial kit for thiobarbituric acid reactive substances (TBARS) (product-10009055, Cayman Chemical, Ann Arbor, MI). Briefly, whole heads or thoraces were homogenized in 250 µl of RIPA buffer. These homogenates were added to a reaction mixture and incubated at 60°C for one hour. Samples were then centrifuged for 1600 *g* for 10 minutes and the absorbance was read at 530 nm in a plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA).

Because we saw no statistically significant differences in MDA content between intermediate-aged (17-18 day old) foragers with free access to flight, flight-restricted foragers, and age-matched nurse bees, we performed a second experiment to assess if lipid damage increases as a function of age. MDA levels were measured in groups of age-matched nurses and foragers up to 40 days of age. For this analysis, dissected brains and flight muscle were used instead of whole heads and thoraces. Levels of MDA in this experiment were measured using a commercially available ELISA (product – Sta-332, Cell Biolabs, San Diego, CA), which has a higher sensitivity for MDA compared to the TBARS assay, to ensure any subtle differences in age-related MDA content would be detected. To determine MDA levels, samples containing 10 µg/ml of protein were added to a 96-well binding plate and incubated at 37°C for two hours, washed, and blocked with

assay diluent. The plate was then washed and incubated with primary and secondary antibodies separately. Following multiple washes the plate was incubated with a color development reagent and the absorbance was read at 450 nm in a plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA). The MDA in each sample was calculated in reference to a MDA curve. The standard curve was generated using MDA/BSA standards.

2.2.8: Measurement of 8-hydroxy-2-deoxyguanosine

To avoid potentially confounding data by changes in fatty acid saturation during flight, we measured a marker of DNA oxidation (8-OHdG). Increased ROS production and/or decreased antioxidant protection forms 8-OHdG leading to age-related impairment (Harman, 1956).

To estimate DNA damage, levels of 8-OHdG were measured in groups foragers and nurses (See Table 1). To control for the effects of flight, free-flight foragers were compared to flight-restricted foragers. To control for physiological differences between nurses workers, foragers were compared to age-matched nurses. DNA was extracted from dissected brains and flight muscle using a DNeasy extraction kit following the manufacturer's instructions (Qiagen, Valencia, CA). 8-OHdG was measured using a commercially available kit (product – 589320, Cayman Chemical, Ann Arbor, MI). Briefly, DNA samples, an antiserum to 8-OHdG, and 8-OHdG linked to an acetylcholine esterase were added to a 96-well binding plate, and incubated at 4°C for 18 hours. After unbound reagents were removed from the plate by washing, the plate was developed using Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) The plate was allowed to

develop for 90 minutes then read at 410 nm in a plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA). The amount of 8-OHdG in each sample was calculated in reference to an 8-OHdG standard curve made from purified 8-OHdG, which also functioned as a positive control. Wells without DNA added were used as negative controls.

2.2.9: Reactive oxygen species measurements

Intracellular ROS generation was measured using 2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (product - C6827, Molecular Probes, Eugene, OR), a chemically reduced form of fluorescein. Upon oxidation by ROS (not incuding superoxide), CM-H_2DCFDA is converted into fluorescent 2', 7'-dichlorofluorescein (DCF). This probe reacts with hydrogen peroxide, hydroxyl radicals, and peroxynitrite. Superoxide production was assessed using MitoSOX (product - M366008**,** Molecular Probes, Eugene, OR), a fluorogenic probe that is rapidly oxidized by superoxide, but not by other ROS generating systems.

Briefly, freshly collected (not frozen) heads and thoraces were homogenized in PBS containing 0.1% Triton X-100 within 20 minutes of collection. Head or thorax (100 µL) homogenate was added to each well (96 well microtiter plate) with 5 µM CM-H_2DCFDA to measure H_2O_2 and $^{\cdot}OH$ or MitoSOX to measure superoxide. To compare samples from multiple microplates, positive controls for each microplate were used. For H_2DCFDA, 100 µM hypoxanthine, 5mU/mL xanthine oxidase, and 0.2 U/mL horseradish peroxidase were used. For MitoSOX, 100 µM hypoxanthine and 5mU/mL xanthine oxidase were used. Negative controls consisted of homogenates with no probe added.

CM-H_2DCFDA samples and negative control were incubated at room temperature for 25 minutes before adding the positive control, which was incubated for an additional 5 minutes at room temperature. MitoSOX samples and controls were incubated at 37°C for 30 minutes. Plates were then read at 485 Ex/535 Em for CM-H_2DCFDA or at 510 Ex/580 Em for MitoSOX on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA).

2.2.10: Glycerol-3-phosphate dehydrogenase activity

GPDH is an enzyme that maintains redox potential across the inner mitochondrial membrane in gylcolysis. In insects, GDPH plays a crucial role in flight performance by cycling NADH to the respiratory chain (Zebe and McShan, 1957). GPDH activity was determined using the method described by Wise and Green (1979). Samples from foragers and nurses were homogenized in a buffer containing 10 mM Tris-HCL (pH 7.4), 0.15 M NaCl and a protease inhibitor cocktail. The assay buffer contained 100 mM triethanolamine, 2.6 mM EDTA, 0.1 mM 2-mercaptoethanol, and 0.120 mM dihydroxyacetone phosphate. The change in absorbance at 340 nm was monitored at 37°C using the cuvette port on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). One unit of enzyme activity corresponds to the oxidation of 1 nmol of NADH/min.

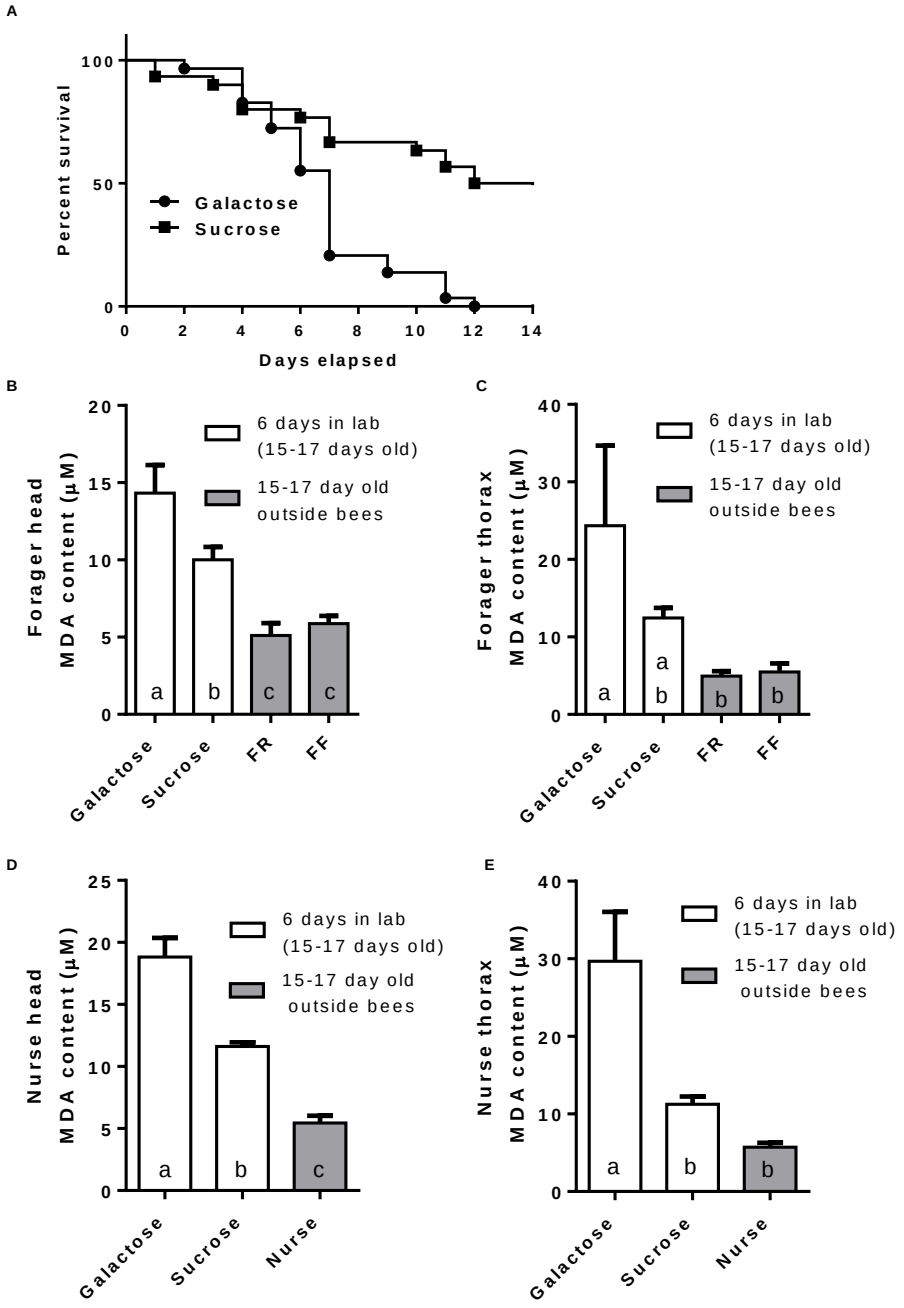
2.2.11: Antioxidant activity

To assess antioxidant protective mechanisms we measured superoxide dismutase and catalase activity. Superoxide dismutase activity was determined using a commercial assay kit (product 706002, Cayman Chemical, Ann Arbor, MI). This enzyme activity assay measures the copper/zinc, iron, and manganese forms of SOD by utilizing a water-

soluble tetrazolium salt (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfo-phenyl)-2*H*-tetrazolium, monosodium salt) (WST-1) to measure superoxide radicals. WST-1 is reduced to a water-soluble formazan dye upon reaction with superoxide anions. SOD calculation for honey bee samples was based on the percent dismutation of superoxide generated by xanthine oxidase and hypoxanthine upon addition of the sample. Samples were prepared by homogenizing individual dissected brains and flight muscle in 500 μL of cold 20 mM HEPES buffer, pH 7.2 containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose. Homogenates were spun at 1500g for 5 minutes at 4°C. Formazan dye absorbance was measured at 450 nm on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). The SOD standard curve functioned as a positive control, and wells without honey bee sample added acted as a negative control.

Catalase activity was measured using a commercially available kit (product - 707002, Cayman Chemical, Ann Arbor, MI). This assay utilizes the peroxidatic function of catalase to react with methanol in the presence of an optimal concentration of hydrogen peroxide to produce formaldehyde. The formaldehyde is then measured spectrophotometrically using purpald (4-amino-3-hydrazino- 5-mercapto-1,2,4-trizazole) as the chromagen. Samples were prepared by homogenizing individual dissected brains and flight muscle in 350 μL of 50 mM potassium phosphate buffer pH 7.2 with 1 mM EDTA. Homogenates were spun at 1000 g for 10 minutes at 4°C. Absorbance was measured at 540 nm on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). The catalase standard curve functioned as a positive control, and wells without honey bee sample added acted as a negative control.

Figure 2.1. D-galactose treatment decreases lifespan and increases MDA content. (A) Circles represent forager bees fed 50% sucrose (w/v) supplemented with 10% D-galactose. Squares represent forager bees fed only 50% sucrose (w/v). MDA was quantified in (B) forager heads, (C) forager thoraces, (D) nurse heads, and (E) nurse thoraces. White bars represent 15-17 day old bees fed 10% D-galactose + 50% sucrose or 50% sucrose for 5 days post capture. Dark bars represent 15-17 day old nurse bees, forager bees, or forager bees that were restricted from taking flights. Bars not connected by the same letters are statistically different. FF= Free-flight; FR = flight-restriction.



2.2.12: Statistical analysis

Statistical analyses were performed in JMP (SAS, Cary, NC), and graphs were prepared in GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). Mixed-model ANOVAs with Tukey's HSD post hoc tests were used to compare the means of experimental groups. Survival curves were prepared in GraphPad Prism 6 and statistical differences between lifespan curves were determined using the Mantel-Cox method. P-values less than 0.05 were considered statistically significant in all statistical tests. Data are represented as mean ± SEM.

2.3: Results

2.3.1: D-galactose treatment decreases forager lifespan and increases MDA content in laboratory-kept nurses and foragers

The mean lifespan of laboratory-caged foragers caged decreased ($p < 0.05$, Mantel-Cox test) when their food was supplemented with 10% D-galactose (Fig. 2.1A) and the median survival time for D-galactose treated bees was 7 days. During the first 5 days of D-galactose treatment, the proportion of bees surviving was within 5% of control sucrose fed bees, but after day 6 the proportion of D-galactose-fed bees alive dropped to 55%, while 77% of sucrose-fed bees were alive. After all of D-galactose treated bees had died the experiment was discontinued, and at this point 50% of the sucrose-only fed bees were still alive.

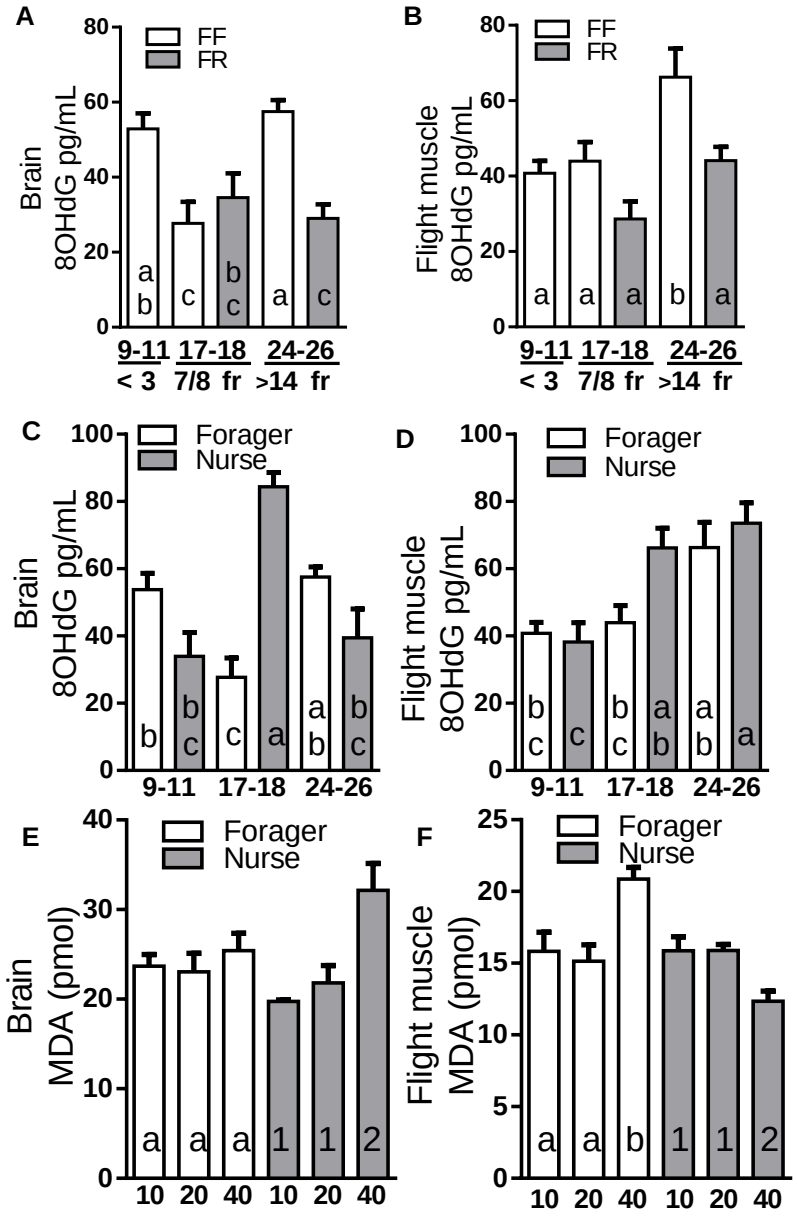
Overall, bees fed D-galactose had higher levels of MDA compared to sucrose fed control bees and age-matched bees collected from SCCs. MDA levels in the heads of caged foragers treated with D-galactose were significantly higher than the MDA levels in

heads of free-flying foragers with 7-9 days of flight experience and age-matched flight-restricted foragers (Fig. 2.1B). MDA levels were higher in the heads of nurse bees fed D-galactose compared to sucrose-fed control bees and age-matched nurses (15-17 days old) collected from a SCC (Fig. 2.1D). Surprisingly, in the heads, *but not the thoraces*, of both nurses and and foragers, MDA levels were elevated in bees kept inside of the laboratory regardless of whether they were fed D-galactose or sucrose only. In forager thoraces, MDA levels were not significantly different in D-galactose fed bees compared to sucrose-fed control bees (Fig. 2.1C), despite a trend in this direction.

Levels of MDA in caged nurse thoraces (Fig. 2.1E) resembled patterns nurse and forager heads, where D-galactose fed nurses showed elevated MDA levels compared to sucrose fed nurses and age-matched nurses collected from a SCC. These MDA data indicate oxidative damage occurs in a tissue-dependent manner and, age has a significant effect at the higher age range of worker bees. In forager brain tissue (Fig. 2.2E), MDA levels were independent of age as there were no statistical differences between 10, 20 or 40 day old foragers.

However, in nurse brain tissue (Fig. 2.2E), MDA was higher in the oldest nurses as MDA levels of 40 day old nurse brains were higher compared to both 10 and 20 day old nurses. Conversely, in the flight muscle of nurses (Fig. 2.2F) the opposite pattern was observed; MDA levels in nurse flight muscle were lower at 40 days of age compared to other ages. MDA levels in 40 day old forager flight muscle were higher compared to 10 and 20 day old foragers (Fig. 2.2F) demonstrating an age-related increase. MDA levels were independent of behavioral group (nurse vs. forager; data not shown) indicating that behavior did not affect lipid peroxidation.

Figure 2.2. Flight activity and age are associated with increased oxidative damage. 8-OHdG was quantified in forager (A) brain tissue and (B) flight muscle. In A and B, white bars represent foragers that had unrestricted access to flight and dark bars represent foragers that were restricted from taking flights after 3 days. The x-axis describes ages and flight experience. Nurse (C) brain tissue and (D) flight muscle were also compared to foragers that had free access to flight. In C and D white bars represent foragers that had unrestricted access to flight and dark bars represent nurse bees. The x-axis represents each group respective age at the time of collection. MDA was quantified in the (E) brain tissue and (F) flight muscle of 10, 20, and 40 day old foragers (white bars) and nurses (dark bars). Bars not connected by the same letter are statistically different from one another. FF= Free-flight; FR = flight-restriction, <3 days of flight.



2.3.2: Forager flight activity and age is associated with increased oxidative damage

High amounts of flight compared to lack of flight (flight-restricted bees), rather than linearly increasing amounts of flight experience, had the largest effect on 8-OHdG accumulation. Aged foragers (24-26 days old) with greater than two weeks of flight experience had higher 8-OHdG levels relative to age-matched flight-restricted foragers (Fig. 2.2A, B); demonstrating flight compared to negligible flight has the largest impact on 8-OHdG levels. In flight muscle, levels of 8-OHdG were significantly higher in aged foragers (24-26 days old) with greater than 14 days flight experience compared to all other groups of foragers, showing a high amount flight is associated with higher 8-OHdG levels.

Nurse bees were compared to age-matched forager bees (only free-flight) to determine the effects of age and behavior on 8-OHdG levels. In nurse brain tissue 8-OHdG levels increased with age (9-11 to 17-18 days old, Fig. 2.2C), but then decreased at the highest age (24-26 days old). In nurse flight muscle, 8-OHdG levels were higher at the oldest age (24-26 days old, Fig. 2.2D). However, in forager bees, 8-OHdG levels did not continue to increase as foragers reached 24-26 days of age. Levels of 8-OHdG were unchanged in nurses compared to foragers with the exception of 17-18 day old nurses, which exhibited elevated 8-OHdG levels relative to same-aged foragers.

2.3.3: Levels of ROS

Levels of ROS, including H_2O_2, ^{-}OH, and $ONOO^{-}$ in forager heads were significantly higher in intermediate aged (17-18 days old) foragers compared to young foragers (9-11 days old) (Fig 2.3A). ROS levels in aged free-flying forager heads (24-26

Figure 2.3. ROS accumulates with flight activity. ROS accumulation associated with flight was measured in the heads (A) and thoraces (B) of different aged (9-11, 17-18, or 24-26 days old) foragers that were allowed free access to flight (white bars: < 3, 7-9, or > 14 days flight experience) or restricted to the hive (dark bars: < 3 days flight experience). ROS accumulation associated with age and behavioral differences in heads (C) and thoraces (D) was measured in forger bees (white bars: 9-11, 17-18, or 24-26 days old) and nurse bees (dark bars: 9-11, 17-18, or 24-26 days old). Values are percentages of positive controls (100 μM hypoxanthine, 5mU/mL xanthine oxidase, and 0.2 U/mL horseradish peroxidase). Bars (mean ± SEM, n=5-7 per bar) with different lowercase letters are significantly different ($p<0.05$, mixed model ANOVA and Tukey's HSD). FF= Free-flight; FR = flight-restriction.

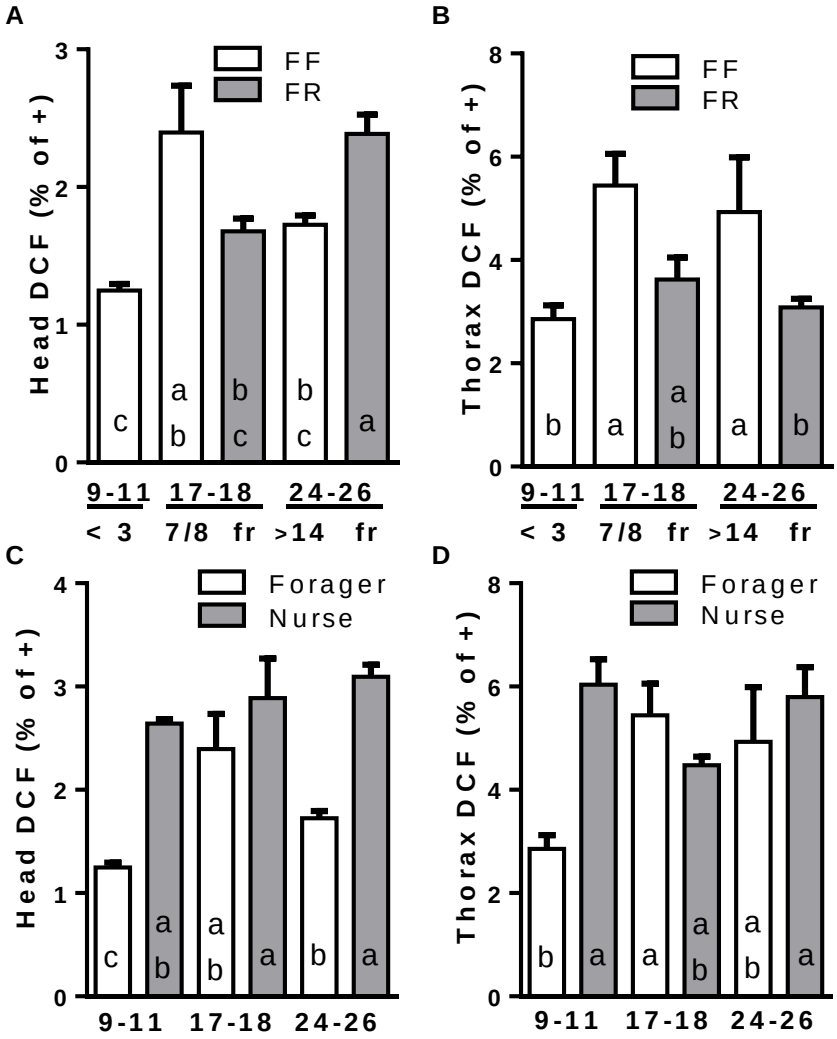
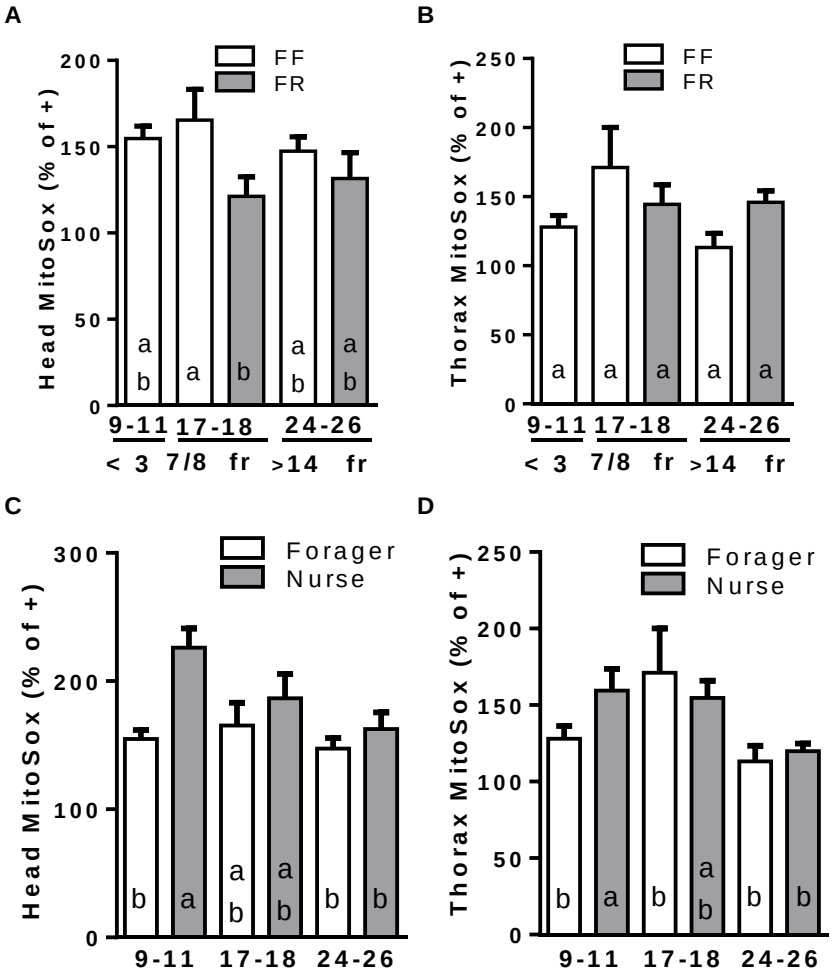


Figure 2.4. Superoxide accumulation in brain and flight muscle. Superoxide accumulation associated with flight was measured in the heads (A) and thoraces (B) of different aged (9-11, 17-18, or 24-26 days old) foragers that were allowed free access to flight (white bars: < 3, 7-9, or > 14 days flight experience) or restricted to the hive (dark bars: < 3 days flight experience). Superoxide accumulation associated with age and behavioral differences in heads (C) and thoraces (D) was measured in forger bees (white bars: 9-11, 17-18, or 24-26 days old) and nurse bees (dark bars: 9-11, 17-18, or 24-26 days old). Values are percentages of positive controls (100 μM hypoxanthine, 5mU/mL xanthine oxidase). Bars (mean ± SEM, n=5-7 per bar) not connected by the same lowercase letter are significantly different ($p<0.05$, mixed model ANOVA and Tukey's HSD). FF= Free-flight; FR = flight-restriction.



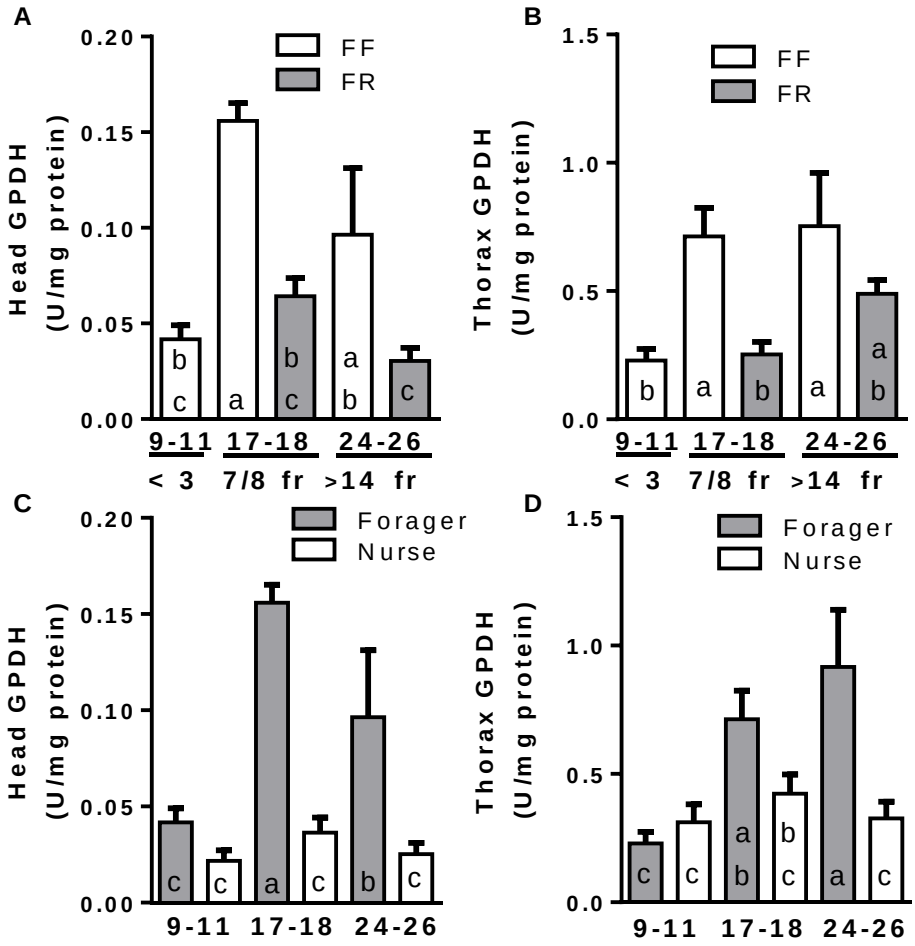
days old), were significantly lower than in age-matched flight-restricted foragers (Fig. 2.3A). Age alone had no effect on levels of ROS in heads (Fig. 2.3C). In contrast to levels of ROS, superoxide levels were affected by the presence or absence of flight rather than amount of flight. Levels of superoxide were significantly higher in intermediate aged (17-18 days old) forager heads compared to heads from age-matched flight-restricted bees (Fig. 2.4A). However, these patterns were not observed in aged (24-26 days old) free-flying and flight-restricted foragers.

Levels of ROS, including H_2O_2, ^{-}OH, and $ONOO^{-}$ in forager thoraces were significantly higher in intermediate aged (17-18 days old) foragers compared to young foragers (9-11 days old) (Fig 2.3B). Together with data from forager heads, these data demonstrate amount of flight has an effect on accumulation of these ROS (Fig. 2.3A and B). Levels of ROS in the thoraces aged foragers (24-26 days old) were significantly elevated compared to age-matched flight restricted foragers (Fig. 2.3B). Age alone had no effect on levels of ROS in thoraces (Fig. 2.3D). In forager thoraces, levels of superoxide were independent of flight activity (Fig. 2.4B) and were higher in 9-11 day old nurses compared to age-matched foragers (Fig. 2.4D). In nurse bee thoraces levels of superoxide decreased with age in both heads and thoraces. This decrease did not occur for foragers, where levels of superoxide were independent of age in both heads and thoraces (Fig. 2.4C, D).

2.3.4: GPDH Activity

GPDH activity was dependent on flight activity. In the heads (Fig. 2.5A, C) and thoraces (Fig. 2.5B, D) of foragers, GPDH activity increased after a week of flight experience. GPDH activity was lower in nurses compared to foragers and lower in flight-

Figure 2.5. GPDH activity is dependent on flight activity. GPDH enzyme activity was measured in the heads (A) and thoraces (B) of different aged (9-11, 17-18, or 24-26 days old) foragers that were allowed free access to flight (white bars: < 3, 7-9, or > 14 days flight experience) or restricted to the hive (dark bars: < 3 days flight experience). GPDH activity associated with age and behavioral differences in heads (C) and thoraces (D) was measured in forger bees (white bars: 9-11, 17-18, or 24-26 days old) and nurse bees (dark bars: 9-11, 17-18, or 24-26 days old). Values are superoxide dismutase activity in U/mol. Bars (mean ± SEM, n=5-7 per bar) not connected by the same lowercase letter are significantly different ($p<0.05$, mixed model ANOVA and Tukey's HSD). FF= Free-flight; FR = flight-restriction.



restricted foragers than free-flying foragers. GPDH activity in intermediate aged (17-18 days old) foragers was higher than in age-matched flight-restricted foragers. Aged foragers had higher GPDH activity compared to aged nurse bees (Fig, 2.5C, D).

2.3.5: Catalase and SOD Activity

In forager brain tissue, catalase activity was higher in intermediate aged foragers (17-18 days old) relative to same-aged flight-restricted foragers (Fig. 2.6A). In flight muscle catalase activity was independent of age and flight experience (Fig. 2.6B). Intermediate aged (17-18 days old) foragers had higher catalase activity in brain tissue compared to same-aged flight-restricted foragers (Fig. 2.6A). Moreover, free-flying foragers had similar catalase activity levels in brain tissue regardless of age or flight experience. However, catalase levels in flight-restricted bees were similar regardless of age (Fig. 2.6A). Catalase activity was statistically independent of age in both forager and nurse brains (Fig. 2.6C). Catalase activity in flight muscle was statistically independent of age, behavioral state and flight experience for both nurses and foragers (Fig. 2.6D).

In forager brains, SOD activity was similar in foragers that had increasing amounts of flight experience and flight-restricted foragers demonstrating SOD activity is independent of flight (Fig. 2.7A). In forager flight muscle, a high amount of flight (<14 days) was associated with a decrease in SOD activity, and flight restriction was associated with higher SOD activity (Fig. 2.7B). Flight muscle SOD activity decreased in 24-26 day old foragers relative to all other nurses and foragers (Fig. 2.7D). SOD activity in the brain was independent of age and behavioral state (Fig. 2.7C).

Figure 2.6. Catalase activity in brain and flight muscle. Catalase enzyme activity was measured in the heads (A) and thoraces (B) of different aged (9-11, 17-18, or 24-26 days old) foragers that were allowed free access to flight (white bars: < 3, 7-9, or > 14 days flight experience) or restricted to the hive (dark bars: < 3 days flight experience). Catalase activity associated with age and behavioral differences in heads (C) and thoraces (D) was measured in forger bees (white bars: 9-11, 17-18, or 24-26 days old) and nurse bees (dark bars: 9-11, 17-18, or 24-26 days old). Catalase breaks down H_2O_2 to yield formaldehyde Values are nmol formaldehyde produced/min/mL. Bars (mean ± SEM, n=5-7 per bar) not connected by the same lowercase letter are significantly different ($p<0.05$, mixed model ANOVA and Tukey's HSD). FF= Free-flight; FR = flight-restriction.

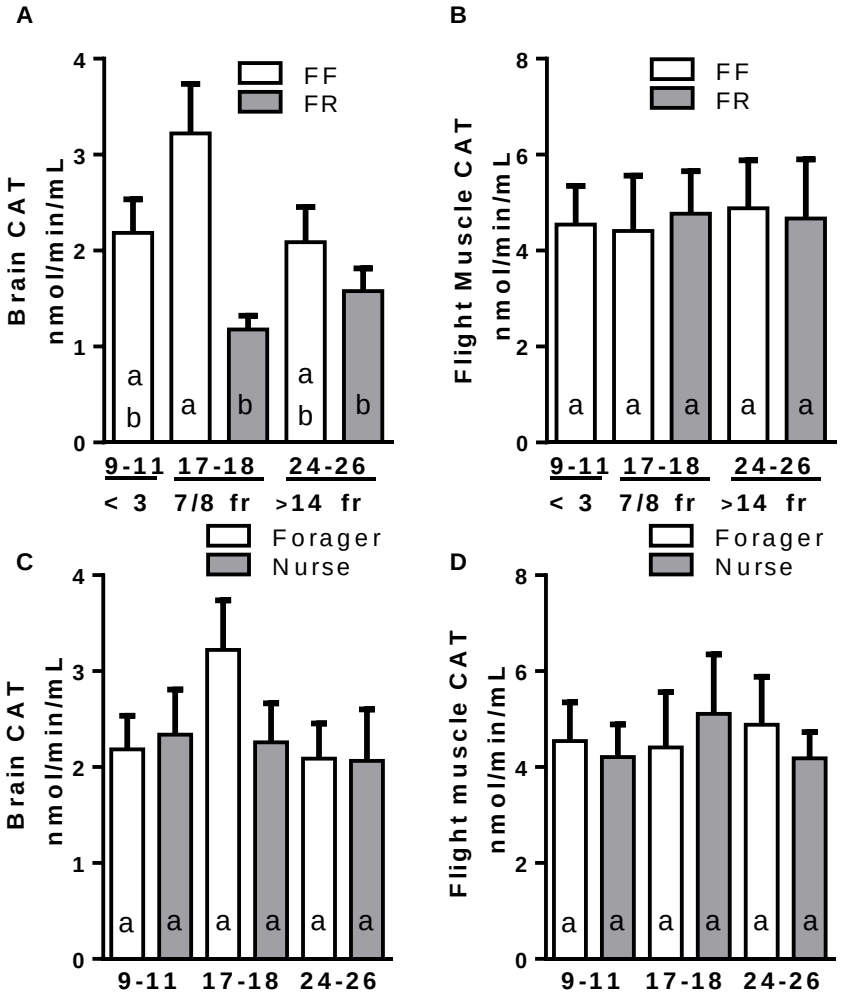
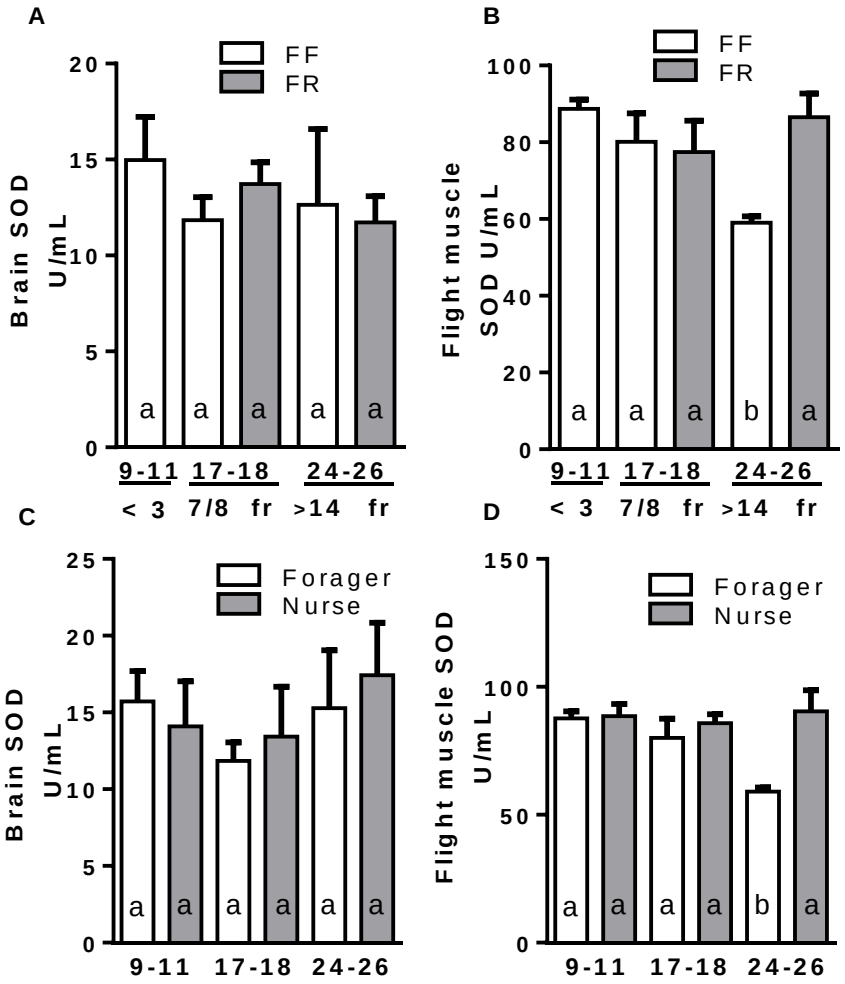


Figure 2.7. Superoxide dismutase activity in brain and flight muscle., Superoxide dismutase (SOD) enzyme activity was measured in the heads (A) and thoraces (B) of different aged (9-11, 17-18, or 24-26 days old) foragers that were allowed free access to flight (clear bars: < 3, 7-9, or > 14 days flight experience) or restricted to the hive (dark bars: < 3 days flight experience). SOD activity associated with age and behavioral differences in heads (C) and thoraces (D) was measured in forger bees (clear bars: 9-11, 17-18, or 24-26 days old) and nurse bees (dark bars: 9-11, 17-18, or 24-26 days old). Values are SOD acitivity in U/mol. Bars (mean ± SEM, n=5-7 per bar) not connected by the same lowercase letter are significantly different ($p<0.05$, mixed model ANOVA and Tukey's HSD). FF= Free-flight; FR = flight-restriction.



2.4: Discussion

The transition to foraging affects both ROS production and mitigation

After transitioning from nurse to forager, oxidative capacity increases to meet the high energetic demands of flight (Harrison, 1986), and increased oxygen intake also presumably augments ROS production. However, superoxide levels in nurse bees, which rarely fly, were higher compared to newly transitioned forager bees, which fly often, suggesting the nurse to forager transition also upregulates oxidative stress defense systems leading to decreased ROS levels. However, catalase and SOD activity did not increase during the nurse to forager transition. Transitioning from nurse to forager induces up-regulation of oxidative stress defense and repair pathways (Margotta et al., 2013; Harrison, 1986; Williams et al., 2008; Wolschin and Amdam, 2007). After flight bouts, antioxidant mechanisms likely clear ROS, but this ability is lost in aged nurses and foragers (Williams et al., 2008). Defense systems other than enzymatic antioxidants, such as glutathione, may clear ROS after metabolically intense foraging bouts, but when oxidative stress mitigation decreases, foraging bees incur oxidative damage (Finkel and Holbrook, 2000). My ROS data indicates the amount of flight regulates ROS levels, but my superoxide, antioxidant, and DNA damage data suggest intense flight vs. little-to-no flight, rather than increasing amounts of flight, play a role in oxidative stress. Thus, the nurse to forager transition likely controls differences in oxidative stress.

Flight compared to negligible flight, rather than amount of flight, increases oxidative stress

Levels of superoxide decreased with age in nurse bees, suggesting mitochondrial function declines with age and lowers ROS production. In fruit flies with free access to flight in vials, mitochondrial respiration and electron transport chain activity decline with age (Ferguson et al., 2005), and forcing flight induces early senescence (Lane et al., *in press*). In my flight comparisons, intermediate aged foragers (17-18 days old) with free access to flight had increased superoxide levels relative to flight-restricted foragers, suggesting flight increases oxidant production. 8-OHdG levels also increased in foragers with greater than two weeks of flight time compared to age- matched flight-restricted foragers. These data, suggest that high flight compared to negligible flight, rather than linear increases in flight, leads to increased oxidative damage.

Contrary to my result showing no difference in MDA between flight-restricted foragers and foragers with free access to flight, Tolfsen and colleagues (2011) surprisingly observed increased MDA levels in the brains of flight-restricted bees. The authors suggest flight activity increases fatty acid saturation levels in the brain, providing protection from lipid damage because saturated fatty acids are less susceptible to oxidation (Haddad et al., 2007). Because my flight-restriction procedure involved caging bees inside of the hive, it is likely flight-restricted foragers still received the signal to forage but were physically constrained from doing so. Tolfsen et al., (2011) used an artificial rain source to restrict foraging, which may have eliminated the entire hive's signal to forage, thus changing fatty acid saturation levels. In both studies, MDA levels

did not increase in foragers with free access to flight; indicating MDA does not accumulate with flight activity.

However, we found no difference in MDA levels between flight-restricted foragers and foragers with free access to flight; indicating lipid damage is not flight-dependent. Paradoxically, we saw an increase in intracellular ROS (H_2O_2 and ^{-}OH, and $ONOO^{-}$) levels but not superoxide levels or SOD activity, which suggests another site of H_2O_2 generation besides the dismutation of superoxide into H_2O_2 by SOD. It is important to note that because the DCF-DA reacts with H_2O_2, ^{-}OH, and $ONOO^{-}$, we are unable to determine which particular ROS increase. Because flight is causal in ROS accumulation, enzymes able to produce ROS that enable sustained flight over time may be an additional site of ROS.

Although the exact mechanisms of ROS generation by mitochondria are not fully understood, most studies suggest the major sites of ROS generation in animals are Complex I and Complex III of the electron transport chain (Turrens, 1997; Hansford et al., 1997; Herrero and Barja, 1997; Barja, 1999). However, in brown adipose tissue and insect flight muscle, GPDH is also a major source of ROS because of reducing equivalents present (Sohal, 1993; Drahota et al., 2002; Miwa et al., 2005). In insects, GDPH plays a crucial role in flight performance by cycling NADH to the respiratory chain (Zebe and McShan, 1957). GPDH-deficient mutant fruit flies fail to sustain flight and eventually lose flight ability, demonstrating GPDH is necessary for flight activity (O'Brien and McIntyre, 1972). We found GPDH activity was dependent on flight activity in forager brain tissue and flight muscle. Because oxidant production was flight-dependent as well, this suggests GPDH activity, not complex I or III, is a major site of

radical production in foraging honey bees. These data suggest increased GPDH activity needed for sustained flight, and not increased oxygen consumption, affects increased ROS production seen in foraging honey bees. However, to unequivocally test this hypothesis, one must measure GPDH activity and ROS production in foragers flying in hypoxic and hyperoxic conditions. Metabolic rates for bees flown in varying levels of oxygen between 10 and 21% are unchanged, but slightly decreased in bees flown in 5% oxygen (Harrison et al., 2001). Therefore, if ROS levels are higher in bees flown under hyperoxic condition, then these results are not due to altered metabolic rate. Measuring ROS production under these conditions may reveal the true source of ROS generation during flight.

Oxidative stress occurs in a tissue-dependent manner

Levels of intracellular ROS increased in aged (24-26 days old) flight-restricted forager heads, aged rarely-flying nurses, and experienced free-flight forager thoraces, but decreased in the heads of experienced free-flight foragers. Because ROS levels decreased only in the heads of experienced foragers, these data may indicate ROS damage brain tissue, resulting in slowed metabolism and less ROS production. We saw no increase in MDA content, indicative of a lack of damage due to lipid peroxidation of aged forager brains. Lack of lipid damage supports data suggesting changes in forager brain fatty acid saturation provide neural protection from lipid damage (Tolfsen et al., 2011). However, decreased metabolism due to ROS accumulation is still a possibility. In contrast, MDA levels increased in aged nurse brain tissue, which has a similar fatty acid saturation level compared to forager bees (Haddad et al., 2007). These data suggest repair mechanisms decline in older nurse bees, becoming a forager is unlikely at 40 days of age.

Surprisingly, MDA levels increased in the brains of foragers and nurses caged inside the laboratory, suggesting the social context of the hive may play a role in fatty acid saturation levels in the brain or neural protection may be conferred by the social interactions inside the hive. The transition to foraging is controlled by the presence of foragers inside of the hive and pheromones they produce (Le Conte et al., 2001). The number of nurses inside the hive is controlled by pheromones released from developed larvae. Removing bees from these physical and chemical cues may alter their ability to mitigate oxidative damage.

If neurons are oxidatively damaged, then ROS production may be lower due to decreased metabolism. ROS differentially affect glial cells/neurons, and because these cells are terminally differentiated they are especially sensitive to ROS thus easily damaged (Gilgun-Sherki et al., 2001). Most insect olfactory input control and learning/memory occurs in the mushroom bodies, paired neuron-dense structures that act as association areas in the honey bee brain (reviewed in Heisenberg, 1998). In foraging honey bees with greater than two weeks of flight experience, mortality sharply increases (Dukas, 2008), olfactory learning decreases (Behrends et al., 2007), and oxidatively damaged proteins accumulate in the brain (Seehuus et al., 2006b). Because oxidative damage increases in the brain and olfactory learning decreases, it is plausible that high amounts of flight lead to mushroom body damage in the brain. However, olfactory learning may be a peripheral sensory effect of senescence, rather than a central effect, and only a small subset of proteins in the brain may become oxidatively damaged. Collectively, these data suggest high amounts of flight activity damage the brain of foragers, but it is unclear if this damage is a product of oxidative stress. Consequently, it

is unlikely that damage to the brain leads to whole-organism functional senescence because foragers still retain some ability to learn (Seehuus et al., 2006).

Catalase is an antioxidant found mainly in peroxisomes and mitochondria (Halliwell and Gutteridge, 1989). Its activity is associated with high concentrations of mitochondria in metabolically active tissues where it is a crucial protective mechanism in highly-metabolic organisms (Powers et al., 1994). In this study, we found that catalase activity was independent of age and behavioral state but saw lower activity in the flight-restricted forager brain tissue (17-18 days old; <3 days flight experience) compared to foragers with free access to flight (17-18 days old; 7-9 days of flight experience). Another study similarly demonstrated catalase protein levels are largely independent of age and behavioral state in the head, but in the thorax catalase levels increased in old nurse bees collected in the evening indicating a diurnal expression pattern (Williams et al., 2008). Because catalase is a high-efficiency enzyme, it is unlikely to become saturated at high H_2O_2 concentrations. In this study, bees were not collected in the evening hours, which may reflect the differences seen in data from older nurse bees. If foragers forgo flight for an extended period of time, the ability to maintain catalase activity in the brain may be lost, possibly due to a decrease in metabolic rate or mitochondrial activity. Honey bee flight muscle contains 39-42% mitochondria per muscle volume (Block, 1994). So, it is unsurprising that flight activity was not associated with a change in catalase activity because additional increases may be impossible.

Together these results suggest the brain is more sensitive to changes in oxidative stress and catalase is a crucially important defense in flight muscle. Future studies, using diutinus bees, overwintered bees that do not fly, will be important to unravel the effects

of long-term natural flight-restriction on antioxidant activity and ROS production. Diutinus bees have the maximum lifespan of worker bees and understanding antioxidant and ROS production in these bees may show have ROS accumulation and mitigation ultimately effects lifespan. Because hypoxic conditions exist inside a winter hive (Van Nerum and Buelens, 1997), diutinus bees may use anaerobic metabolism during shivering thermogenesis, thus reducing ROS generation. Additionally, understanding why senescence is delayed is diutinus bees will provide insight into how flight activity and senescence are related.

Forager flight activity may contribute to senescence when foragers accumulate more than 10 days of flight experience. As foragers age, glycogen synthesis slows and bees that take more flights per day reach a point where the ability to synthesize glycogen decreases (Neukirch, 1982) suggesting these enzymes involved in carbohydrate metabolism may be damaged in foragers with high flight experience. High levels of ROS preferentially damage enzymes in the pathway that synthesizes glycogen (England et al., 2004; Shanmuganathan et al., 2004; Kashiwagi et al., 1996). Foragers with greater than two weeks foraging experience perform poorly in olfactory learning tests compared to bees with up to 13 days of foraging time (Behrends et al., 2007), demonstrating cognitive function also declines with increasing flight experience. Furthermore, oxidative carbonylation and nitration increases independently of age in the brains of forager bees (Seehuus et al., 2006a), and markers of oxidative stress also increase in foragers with greater than 14 days of flight experience. These prior studies collectively show oxidative damage is mechanistically involved in the decline of flight muscle performance and cognition required for finding flowers and returning to the hive as time spent flying

increases. My data extend these findings and determines that flight muscle may be more susceptible to oxidative damage compared to brain tissue. Previous research suggests brain tissue is protected in honey bees.

Flight induces oxidative damage

Several lines of evidence support a link between increased oxidative tissue damage and biological aging (reviewed in Finkel and Holbrook, 2000), and behavior may actually magnify the amount of oxidative stress an organism undergoes. During acute bouts of activity or long term exercise, heat shock proteins and antioxidant enzymes are upregulated to reduce exercise-induced damage (Ji, 1993; Hernado and Manso, 1997; Higuchi, 1985; Jenkins et al., 1984; Salo et al., 1991), but the ability to counteract oxidative stress decreases with age (Banerjee et al., 2003; Ebbeling and Clarkson, 1989; Ji, 2002; Liu et al., 2004). Furthermore, vigorous or unexpected exercise can increase oxidative muscle damage regardless of age (Vollaard et al., 2005). Houseflies prevented from flying live 3x longer and have reduced mitochondrial damage compared to flies permitted to fly (Yan and Sohal, 2000) demonstrating physical activity can accelerate senescence. In this study, bees treated with D-galactose, MDA levels increased establishing a cause-effect relationship between an oxidative insult and increased oxidative damage via lipid peroxidation in honey bees. Levels of 8-OHdG increased in free flying foragers with greater than two weeks of flight time compared to age-matched flight-restricted foragers. Similarly in houseflies, 8-OHdG levels were lower in flies with decreased physical activity and the authors showed a strong correlation between activity levels, 8-OHdG levels, and decreased survival (Agarwal and Sohal, 1994). Because MDA and 8-OHdG levels increased in the oldest bees and foragers with greater than two

weeks of flight experience, the time point at which senescence reaches nearly 100% in foraging bees, these data suggest increased oxidative stress contributes to decreased longevity. Collectively these studies demonstrate that highly-metabolic behaviors are positively associated with oxidative damage and decreased lifespan.

Antioxidants, especially SOD, are increased in muscle and other body parts of numerous organisms, including honey bees, during acute and chronic exercise (Powers et al., 1999; Vollaard et al., 2005; Williams et al., 2008) demonstrating these enzymes are crucial for preventing oxidative damage to highly metabolic tissues. Exercise capacity is decreased in mice heterozygous for manganese SOD (Kinugawa et al., 2005) indicating SOD contributes to an organism's ability to perform highly aerobic behaviors. Additionally, endurance training in rats increases manganese SOD activity primarily in type IIa muscle, the same muscle type that drives honey bee flight (Hollander et al., 1999). Similar to catalase, SOD activity was independent of age and flight activity in forager and nurse brain tissue, but in flight muscle, 24-26 day old forager SOD activity was lower activity inthan age-matched nurses. Furthermore, SOD activity was lower in 24-26 day old foragers with free-access to flight compared to flight restricted foragers, indicating increased flight is associated with a decrease in SOD activity in flight muscle. Together, these data suggest SOD is directly related to an organism's locomotor ability. As honey bees spend more time flying their locomotor ability declines demonstrated by decreased flight capacity (Vance et al., 2009), longer duration flights from a known distance (Tofilski, 2000), and decreased pollen and nectar loads (Schmid-Hempel et al., 1985). Loss of flight ability in older forager bees is an unequivocal marker of whole-organism senescence; therefore, we suggest oxidative damage due to decreased SOD

activity in flight muscle contributes directly to whole-organism senescence in aged forager bees.

2.4.5: Conclusions

Numerous studies suggest flight activity is the underlying cause of decreased longevity and aging in honey bees (Behrends et al., 2007; Neukirch, 1982; Seehuus et al., 2006a; Williams et al., 2008). My study provides new information on the mechanisms underlying this effect; identifying a causal relationship between oxidative insults and oxidative damage establishing that behavioral state, flight experience, and chronological age influence oxidative damage in a tissue-dependent manner likely contributing significantly to functional senescence. Although the fundamental aspects of Harman's theory, which states increased ROS production leads to oxidative damage, hold true, the actual source of ROS in honey bee foragers may not fit predictions of the ROS theory. My data suggest ROS generation associated with oxidative damage in honey bees is GPDH dependent, rather than oxygen dependent, which contradicts the ROS theory. Because foraging honey bees have metabolic rates higher than most animals, it is difficult to conclude that oxidative stress is the foremost cause of aging in non-flying nurse bees and other animals with metabolic rates typical of mammals. Oxidative stress in foraging honey bees, however, may be analogous to late-life oxidative stress. While oxidative damage may not induce the aging process, it may significantly hasten senescence due to chronic sub-lethal damage that progressively increases with age. However, we are unable to determine from this work if negligible flight in nurses produces sub-lethal damage that affects forager lifespan. In the future, understanding how low-level oxidative damage in

nurses influences more extreme damage associated with foraging will yield insight into whether early-life behaviors can decrease lifespan.

CHAPTER 3

PARAQUAT AS A MODEL OF FLIGHT-INDUCED OXIDATIVE DAMAGE

3.1: Introduction

In honey bees, senescence occurs as a function of flight time accrued, rather than a function of age (Neukirch, 1982). Honey bees remaining inside the hive caring for young, or nurse bees, show negligible senescence, and nurses live nearly 6 months when their behavioral transition away from nursing is delayed (Reuppell et al., 2007). However, bees leaving the hive to search for food, or forager bees, typically die within 10 days of their first foraging flights (Neukirch, 1982). Data suggest this phenomenon is partially due to oxidative damage accruing as a result of intense flight while foraging for food (Neukirch, 1982; Seehuus et al., 2006a; Behrends et al., 2007; Williams et al., 2008). However, to fully test the hypothesis that high amounts of honey bee flight leads to oxidative damage, manipulating levels of oxidative damage critical. The ideal approach to determine if flight is causative in oxidative damage is to compare foragers forced to take more flights to normal foragers. However, this manipulation has not yet been successfully done in free-living foragers. Consequently, chemically manipulating levels of oxidative stress mimics the effects of high flight.

The herbicide paraquat, 1, 1'-dimethyl-4, 4'-bipyridium dichloride, functions as a redox cycler to stimulate superoxide radical production in organisms (Bus and Gibson, 1984; Hassan, 1984). Paraquat, which is a dication (PQ^{2+}), accepts an electron from a reductant to form a paraquat monocation radical ($PQ\cdot^{+}$), which then reacts with oxygen to form superoxide radicals (O_2^{-}). The major site of superoxide generation during paraquat exposure is complex I of the electron transport chain (Cocheme and Murphy, 2007).

Because superoxide is highly reactive within the cell, paraquat causes extensive oxidative damage to mitochondria and macromolecules throughout the cell. Along with damage to macromolecules, paraquat also induces apoptosis, depletes cellular NADPH, inhibits cellular antioxidants, and induces cytoskeletal damage (reviewed in Fukushima et al., 2002). Although the production of oxygen radicals and subsequent oxidative damage is well-established, it is unclear if paraquat-induced cytotoxicity results directly from the radicals produced or the radicals trigger other processes that kill the cell (Fukushima et al., 2002).

Although paraquat has been used in honey bees to induce oxidative stress (Corona et al., 2007; Seehuus et al., 2006a), it is unclear whether the typical course of oxidative damage follows that of other model systems. Because honey bee flight is highly metabolic (Suarez et al., 1999) and likely produces high amounts of ROS (Williams et al., 2008), mechanisms to mitigate damage from increased ROS production are likely substantial. Two previous studies used paraquat as an oxidative stressor to examine the effects of vitellogenin (Seehuus et al., 2006; Corona et al., 2007), a yolk precursor protein that acts as an antioxidant in honey bees, on lifespan. In both of these studies, worker bees with higher levels of vitellogenin had substantially increased survival when exposed to paraquat, but no positive indicators of oxidative damage were presented in either study. Furthermore, honey bee brains in paraquat exposed bees had similar levels of oxidative carbonylation and nitration compared to controls (Seehuss et al., 2006a). In both studies, paraquat dosage was at least 30x higher than typically used in mammalian oxidative stress studies. Because bees experience increased mortality shortly after removal from the social context of their colony (Huang and Robinson, 1992), this dosage

ensured fast mortality. Therefore, it is unclear whether increased mortality from paraquat exposure in honey bees is truly due to oxidative stress or other cytotoxic effects that may be associated with high dosage.

In this study, we determined if high dose paraquat injections increase oxidative damage, ROS levels, and enzymatic antioxidant activity in honey bee foragers and nurses. We injected both forager and nurse bees with a dosage of paraquat similar to previous honey bee studies and measured levels of DNA oxidation, superoxide dismutase activity, catalase activity, superoxide production, and hydrogen peroxide/hydroxyl radical production. To rule out a confounding effect of the injection procedure on my measurements, I also included a non-injection control that underwent the same preparation as the injected bees. We found paraquat exposure produces no change or increased oxidative stress in the heads and thoraces of both foragers and nurses. Bees injected with the vehicle showed a positive antioxidant response compared to the non-injected controls indicating injections may induce an antioxidant response similar to the paraquat antioxidant response. Based on these data, we conclude paraquat injections are not an effective oxidative stressor in honey bees because of the confounding effect of the injection and other possible cytotoxic effects.

3.2: Methods

3.2.1: Single-cohort colonies

Honey bees for this study were reared at the University of Nevada, Las Vegas apiary. Bees from 6 source colonies headed by naturally-mated queens were used to obtain age-matched workers to start 4 single-cohort colonies (SCCs). The frames were

placed in an incubator (35°C, 75% relative humidity RH, 24 h dark cycle) and newly eclosed bees were removed from the frames every 24 h. SCCs were formed by housing approximately 2000 single, day-old workers, which eclosed over 2 consecutive days, with a naturally mated queen. These bees were placed in 4 nucleus colonies containing one frame each of pollen and honey and 3 empty frames to allow the queen to lay eggs. The dorsal thorax of each bee was marked with a single dot of paint (Testors, Rockford, IL) to indicate age prior to placing them in their SCC. The SCC was kept in the laboratory for 5 days post-adult emergence to allow for adult maturation and queen egg laying before being moved to the outdoor apiary on the UNLV campus. A single-cohort colony uses skewed colony age demography to induce precocious foraging in approximately 10% of the bees at 7-10 days, a full two weeks earlier than in a natural colony (Giray and Robinson, 1994). Through these experimental manipulations of the honey bee colony, the effects of age and behavior can be decoupled, allowing for analysis of same aged bees with drastically different flight histories.

3.2.2: Flight experience

Once a SCC was placed outdoors, the colony was observed until the onset of first foraging, which occurred between 8 to 11 days of age in this study. Once a bee was identified as a forager an additional dot of paint was placed on the posterior end of the abdomen. Foragers were marked continuously throughout the day until all foragers returning were consistently marked. This process was continued each day with a new color paint mark for each day until the 14^{th} and 15^{th} days after eclosion. Only marked foragers were collected in this study.

3.2.3: Paraquat injections

Foragers (<3 days flight experience) and nurses were collected from SCCs at 9 to 11 days of age. These bees were divided into 3 groups: paraquat injections, vehicle injections, and no-injection controls. All 3 groups were immobilized by placing bees in a 4°C refrigerator until movement ceased. Paraquat was injected following the methods of Seehuus and collegaues (2006a). Briefly, bees were injected with 150 μg/g per bee (wt/wt), which caused the majority of bees to die within 3 days. Typically, mammals are injected with 30x less paraquat, but the concentration chosen ensures bees die before any confounding social stress effects arise. Paraquat (2 μl) was injected between the 5^{th} and 6^{th} abdominal segments with a microsyringe and care was taken to avoid puncturing any abdominal organs by injecting parallel to the body cavity. Controls were injected with the vehicle (sterile water) or not injected but chilled to reduce movement. After injections bees were kept inside an incubator at 37°C in cages with sucrose syrup and water. Bees were collected for analysis after 48 hours. Another set of paraquat injected and control bees (30 bees/group) were prepared for a survival analysis. Every 24 hours, thsese bees were checked for viability and dead bees were removed.

3.2.4: Reactive oxygen species measurements

Intracellular ROS generation was measured in heads and thoraces using 2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Molecular Probes, Eugene, OR), a chemically reduced form of fluorescein. Upon oxidation by hydrogen peroxide and hydroxyl radicals (but not superoxide), CM-H_2DCFDA is converted into highly fluorescent 2', 7'-dichlorofluorescein (DCF). This probe reacts with hydrogen peroxide, hydroxyl radicals, and peroxynitrite. Superoxide production was assessed separately

using MitoSOX (Molecular Probes, Eugene, OR), a fluorogenic probe that is rapidly oxidized by superoxide, but not by other ROS generating systems.

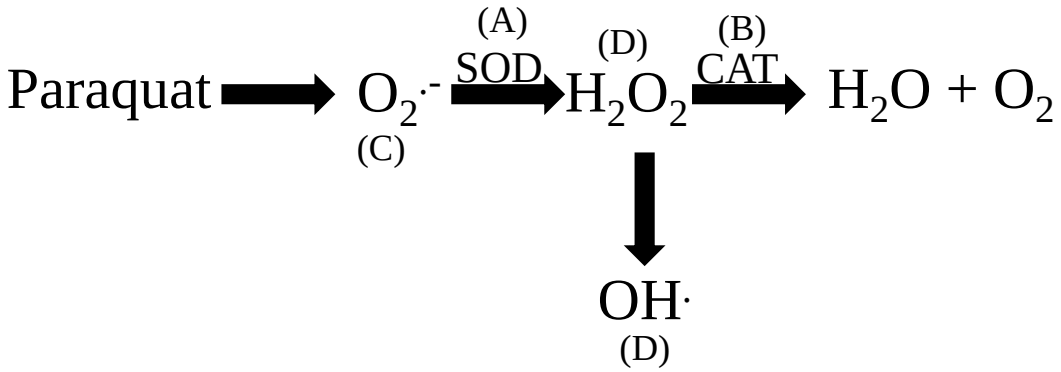
Briefly, freshly collected (not frozen) heads and thoraces were homogenized within 20 minutes in PBS containing 0.1% Triton X-100. Head or thorax (100 µL) homogenate was added to each well (96 well microtiter plate) with 5 µM CM-H_2DCFDA to measure H_2O_2, $^{-}$OH, and $ONOO^{-}$, or MitoSOX to measure superoxide. To compare samples from multiple microplates, positive controls for each microplate were used. For H_2DCFDA, 100 µM hypoxanthine, 5mU/mL xanthine oxidase, and 0.2 U/mL horseradish peroxidase were used. For MitoSOX, 100 µM hypoxanthine and 5mU/mL xanthine oxidase were used. Negative controls consisted of homogenates with no probe added. CM-H_2DCFDA samples and negative control were incubated at room temperature for 25 minutes before adding the positive control, which was incubated for an additional 5 minutes at room temperature. MitoSOX samples and controls were incubated at 37°C for 30 minutes. Plates were then read at 485 Ex/535 Em for CM-H_2DCFDA or at 510 Ex/580 Em for MitoSOX on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA).

3.2.5: Antioxidant activity

Superoxide dismutase (SOD) activity was determined using a commercial assay kit (product 706002, Cayman Chemical, Ann Arbor, MI). This assay measures the copper/zinc, iron, and manganese forms of SOD by utilizing a water-soluble tetrazolium salt [2-(4-iodophenyl) -3-(4-nitrophenyl)- 5-(2,4-disulfo-phenyl) -2*H*- tetrazolium, monosodium salt] (WST-1) to measure superoxide radicals. WST-1 is then reduced to a water-soluble formazan dye upon reaction with superoxide anions. SOD calculation for

honey bee samples was based on the percent dismutation of superoxide generated by xanthine oxidase and hypoxanthine upon addition of the sample. Samples were prepared by homogenizing individual dissected brains and flight muscle in 500 μL of cold 20 mM HEPES buffer, pH 7.2 containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose. Homogenates were spun at 1500g for 5 minutes at 4°C. Formazan dye absorbance was measured at 450 nm on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). The SOD standard curve functioned as a positive control, and a well without honey bee sample added acted as a negative control.

Figure 3.1: Scheme of major cellular oxidants and enzymatic antioxidants. Paraquat is a redox cycler which produces superoxide radicals. Superoxide dismutase readily dismutates these radicals into hydrogen peroxide, which is then broken down intowater and oxygen by catalase. In the presence of ferrous iron, hydrogen peroxide may also form hydroxyl radicals through the Fenton reaction.



Catalase activity was measured using a commercially available kit (product 707002, Cayman Chemical, Ann Arbor, MI). This assay utilizes the peroxidatic function of catalase to react with methanol in the presence of an optimal concentration of hydrogen peroxide to produce formaldehyde. The formaldehyde is then measured spectrophotometrically using purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-trizazole) as the chromagen. Samples were prepared by homogenizing individual dissected brains

and flight muscle in 350 μL of 50 mM potassium phosphate buffer pH 7.2 with 1 mM EDTA. Homogenates were spun at 1000 g for 10 minutes at 4°C. Absorbance was measured at 540 nm on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). The catalase standard curve functioned as a positive control, and a well without honey bee sample added acted as a negative control.

3.2.6: 8-hydroxy-2'-deoxyguanosine measurement

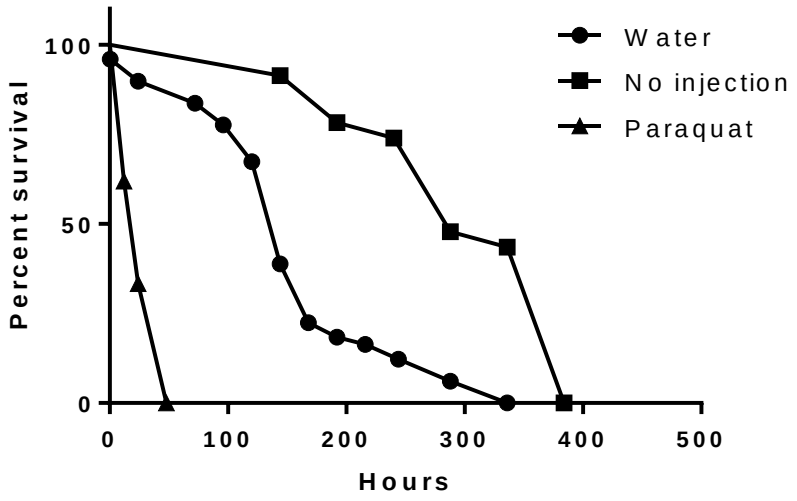
Levels of 8-OHdG were measured using an ELISA with an 8-OHdG antibody. DNA was extracted from dissected brains and flight muscle using a DNeasy extraction kit (Qiagen). 8-OHdG was measured using a commercially available kit (Cayman Chemical, Ann Arbor, MI). Briefly, DNA samples, an antiserum to 8-OHdG, and 8-OHdG linked to an acetylcholineesterase was added to a 96-well binding plate and incubated at 4°C for 18 hours. Unbound reagents were removed from the plate by washing and the plate was developed using Ellman's reagent. The plate was allowed to develop for 90 minutes then read at 410 nm on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). The amount of 8-OHdG in each sample was calculated in reference to an 8-OHdG standard curve.

3.2.7: Statistical analysis

Statistical analyses were performed in JMP (SAS, Cary, NC) and graphs were prepared in GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). Mixed-model ANOVAs with Tukey's HSD post hoc tests were used to compare the means of experimental groups. Survival curves were prepared in GraphPad Prism 6 and statistical differences between lifespan curves were determined using the Mantel-Cox method. P-

values less than 0.05 were considered statistically significant. Data are represented as mean ± SEM.

Figure 3.2: Water and paraquat injections decrease the lifespan of foragers caged inside the laboratory. Foragers were injected with 15 μg (wt/wt) paraquat or injected with the vehicle. A third group of foragers underwent the same preparation, but were not injected. Each group consisted of 30 foragers gathered from the entrance of a typical honey bee colony. Dead foragers were removed every 24h. Log-rank test (Mantel-Cox) for differences in survival p=<0.05.



3.3: Results

3.3.1: Both paraquat and water injections decreased forager lifespan

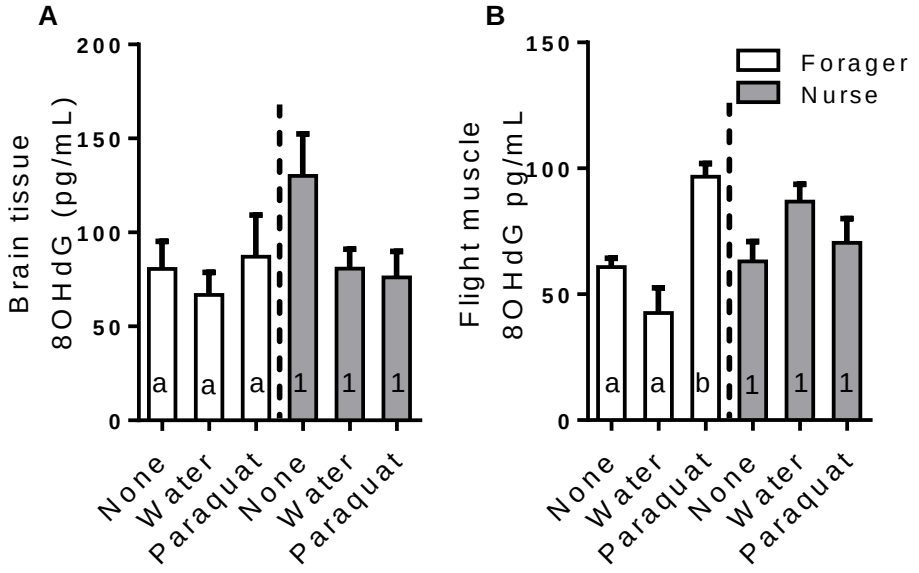
Groups of foragers collected from a typical honey bee colony were divided into 3 groups and two groups were injected with 15 μg of paraquat or sterile water while the other group underwent the same handling procedure but did not receive an injection. Survival was significantly reduced in foragers injected with paraquat compared to sterile water injections or no injection controls (Fig. 3.2). All bees injected with paraquat died within 48 hours of injection, but survivorship in bees injected with the vehicle also

decreased compared to the no injection control. Because lifespan was decreased in both water and paraquat injected bees, these data indicate that injections, regardless of the substance injected, decrease survivorship in laboratory-kept forager bees.

3.3.2: 8-hydroxy-2'-deoxyguanosine is increased forager flight muscle

Because paraquat injections significantly decreased forager survival in the laboratory, we next measured a well-established biomarker of oxidative DNA damage, 8-OHdG, to determine if oxidative damage increases with paraquat exposure. To exclude the effects of age and flight experience on 8-OHdG accumulation, we analyzed age-matched nurses and foragers that were collected from SCCs 9-11 days post-eclosion with less than 3 days of flight experience.

Figure 3.3: 8-OHdG levels in nurses and foragers injected with paraquat, sterile water, or no injection controls. Levels of 8-OHdG, a marker of oxidative DNA damage, were quantified in the brain tissue (A) and flight muscle (B) of age-matched foragers (white bars) and nurses (grey bars). Bees were collected from SCCs at ages 9-11, injected (or same preparation), and collected for analysis 48 hours later. Bars not connected by the same letter or number are statistically different: mixed-model ANOVA with Tukey's post hoc test ($p<0.05$).

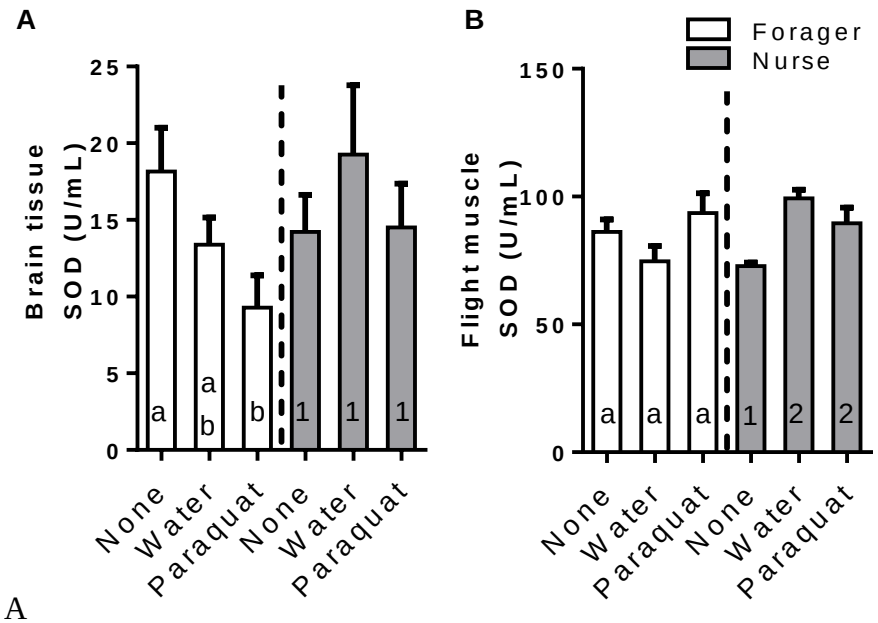


In the brain tissue of both foragers and nurses (Fig. 3.3A) 8-OHdG was not significantly different between paraquat injected, sterile water injected, or no injection controls. Similarly, nurse bees injected with paraquat or sterile water and no injection controls had comparable 8-OHdG levels in flight muscle (Fig. 3.3B). However, 8-OHdG levels in levels in forager flight muscle (Fig. 3.3B) were significantly increased in paraquat injected bees compared to both control groups. A significant increase in 8-OHdG was only observed in the flight muscle of foragers. Because we used 150 μg/g of bee (wt/wt), which is 30 fold higher than typically used in mammals, these data suggest that paraquat treatment does not ubiquitously induce oxidative damage between tissues and among behaviors as in other model systems.

3.3.3: Paraquat and water injections decrease superoxide dismutase activity in forager brains but increase activity in nurse flight muscle

To determine if increased 8-OHdG levels in the flight muscle of paraquat-injected foragers was associated with an imbalance of ROS/antioxidants, we first measured the enzymatic activity of superoxide dismutase, a key antioxidant in dismutating superoxide radicals (See Fig. 3.1), which is the main free radical produced by paraquat exposure. In forager brain tissue, SOD activity was lower with paraquat injections (Fig. 3.4A) compared to the no injection controls, but sterile water injected forager brain tissue had intermediate levels of SOD activity, which suggests injecting foragers with sterile water elicits an antioxidant response in forager brains.

Figure 3.4: Superoxide dismutase activity in nurses and foragers injected with paraquat, sterile water, or no injection controls. The enzymatic activity of SOD was quantified in the brain tissue (A) and flight muscle (B) of age-matched foragers (white bars) and nurses (grey bars). Bees were collected from SCCs at ages 9-11, injected (or same preparation), and collected for analysis 48 hours later. Bars not connected by the same letter or number are statistically different: mixed-model ANOVA with Tukey's post hoc test ($p<0.05$).



A

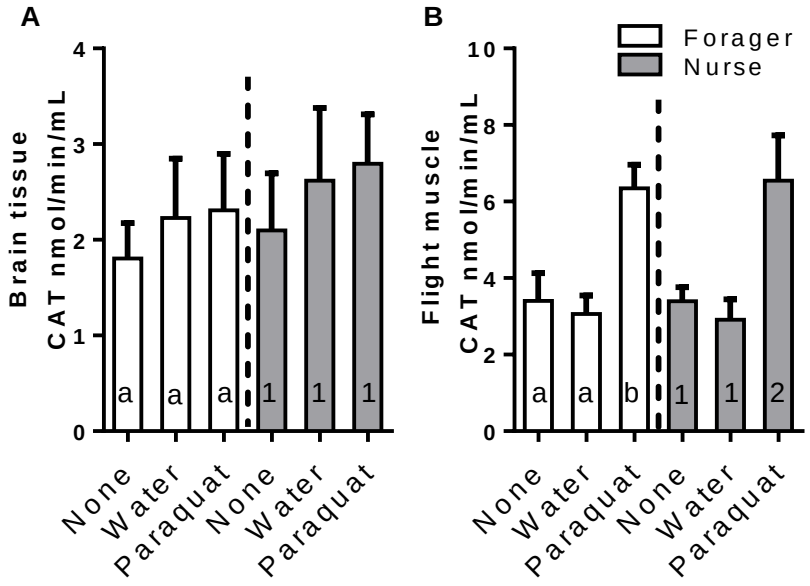
Although, 8-OHdG levels rose in forager flight muscle following paraquat exposure, we did not see similar response in SOD activity. In forager flight muscle (Fig. 3.3B), no differences in SOD activity between paraquat injections and controls were detected. However, in nurse flight muscle (Fig. 3.4B), paraquat and sterile water injections elicited an increase in SOD activity, suggesting injections alone lead to an antioxidant response in nurse flight muscle. Taken together these data indicate the effects of paraquat are confounded, possibly by an antioxidant response resulting from an injection.

3.3.4: Catalase activity is increased in the flight muscle of foragers and nurses injected with paraquat.

In addition to SOD, catalase is a vital part of the enzymatic antioxidant protective system in animals (Fig. 3.1). Hydrogen peroxide, which is produced as SOD dismutates superoxide radicals, is broken down into water and oxygen by catalase. To maintain homeostasis between pro- and antioxidants, both SOD and catalase must be up-regulated in response to higher levels of ROS production, which may result from increased metabolic expenditure or exposure to paraquat.

In the brain tissue of both foragers and nurses (Fig. 3.5A) no statistical difference in catalase activity was detected between paraquat injected or control bees. However, in the flight muscle of nurses and foragers (Fig. 3.5B), catalase activity was significantly increased in only bees injected with paraquat. Control injections did not elicit an increase in catalase activity suggesting catalase may not be part of the general antioxidant response apparently induced during injection.

Figure 3.5: Catalase activity in nurses and foragers injected with paraquat, sterile water, or no injection controls. The enzymatic activity of catalase was quantified in the brain tissue (A) and flight muscle (B) of age-matched foragers (white bars) and nurses (grey bars). Bees were collected from SCCs at ages 9-11, injected (or same preparation), and collected for analysis 48 hours after injections. Bars not connected by the same letter or number are statistically different: mixed-model ANOVA with Tukey's post hoc test ($p<0.05$).

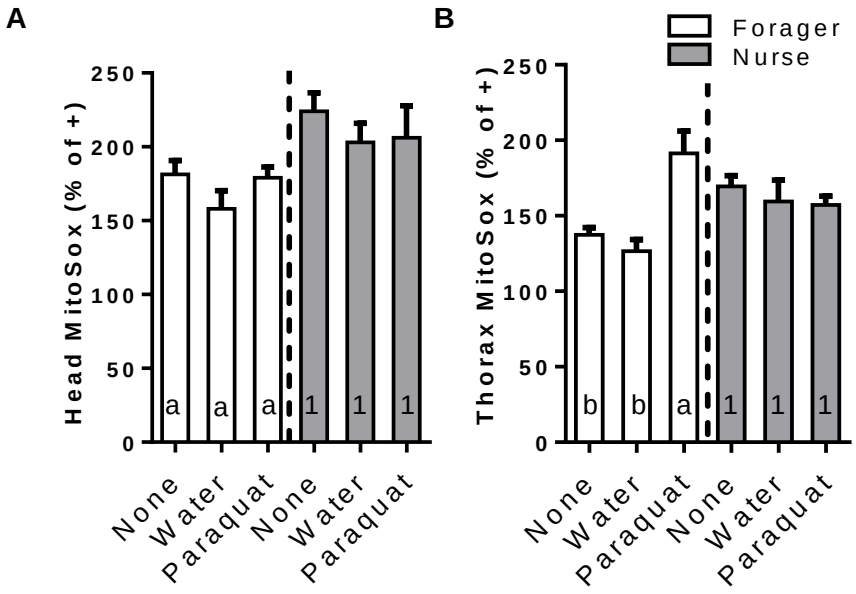


3.3.5: Superoxide levels are increased in the flight muscles of foragers injected with paraquat.

To understand why high concentrations of paraquat only increased 8-OHdG levels in forager flight muscle, it is important to establish ROS accumulation and antioxidant activity in both brains and flight muscle. The patterns of MitoSox fluorescence observed between tissues and behaviors were similar to 8-OHdG levels. In forager and nurse brain tissue (Fig. 3.6A), superoxide levels were similar in paraquat injected and control bees. Similar to catalase and SOD activity, superoxide levels were not increased as predicted in nurse or forager brain tissue. Additionally, in nurse flight muscle (Fig. 3.6B) superoxide levels remained constant in injected and controls bees but superoxide levels increased in the flight muscle of foragers injected with paraquat relative to water and no injection

controls. These data indicate paraquat-induced superoxide formation acts in a tissue-specific manner.

Figure 3.6: MitoSox levels in nurses and foragers injected with paraquat, sterile water, or no injection controls. The fluorescence of MitoSox, which is specific for superoxide radicals was quantified in the brain tissue (A) and flight muscle (B) of age-matched foragers (white bars) and nurses (dark bars). Bees were collected from SCCs at ages 9-11, injected (or controls), and collected for analysis 48 hours later. Bars not connected by the same letter or number are statistically different: mixed-model ANOVA with Tukey post hoc test ($p<0.05$).



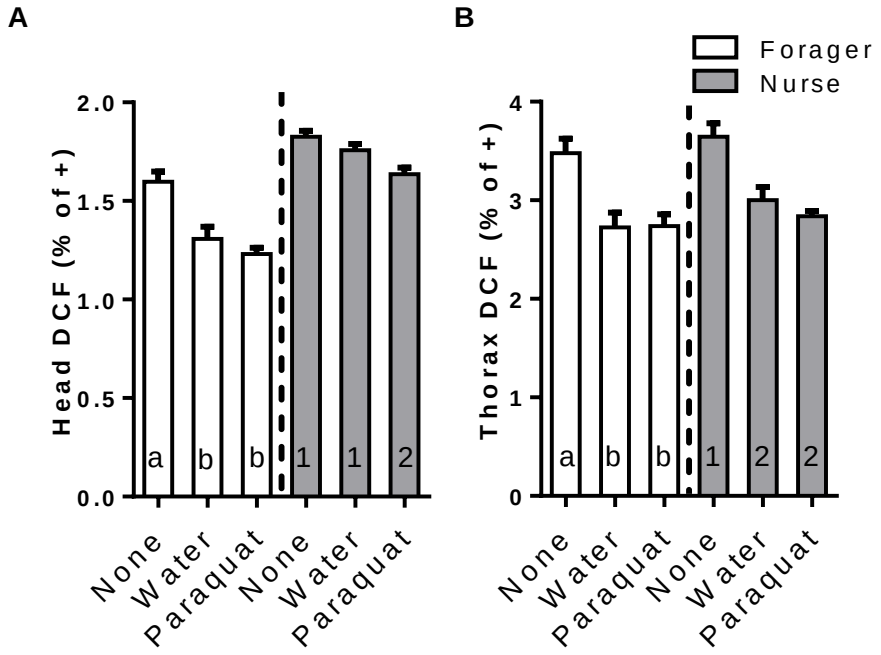
3.3.6: Hydrogen peroxide/ hydroxyl radical levels are decreased in injected bees.

Once SOD dismutates superoxide, hydrogen peroxide is produced, which also acts as an oxidant if not broken down into water and oxygen by the enzymatic

antioxidant, catalase. Hydrogen peroxide, if not acted upon by catalase, in the presence of ferrous iron reacts to produce highly reactive hydroxyl radicals though the Fenton reaction. We measured these ROS using a florescent derivative of fluorescein, 2',7'-

dichlorodihydrofluorescein diacetate (DCF-DA), which reacts with both hydrogen peroxide and hydroxyl radicals. In forager heads, (Fig. 3.7A) levels of ROS decreased in paraquat and sterile water injected bees, but in nurse heads (Fig. 3.7A). ROS levels decreased in only paraquat injected bees. In forager and nurse flight muscle (Fig. 3.7B) ROS levels were decreased by both paraquat and sterile water injections. These data suggest that an antioxidant response invoked by the injection procedure leads to decreased ROS levels.

Figure 3.7: DCF-DA levels in nurses and foragers injected with paraquat, sterile water, or no injection controls. The fluorescence of DCF-DA, which is specific for hydrogen peroxide and hydroxyl radicals was quantified in the brain tissue (A) and flight muscle (B) of age-matched foragers (white bars) and nurses (grey bars). Bees were collected from SCCs at ages 9-11, injected (or same preparation), and collected for analysis 48 hours later. Bars not connected by the same letter or number are statistically different: mixed-model ANOVA with Tukey post hoc test ($p<0.05$).



3.4: Discussion

To fully understand the relationship between honey bee flight and oxidative stress, manipulating levels of oxidative damage is critical. However, no studies in honey bees show that paraquat exposure, which is the most common method used to induce oxidative damage, actually increases markers of oxidative damage and ROS. It is unclear if paraquat ubiquitously induces oxidative stress in insects with high metabolic rates as in mammals.

We demonstrate high dose (150 μg/g wt/wt) paraquat injections do not produce the expected result of ubiquitously inducing oxidative DNA damage across behaviors and between tissues. Water injections into honey bee abdomens increase antioxidant activity and ROS generation, likely confounding the effects of paraquat exposure. Furthermore, nurse bees may be resistant to paraquat-induced oxidative stress, but may succumb to other cytotoxic effects associated with a high dose of paraquat. This response may explain previous studies that relied on death as an indicator of oxidative stress.

Paraquat is a commonly used method of inducing oxidative stress, but insects are likely well-equipped to handle high superoxide generation due to high aerobic expenditure during flight, which produces high amounts of ROS. But, insects have substantial antioxidant defenses (reviewed in Felton and Summers, 1995) expressed at high levels to combat the oxidative burden associated with flight. In addition to the effects of normal aerobic respiration, insects have added burdens that may increase oxidative insults further requiring an enhanced ability to mitigate oxidative damage relative to mammals. Insect tracheal systems deliver oxygen directly to tissues using gas

phase diffusion. The demand for oxygen is high during flight (Krogh and Weiss-Fogh, 1951), especially during honey bee flight, which has the highest mass specific metabolic rate measured thus far (Suarez et al., 1999). In the house fly, *Musca domestica*, which also has a high metabolic rate during flight, superoxide is generated at a rate 6 times higher than in mice, and SOD activity increases as superoxide levels rise (Sohal et al., 1989). In my experiments, paraquat exposure only increased superoxide levels in forager thoraces, which contain the flight muscles. However, paraquat is toxic to both nurses and foragers indicating paraquat does not ubiquitously increase ROS production as in other animals. Therefore, factors other than oxidative tissue damage likely cause paraquat toxicity in honey bees.

Because the ROS theory of aging predicts damage to lipids, proteins, or DNA leads to senescence, all markers of oxidative damage are relevant. The mechanisms of paraquat toxicity are debated (reviewed in Fukushima et al., 2002), but studies in houseflies suggest paraquat toxicity is not due to increased lipid damage, because lipid peroxides do not increase in flies exposed to paraquat (Allen et al., 1984). Although we did not measure lipid oxidation in paraquat exposed honey bees, we measured another marker of oxidative damage to DNA, 8-OHdG. In mammals, both lipid damage and DNA damage increase after paraquat exposure (Chen et al, 2010) demonstrating both markers of oxidative damage should increase after paraquat exposure. Because levels of 8-OHdG only increased in forager thoraces, but mortality was high in both nurses and foragers exposed to paraquat, these data along with the housefly data suggest oxidative damage is not the primary mechanism of paraquat toxicity in insects with high metabolic rates.

Although evidence suggests paraquat toxicity is due to oxidative damage, evidence also demonstrates paraquat toxicity is due to mechanisms other than oxidative stress. Paraquat induces lipid peroxidation in mouse lung microsomes, but not rat lung microsomes, and lipid peroxidation levels do not increase in paraquat-treated mice fed fat-free diets, although mortality is high (Shu et al., 1979). Paraquat also paradoxically inhibits iron-mediated lipid peroxidation in bovine lung microsomes (Misra and Gorsky, 1981). Permeability of the inner mitochondrial membrane increases during paraquat exposure leading to membrane depolarization, uncoupling, and matrix swelling (Constantini et al., 1995). Studies also indicate NADPH, but not NADH, depletion by paraquat leads to alveolar epithelial cell damage (Forman et al., 1980; Keeling and Smith, 1982; Nagata et al., 1987), which may be analogous to tracheal damage in insects. Additionally, paraquat either directly or indirectly damages the cytoskeleton (Milzani et al., 1997; Tomita et al., 2001) leading to cellular death. Although paraquat exposure unequivocally increases oxidative damage in many model systems, other cytotoxic stress is induced. Whether oxidative damage, and/or other cytotoxic stressors cause mortality during paraquat exposure remains unclear.

In addition to confounded mechanisms of paraquat toxicity, abdominal water injections likely induce an antioxidant response. Levels of ROS decreased in all water and paraquat injected tissues, except for nurse heads where ROS levels decreased only in paraquat injected. Stressful events in insects, such as injections, boost the immune system and activate stress defense systems (Baines et al., 1992; Mowlds et al., 2008). In bacteria-challenged mosquitoes, two oxidative stress defense enzymes, thioredoxin reductase and thioredoxin peroxidase, are present in expressed sequence tags of immune-activated

hemocytes (Bartholmay et al., 2004). Injections may induce an immune response similar to an oxidative stress response, because oxidant-based defenses, such as peroxidases, are crucial parts of insect immunity. Increased immune responses after injections likely confound the effects of paraquat exposure (Nappi and Christensen, 2005). Although a link between stress and immune responses has yet to be established in honey bees, several pieces of data suggest a connection between the two (Reviewed in Even et al., 2012; Alaux et al., 2010; Aufauvre et al., 2012; Köhler et al., 2012;). For example, honey bees previously infected with *Nosema ceranae* have increased mortality when exposed to sublethal doses of synthetic pesticides (Vidau et al., 2011). Furthermore, bacteria are likely introduced into the hemolymph during injections because of a wounding, possibly invoking an immune response.

Previous work with paraquat in honey bees demonstrates increased survival in worker bees with higher hemolymph vitellogenin titers when exposed to paraquat, and vitellogenin is preferentially carbonylated over other proteins during exposure (Seehuus et al., 2006a). Although vitellogenin is preferentially oxidized in response to paraquat exposure, no causal relationship shows vitellogenin prevents oxidative damage or mitigates senescence after damage occurs. Another study demonstrates vitellogenin binds directly to live cells, which may provide protection from oxidative damage, but also recognizes damaged cells, suggesting vitellogenin may recognize cell stress to help invoke a stress response (Havukainen et al., 2013). Vitellogenin-correlated survival during paraquat exposure is likely partially due to oxidative stress protection conferred by vitellogenin, but also from an increased general stress response from vitellogenin's cellular damage detection ability. High mortality in foragers bees exposed to paraquat is

not surprising, because forager bees typically have low vitellogenin titers compared to nurse bees (Amdam et al., 2005).

In conclusion, the results of this study indicate abdominal paraquat injections in honey bees produce only a partial oxidative stress effect and increased mortality is likely due to both oxidative damage and other cytotoxic factors associated with paraqaut exposure. However, it remains unclear if cytotoxic effects of paraquat, other than oxidative damage, are caused by increased production of superoxide and other ROS. Therefore, my data indicates injection induce an antioxidant response confounding the oxidative stress effect of paraquat. In addition to houseflies, I suggest insects with high metabolic rates may be uniquely situated to mitigate the oxidative effects of paraquat, but succumb to other cytotoxic effects. We conclude paraquat does not function as an adequate mimic of flight-induced oxidative stress. Flying bees likely evolved unique mechanisms of oxidative stress protection. So elucidating the actual mechanisms that are associated with an increase in oxidative damage accrued from honey bee flight may uncover additional ways to mitigate stress.

CHAPTER 4

EFFECTS OF FLIGHT ON GENE EXPRESSION AND AGING IN THE HONEY BEE BRAIN AND FLIGHT MUSCLE

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4.1: Introduction

Aerobic cellular respiration inevitably produces reactive oxygen species (ROS) that damage DNA, proteins, and lipids if antioxidant and repair systems are overwhelmed (Hulbert et al., 2007; Constantini, 2008). These accumulated effects of oxidative stress are the basis for the free radical theory of aging, which is widely researched, but often disputed (Harman, 1956, but see Lapointe and Hekimi, 2010; Salmon et al., 2010; Sanz and Stefanatos, 2008). Studies in social insects reveal ROS damage is important in aging, but only a single part of a more complex phenomenon (Parker, 2010). Despite the controversy among theories of aging, evidence suggests that damage to biological macromolecules readily leads to premature aging, cell death, and senescence (Chen et al., 2007) unless defense systems can be up-regulated. However, individuals with increased antioxidant capacity are not necessarily better prepared to mitigate damage from ROS (Monaghan et al., 2008). Consequently, an organism's ability to mitigate the effects of ROS changes ontogenetically across its lifetime and is affected by diet and other environmental conditions (Metcalfe and Alonso-Alvarez, 2010). While metabolically-intensive behaviors and other secondary sexual traits requiring greater ability to mitigate

ROS may be selected for as honest signals of fitness (Metcalfe and Alonso-Alvarez, 2010; Von Schantz et al., 1999; Alonso-Alvarez et al., 2007), how these physical and behavioral traits contribute to senescence is not well-known. Understanding how senescence occurs and how it is influenced by behavioral development and behavioral intensity may reveal how behavior itself can damage a cell and consequently limit lifespan.

Few studies link variation in metabolically-intensive, naturally-occurring behaviors to oxidative stress, fitness, and lifespan (Metcalfe and Alonso-Alvarez, 2010). In this study, we use the experimental tractability of the honey bee (*Apis mellifera*), to examine links between behavior, oxidative stress, and senescence. Senescence in traditional model organisms such as mice, rats, flies, and nematodes is characterized by irreversible aging as time progresses, while honey bee aging is directly related to behavioral state. The non-reproductive female worker caste exhibits a behavioral plasticity; termed "temporal polyethism (see Fig. 1.1) (Elekonich and Roberts, 2005)," where "nurses" transition to "foragers" in response to a multitude of environmental and physiological factors (Robinson, 1987; Pankiw et al., 1998). The pace of these transitions can be increased, decreased, or reversed by controlling these cues (Elekonich and Roberts, 2005).

Typically, during the first 2–3 weeks of adult life, female workers perform hive maintenance and brood care, or nursing, during which they rarely fly. After transitioning to foraging, workers can fly long distances (up to 8 km) gathering nectar and pollen for several hours per day (Winston, 1987). Once honey bees begin to forage for pollen and nectar, their aerobic metabolism greatly increases. Foraging honey bees have a metabolic rate of 100–120 mL O_2 g^{-1} h^{-1}, which is the highest mass-specific metabolic rate known

and is 10–100 times higher than in nurse bees, which fly much less often (Suarez et al., 1996). Previous experiments from my laboratory suggest that the elevated metabolism of flying honey bees likely produces high levels of ROS that, coupled with an age-dependent decrease in antioxidant activity, negatively affects longevity (Williams et al., 2008). Additionally, foraging bees show a decline in flight capacity with age (Vance et al., 2009) and time spent flying is negatively correlated with survivorship (Visscher and Dukas, 1997). When the transition from nurse to forager is delayed, bees that stay in the hive can live up to 8 times longer than bees that transition naturally (Rueppell et al., 2007).

Another aspect of the plasticity of honey bee aging is the ability to revert from foraging tasks to in-hive nursing duties (Robinson et al., 1992; Huang and Robinson, 1996), with an accompanying reversion of many physiological characteristics. During behavioral reversion, hypopharyngeal glands (which produce food that young larvae consume and that atrophy in foraging bees) redevelop (Amdam et al., 2004; Baker et al., 2012), juvenile hormone titers drop (Robinson et al., 1992; Huang and Robinson, 1996), and vitellogenin levels increase (Amdam et al., 2005). Reverted nurse bees also undergo a reversal of the immunosenescence observed in foraging bees (Amdam et al., 2005). Foragers' age-related learning deficits also reverse during behavioral reversion (Baker et al., 2012). Despite exhibiting many of the physiological traits of typical nurse bees, reverted nurse bees are not identical to typical nurse bees. Reverted nurse bees have a mixed proteomic profile that is similar to both nurses and foragers (Wolschin and Amdam, 2007). Additionally, some foragers appear to reach a threshold where they are unable to revert and continue to progress towards functional senescence (Wolschin and

Amdam, 2007). It remains unclear if reverted nurse bees revert at the genomic level or if the effects of extended flight bouts on gene expression are permanent.

Here we examine how performance of behaviors with low *vs.* high metabolic cost affects gene expression in the flight muscle and the brain as bees transition to foraging and during reversion from forager to nurse. We chose to do this analysis on both tissues because brain tissue is particularly susceptible to oxidative stress (Bowling and Beal, 1995) and flight muscle experiences oxidative stress resulting from flight (Williams et al., 2008). In these experiments, we compared whole-genome transcriptional profiles of nurse bees and forager bees of different ages with different flight histories, including reverted nurse bees. We found that patterns of transcription differ between tissues in response to flight and that these changes can partially revert. We identified particular transcripts involved in stress response pathways that are differentially expressed between bees of various ages and with various flight experiences.

4.2: Methods

4.2.1: Field methods

Honey bees (*Apis mellifera* L.) used for this project were reared at the University of Nevada, Las Vegas apiary. For the microarray experiments and follow-up mRNA quantification of immune genes, 4 source colonies headed by unrelated single-drone inseminated queens (Glenn Apiaries, Fallbrook, CA, USA) carrying the Minnesota (MN) varroa sensitive hygienic (VSH) genotype were used to obtain age/genotype-matched workers to start single-cohort colonies (SCCs). Single, drone-inseminated queens were chosen to head the source colonies for the microarray experiment to decrease the genetic

variability between worker bees. From these source colonies, eight SCCs were created using a frame of pollen, an open frame for the SCC's queen to lay in and a frame of honey. Each SCC contained approximately 2,000 bees originating from multiple source colonies. A SCC uses skewed colony age demography to induce precocious foraging in approximately 10% of the bees at 7–10 days of age, whereas the onset of foraging normally takes place at 21–25 days of age (Giray and Robinson 1994). The SCC allows the effects of age and behavior to be decoupled, permitting comparison of same-aged bees with drastically different flight histories and bees of different ages with the same behavioral activity.

Reversion colonies were made from 4 of the original SCC colonies (3 in June and 1 in July). Reversion colonies were made by collecting 1,000–3,000 19–22 day old foragers from a SCC colony's entrance and placing that colony's foragers into a new nucleus hive containing one frame of open larvae (1st–5th instar) with occasional capped brood, one frame each of honey and pollen, one sugar water in-hive feeder (half filled), a small pollen patty, and a Bee Boost strip at the center as source of queen mandibular pheromone. Foragers from separate colonies were not mixed together. This was done for each of the 4 colonies in turn resulting in 4 reversion colonies. The original colonies and their queens were moved out of the bee yard. The reversion colonies were moved to the location of the parent colonies and kept closed for three days to keep the foragers inside and force some to revert. Although unconventional, we chose to confine bees to the colony for three days to induce more bees to detectably revert (as suggested by Zachary Huang pers. comm.). Using my altered methodology, more foragers reverted and reverted nurses were more clearly attending to brood compared to my pilot study, which used

previously described methods that do not include confinement (Amdam et al., 2005; Baker et al., 2012; Wolschin and Amdam, 2007). This set of manipulations induced a small portion of the foragers in each of the 4 colonies to revert to nursing tasks (Robinson et al., 1992; Huang and Robinson, 1996; Page et al., 1992). Reverted nurse bees were then identified and collected.

In a separate follow-up experiment, additional SCCs were made to measure mRNA levels in genes known to play a role in aging (Imai et al., 2000; Wu et al., 1999; Rogina et al., 2000). Two source colonies headed by naturally-mated queens were used to obtain workers to start four SCCs. Each SCC contained bees from multiple colonies. Naturally-mated queens were used in this experiment to mimic typical hive conditions, which naturally have high genetic variability. Because the SCCs for the experiment above were made from only four source colonies headed by SDI queens, those colonies would represent at most four patrilines and four matrilines. In contrast, these SCCs made from source colonies with naturally mated queens like had 10–40 times more genetic variability than SCCs in the first experiment. The SCCs were created using seven frames of brood from the source colonies. The frames were placed in an incubator (32 °C, 75% relative humidity RH, 24 h dark cycle) and newly-eclosed bees were removed from the frames every 24 h. A SCC was formed by housing approximately 2,000 single, day-old workers from, which eclosed over two consecutive days, with a naturally mated queen (Koehnen Apiaries, CA, USA). These bees were placed in a nucleus colony containing one frame each of pollen and honey and three empty frames to allow the queen to lay eggs. The dorsal thorax of each bee was marked with a single dot of paint (Testors, Rockford, IL, USA) to indicate age, prior to placing them in their SCC. Each SCC was

kept in the laboratory for five days post-adult emergence to allow for young worker adult maturation and queen egg laying before being moved to the outdoor apiary on the UNLV campus.

4.2.2: Behavioral groups

Foraging bees were identified as bees returning to the hive with a pollen load or distended abdomens from nectar. Nurse bees were identified as individuals placing their heads into frame cells containing an egg or larva (Robinson, 1987). Once identified behaviorally, bees were marked with a dot of paint, and only marked foragers and nurses were used for these analyses. Sample sizes collected were n = 5–6 per group for the microarray and immune gene experimental colonies and n = 12 per group for the aging gene experiments. Bees were immediately placed in liquid nitrogen upon collection and stored at −80 °C until processing so that mRNA levels accurately reflected natural differences in gene expression.

To separate the effects of age and behavior, nurse bees and forager bees were collected at various time points (8–10 days, 19–22 days, and 25–26 days) with various flight experiences ranging from less than one day of flight experience to over 25 days (See Table 1 for more details). For the aging genes experiment, the same collection regime was followed except collection days for nurses and foragers were extended until 40 days past eclosion so that any pronounced changes in gene expression (mRNA levels) could be detected. Because this second experiment was concerned with expression of genes involved in aging, we did not create reversions. During all experiments, capped brood was removed and replaced with empty frames to encourage the queen to lay eggs

normally and to prevent any new bees from eclosing and changing the demography of the SCCs.

Table 4.1. Behavioral Groups. Group of nurse bees and forager bees with varying amounts of flight were used in the microarray and real-time PCR experiments. Abbreviations are used throughout experiments.

Behavioral Group	Abbreviation	Age (days)	Days of flight
Young nurse	YN	8 to 10	< 1
Precocious forager	PF	8 to 10	2 to 3
Older nurse	ON	19 to 22	< 1
Forager - low flight	TH	19 to 22	2 to 3
Forager - high flight	TL	19 to 22	7 to 9
Forager - old	OF	25 to 26	10 to 12
Reverted nurse	RN	25 to 26	7 to 9

4.2.3: RNA extraction

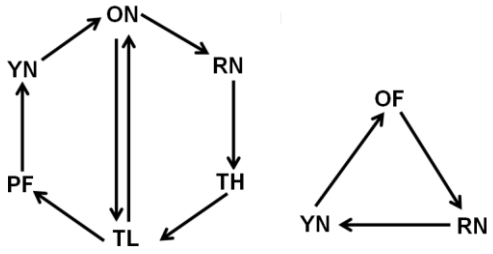
Whole bee heads and thoraces were partially lyophilized at −70 °C to facilitate dissection (36). Heads and thoraces were then dissected on dry ice to prevent RNA degradation. Care was taken to avoid including the hypopharyngeal or subesophageal glands in brain samples (leaving the optic lobes, antennal lobes, and mushroom bodies) and to precisely obtain the primary flight muscles of the thoraces. The high yields of RNA needed for microarray experiments were extracted from dissected brains and thoraces using PicoPure RNA Isolation kits according to the manufacturer's protocol

(Molecular Devices, Sunnyvale, CA, USA). For the aging genes real-time PCR experiment, which required less RNA than the microarray experiment, RNA was extracted using 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

4.2.4: Microarray hybridization

Brains and thoraces from the same individuals of all behavioral groups were compared on a total of 132 arrays. The samples were hybridized against each other using a loop design (Grozinger et al., 2003) (See Fig. 4.1 for experiment design). The microarray hybridization procedure followed previously described methods (38). Extracted total RNA, cDNA, and labeled aRNA sample concentrations were quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Five hundred nanograms of RNA were amplified using the MessageAmp II aRNA amplification kit (Ambion, Austin, TX, USA). For each individual, 4 micrograms of brain RNA were labeled with cy3 and 4 micrograms of thorax RNA were labeled with cy5 using a Kreatech Labeling Kit (Applied Biosystems, Salt Lake City, UT, USA). The directionality of the dye labeling was switched between replicates to avoid a dye bias. Whole transcriptome oligonucleotide arrays (W.M Keck Center for Comparative and Functional Genomics, University of Illinois, Urbana-Champaign) were hybridized with 120 picomoles of each labeled tissue's probes. Arrays were then scanned with a GenePix scanner (Molecular Devices, Sunnyvale, CA, USA) and visualized using GenePix software (Agilent Technologies, Santa Clara, CA, USA). Prior to data analysis, each array was visually inspected for any inconsistencies in dye incorporation.

Figure 4.1. Microarray experimental design. The microarray experiment was designed with 132 arrays in a "round-robin" style. Each arrow represents 6 arrays. **YN** = young nurse (10 days-old; <1 day flight); **RN** = reverted nurse (25–26 days-old; 7–9 days flight); **OF** = old forager (25–26 days-old; 10–12 days flight); **TH** = typical-aged forager-high flight (19–22 days old; 7–9 days flight); **ON** = older nurse (19–22 days old; <1 day flight); **TL** = typical-aged forager-low flight (19–22 days old; 2–3 days flight); **PF** = precocious forager (8–10 days old; 2–3 days flight).



4.2.5 Microarray data analysis

Analysis was implemented in R version 2.8 (R Core Development Team, 2011). Data was normalized using the Limma package in R (Smyth and Limma, 2005). Spots were log-transformed and within-slide normalization was conducted using a print-tip Loess to correct for differences between print-tips on the array printer and variation during the print run (Smyth and Limma, 2005). No background correction was used for within-slide normalization. For between-slide normalization, a quantile normalization method was used (Smyth and Speed, 2003). Pre- and post- normalization MA plots were generated to ensure each array was of acceptable quality to use. To detect differential expression, we used a mixed-model two-way ANOVA from the MAANOVA package in R (Cui and Churchill, 2003; Cui et al., 2005). The model was fit with treatment and dye as fixed effects and array as a random effect. Contrast statements were used to identify transcripts that were differentially expressed between different tissues. Transcripts were considered

differentially expressed when their interactions yielded an adjusted p-value (FDR) of less than 0.05 (Storey et al., 2003). Gene ontology analysis was conducted using ArrayTrack (Tong et al., 2003). The microarray data for this manuscript were submitted to the NCBI Gene Expression Omnibus database (accession number: GSE40650).

4.2.6: Quantitative real-time polymerase chain reaction

We performed quantitative real-time PCR on flight muscle for 6 immunity genes and on brains and flight muscle for 3 aging genes that yielded statistically significant results in the microarray experiments. For the immunity genes, cDNA was synthesized from 200 ng of RNA used for the microarray experiments and for the aging genes, 200 ng of RNA was used following manufacturer's instructions using a qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). qRT-PCR was performed on an iCycler iQ (BioRad, Richmond, CA, USA) using the SYBR green detection method (Quanta Biosciences, Gaithersburg, MD, USA). Five individual thoraces were used for the immunity gene analysis and brains and thoraces from five different individual bees were used for the aging genes experiment. As a transcriptional control, mRNA levels of the genes of interest were measured relative to the housekeeping gene, ribosomal protein 49 (*rp49*). Statistical analyses were done using delta Ct values using a two-way analysis of variance with age and behavior as the main effects and colony and PCR plate as random effects in JMPv8.0 (SAS Institute, Cary, NC, USA)

4.3: Results and discussion

4.3.1 Behavior induces dramatic changes in global gene expression of A. mellifera brains and thoraces

In this study, we compared age-matched nurse bees and forager bees with differing amounts of prior flight activity to examine gene expression associated with flight in brain tissue and flight muscle. Using whole-genome oligonucleotide arrays, either 6 or 12 biological replicates (Fig. 4.1) were used for each group involved in this analysis. By using a manipulation that causes forager bees to revert back to nurse bees, we were able to determine the reversibility of the transcriptional profile of these bees (Fig.4.2A). Groups more closely related in age had the most similar transcriptional patterns (Fig. 4.2B). Similar to the findings of a previous study looking at brain transcriptional patterns (Whitfield et al., 2003), we found that honey bee flight induces unique patterns of expression across the genome in brain tissue, but in the flight muscle we saw age-related patterns of expression similar to expression patterns in *Drosophila* thoraces (Giradot et al., 2006).

In brain tissue (Fig. 4.2A), gene expression patterns between young, aged-matched (8–10 days old) nurses and foragers were the least similar. These expression differences were not as pronounced between older nurses (19–22 days old; <1 day flight experience) and foragers (19–22 days old; 2–3 days flight experience or 7 to 9 days flight experience). In flight muscle (Fig. 4.2A), reverted nurse transcriptional patterns were most related to older nurses (19–22 days old; <1 day flight experience) followed by typical-aged foragers (19–22 days old with either 2–3 days flight experience or 7 to 9 days flight experience). We found no effect of the number of days spent flying,

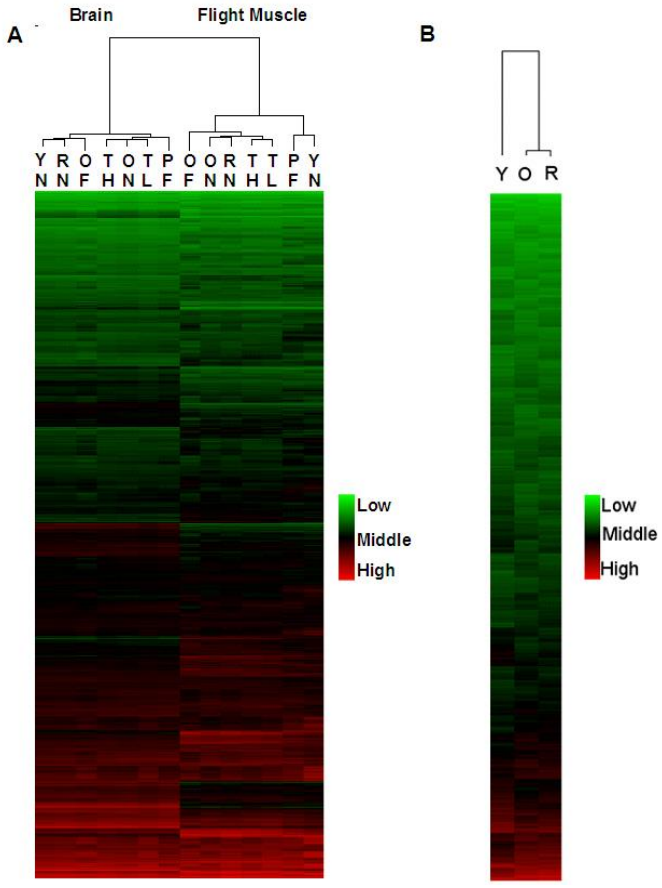
independent of age, in either tissue. This suggests that the transition to foraging behavior and the interaction between age and frequent flight have more impact on gene expression and senescence than the actual amount of flight. Over the course of an entire foraging day, acute effects of flight have an effect on levels of heat shock proteins and antioxidant activity, but behavioral state (nurse or forager) does not have an effect (Williams et al., 2008). Consequently, behavioral state affects many transcripts, such as those involved in signaling pathways. Because no transcriptional changes between high and low flight foragers were detected, but changes between age-matched nurses and foragers were present, this suggests the transcriptional differences are due to both flight and caste-specific behavioral/physiological differences. To fully separate acute effects of flight and effects of physiology/behavior, nurse bees with high amounts of flight time would be ideal. However, obtaining these bees in a natural hive or SCC is difficult and likely unfeasible at a large scale. To more closely examine the effects of flight, my ongoing studies are examining foragers restricted from flying, foragers with high and low amounts of flight time, and nurse bees. Longevity in honey bees is directly related to flight activity (Neukirch, 1982). As the transition from in-hive nursing tasks to outside foraging tasks occurs, senescence accelerates, and time spent foraging is negatively correlated with survivorship (Visscher and Dukas, 19978). After approximately 14 days of foraging, foragers experience cognitive decline (Behrends et al., 2007) and oxidatively-damaged proteins accumulate in the brain (Seehuus et al., 2006). As my lab has previously shown, a decrease in antioxidant activity also occurs in forager and nurse flight muscle, but not the brain, of bees greater than 30 days of age (Williams et al., 2008). As foraging time increases bees show a decline in flight capacity (Vance et al., 2009). However, longevity

increases when the transition from nurse to forager is delayed (Rueppell et al., 2007). Together, the results of previous studies and the results from this study suggest that brain tissue and flight muscle respond differently to cellular stress. These results also imply that the brain is perhaps more effectively able to mitigate flight associated cellular stress. Flight muscle is the most metabolically active tissue in the honey bee (Suarez et al., 1996); hence it experiences greater levels of ROS production. As a result, the honey bee's ability to mitigate stress in this tissue may become compromised as foraging time increases. Indeed our previous work suggests older foragers are less able to mitigate that stress (Williams et al., 2008). Once the flight muscle is compromised, the honey bee forager is essentially ecologically dead even if the brain is not yet showing signs of senescence. Because brain tissue is particularly susceptible to stress (Bowling and Beal, 1995), the honey bee brain may possess additional mechanisms to mitigate flight-associated stress that are absent in flight muscle. However, even protective mechanisms in the brain may be eventually overcome leading to cognitive or whole-organism functional senescence.

Previous studies show that under certain conditions forager bees can behaviorally revert and return to nurse behaviors (Robinson et al., 1992; Huang and Robinson, 1996) and that this reversion is also accompanied by physiological reversions. During reversion, JH levels drop (Robinson et al., 1992; Huang and Robinson, 1996), vitellogenin levels increase (Amdam et al., 2004), hypopharyngeal glands redevelop and immunosenescence reverses (Amdam et al., 2005). In general, my results reveal that, at the transcriptional level, there also exists a reversion, but some aspects of forager gene expression patterns persist (Fig. 4.2A). This intermediate pattern of transcription is likely due to the

combined effects of both behavioral/physiological differences between nurses and foragers as well as the effects of flight. In brain tissue, reverted nurse transcriptional patterns are most closely related to those of young nurses (8–10 days old; <1 day flight experience). However, in flight muscle, reverted nurse transcriptional patterns were most

Figure 4.2. Global expression analysis. Hierarchical clustering was performed to visualize global patterns of gene expression. Replicates were averaged before analysis. Each graph represents over 12,000 transcripts and each row of the graphs represents one transcript. (**A**) Unique patterns of transcription were seen between groups that represented various ages and behavioral groups with varying amounts of flight experiences. Unique expression patterns were also seen between tissues. (**B**) Hierarchical clustering reveals groups more closely related in age are most related. **YN** = young nurse (8–10 days-old; <1 day flight); **RN** = reverted nurse (25–26 days-old; 7–9 days flight); **OF** = old forager (25–26 days-old; 10–12 days flight); **TH** = typical-aged forager-high flight (19–22 days old; 7–9 days flight); **ON** = older nurse (19–22 days old; <1 day flight); **TL** = typical-aged forager-low flight (19–22 days old; 2–3 days flight); **PF** = precocious forager (8–10 days old; 2–3 days flight). **Y** = young bees (8–10 days-old); **O** = old bees (25–26 days-old foragers; 19–22 day-old nurses); **R** = reverted nurse bees (25–26 days-old; 7–9 days flight).



related to older nurses (19–22 days old; <1 day flight experience) followed by typical aged foragers (19–22 days old with either 2–3 days flight experience or 7 to 9 days flight experience). This result suggests the brain has more transcriptional plasticity than flight muscle, which may allow the brain to more effectively mitigate stress.

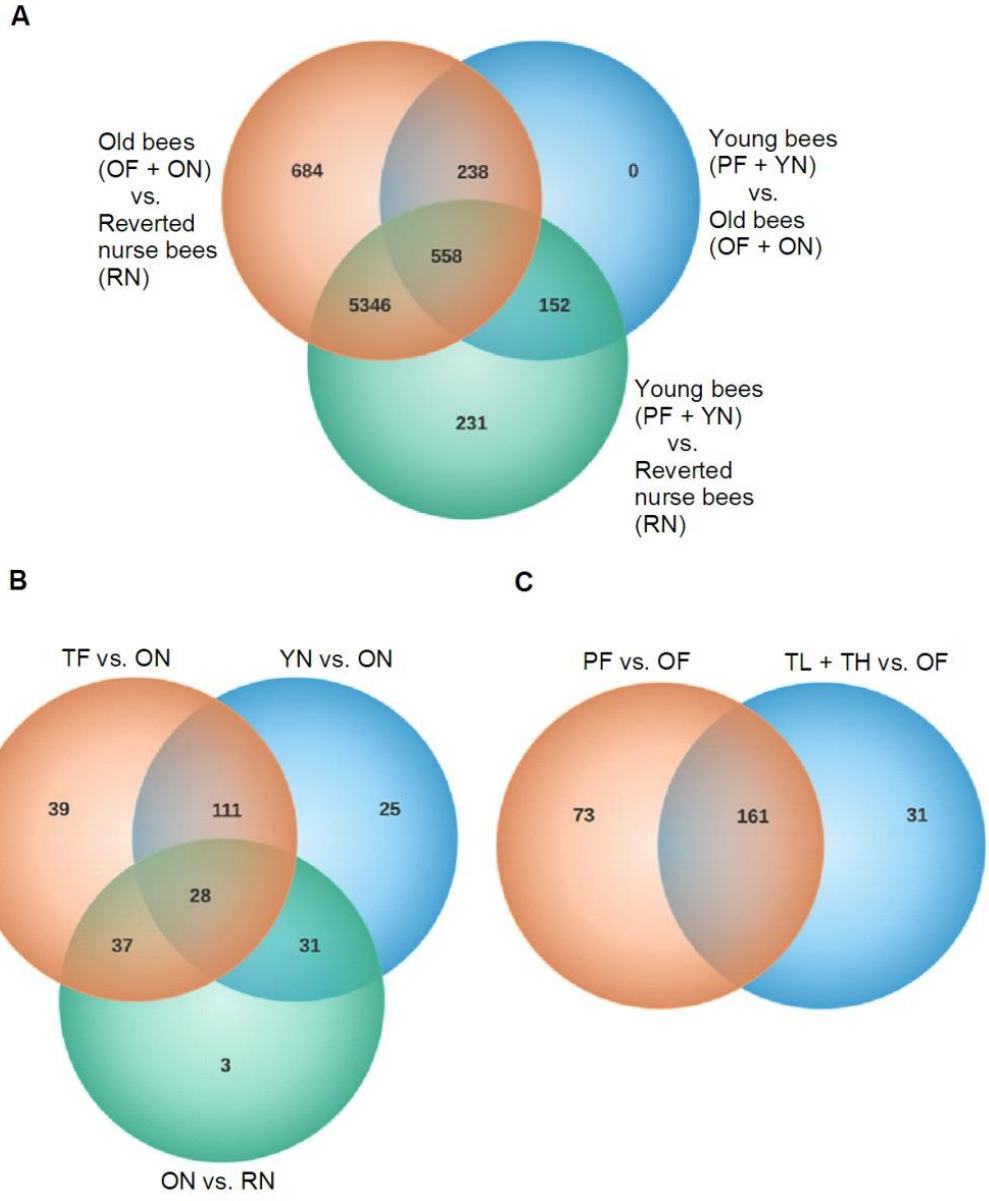
After a small pilot study using the reversion methods of previous studies (Robinson et al., 1992; Huang and Robinson, 1996; Amdam et al., 2005; Baker et al., 2012; Wolschin and Amdam, 2007) yielded only a small number of reverted foragers exhibiting clear nursing behavior, we chose to modify the reversion method as noted above. Our modified method used queen pheromone in place of a queen, and confinement of foragers to the hive during the reversion time. This strategy induced more frequent and less ambiguous reversion perhaps because closing the colony blocks out photoperiod, allowing the reverted foragers to more easily assume a more nurse-like circadian rhythm. Despite these differences, our transcriptional data agree with Wolschin et al. (2007), which show the proteomic signature of reverted nurses is a mix between nurses and foragers. Additionally our transcriptional data and the proteomic data of Baker et al. (2012) show reversion affects levels of cellular stress transcripts or proteins. Because of experimental agreement between our results and others, we conclude that our reversion technique and collection regime, albeit slightly different than other reversion techniques (Amdam et al., 2005; Milojevic, 1940), produced a comparable reversion. While it is possible that confining foragers to the hive during reversion induced a response that altered transcriptional patterns, this response may also be present in other reversion techniques as reversion may be a response to colony level stress. Additionally, Baker et al. (2012) and Wolschin et al. (2007) allowed the reversion to continue longer than three days before

collection. Thus, it is possible that some effects of reversion that do not occur immediately were undetectable in this study.

4.3.2: Unique and shared transcripts between behaviors and ages

We used a two-way ANOVA to find transcriptional differences between young bees, older bees and reverted nurse bees as well as between different aged nurses and between different aged foragers. Statistically significant differences in transcript expression were found by using the false discovery rate for multiple comparisons. Fig. 4.3A shows which differentially expressed transcripts were unique to each age comparison and which transcripts overlapped. We found 684 differentially expressed transcripts unique to young bees (PF and YN) compared to old bees (OF and ON) and 231 transcripts unique to young bees (PF and YN) compared to reverted nurse bees (RN). This result implies only a small subset of the genes differentially transcribed during the nurse-to-forager transition do not revert during reversion from forager to nurse. There were no unique transcripts when old (OF and ON) bees were compared to reverted nurse bees (RN). When we analyzed what transcripts were unique within nurses and within foragers (Fig. 4.3B,C), we found that foragers had more differentially expressed transcripts than aged-matched nurses, indicating that gene expression varies with age and behavior but flight has a greater effect on gene expression than age. This result is not surprising because flight produces high levels of reactive oxygen species and flight muscle antioxidant capacity decreases with age (Williams et al., 2008), both of which may lead to changes in gene expression.

Figure 4.3. Unique and shared transcripts. Venn diagrams were used to visualize transcripts unique and shared between each group. Numbers represent transcripts differentially expressed (FDR < 0.05). (**A**) Transcripts differentially expressed between young bees, old bees and reverted nurse bees. (**B**) Transcripts differentially expressed between nurse bees. (**C**) Transcripts differentially expressed between forager bees. **YN** = young nurse (8–10 days-old; <1 day flight); **RN** = reverted nurse (25–26 days-old; 7–9 days flight); **OF** = old forager (25–26 days-old; 10–12 days flight); **TH** = typical-aged forager-high flight (19–22 days old; 7–9 days flight); **ON** = older nurse (19–22 days old; <1 day flight); **TL** = typical-aged forager-low flight (19–22 days old; 2–3 days flight); **PF** = precocious forager (8–10 days old; 2–3 days flight).



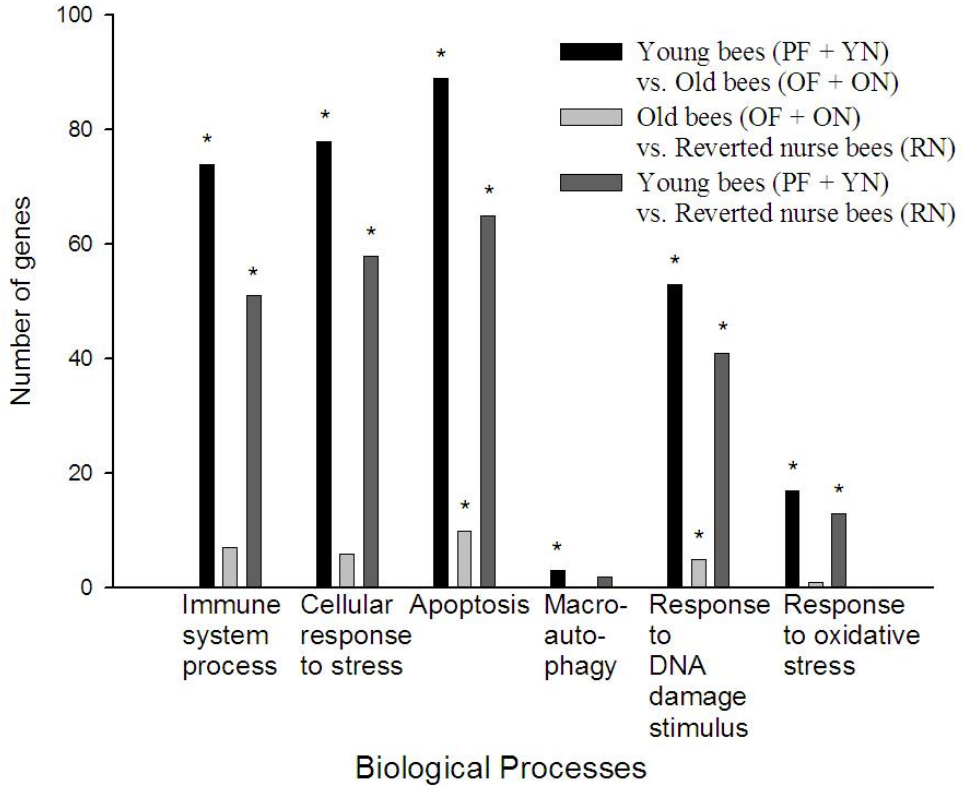
4.3.3: Gene ontology analysis reveals age causes a decrease in certain stress and immune processes in *A. mellifera*

To put the changes in gene expression we identified into a functional context, we performed a gene ontology and biological pathway analysis in ArrayTrack. Fig. 4.4 shows six biological processes relevant to immune function and aging/senescence. In general, young bees compared to old bees had the most differentially expressed transcripts involved in any particular biological process, while young bees (PF and YN) compared to reverted nurse bees (RN) had considerably fewer differentially expressed transcripts between each other, if any at all. All three comparison groups yielded statistically significant enrichment of response to DNA damage stimulus (GO:0006974) and apoptosis (GO:0006915).

In our specific comparison of old bees (ON and OF) *vs.* reverted bees (RN), we found two gene ontology categories, apoptosis (GO:0006915) and response to DNA damage stimulus (GO:0006974), statistically enriched. These results may suggest that reversion from forager to nurse may increase DNA damage repair thus decreasing apoptosis and increasing lifespan. In *Drosophila*, apoptosis increases with age, limiting lifespan (Zheng et al., 2005), and so honey bees may employ a mechanism during behavioral reversion that could decrease apoptosis to delay functional senescence. Additionally, macroautophagy (GO:0016241) was significantly enriched in young (PF and YN *vs.* old bees (OF and ON) but not in any other comparisons. Under most cellular conditions, autophagy promotes cell survival by adapting the cell to various conditions of stress. However, if autophagic activity is lost, cell death is accelerated leading to functional

senescence (Vicencio et al., 2008). The transition to foraging behavior contributes to a loss of autophagy, thus increasing senescence in the honey bee.

Figure 4.4. Comparison of biological processes. Gene ontology analysis revealed a series of biological processes involved in immune function and aging/senescence that were enriched in each comparison. The x-axis represents the number of genes differentially expressed for the biological processes represented on the y-axis. Stars represent a Fisher p-value <0.05. **YN** = young nurse (8–10 days-old; <1 day flight); **RN** = reverted nurse (25–26 days-old; 7–9 days flight); **OF** = old forager (25–26 days-old; 10–12 days flight); **ON** = older nurse (19–22 days old; <1 day flight); **PF** = precocious forager (8–10 days old; 2–3 days flight).

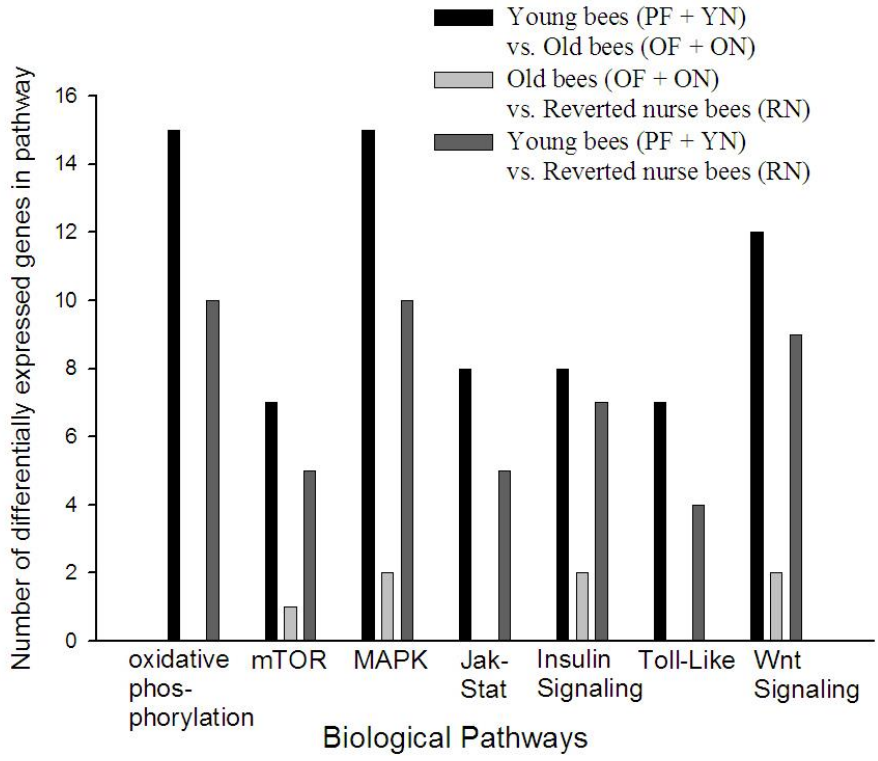


4.3.4. KEGG pathway analysis reveals age alters specific signaling pathways in A. mellifera

Along with the gene ontology analysis, we conducted a biological/biochemical pathway analysis to putatively identify pathways that differed in the expression of their

constituents between age/behavioral groups (Fig. 4.5). The analysis was implemented in ArrayTrack using the KEGG database of pathways. Similar to biological processes, young bees compared to old bees had the most differentially expressed transcripts in each pathway, while young bees compared to reverted nurse bees had far fewer differentially expressed transcripts and in some cases none at all. The oxidative phosphorylation, Jak-Stat, and Toll-like receptor pathway signaling pathways were not represented in old bees (OF and ON) compared to reverted nurse bees (RN), but pathways such as MAPK and mTOR were represented. These results suggest a number of distinct biochemical pathways are activated or repressed with age and the reversion from forager to nurse.

Figure 4.5. Comparison of signaling pathways. The KEGG database revealed pathways and the number of differentially expressed (FDR < 0.05) transcripts. The x-axis represents the number of genes differentially expressed for the biological pathway represented on the y-axis. **YN** = young nurse (8–10 days-old; <1 day flight); **RN** = reverted nurse (25–26 days-old; 7–9 days flight); **OF** = old forager (25–26 days-old; 10–12 days flight); **ON** = older nurse (19–22 days old; <1 day flight); **PF** = precocious forager (8–10 days old; 2–3 days flight).

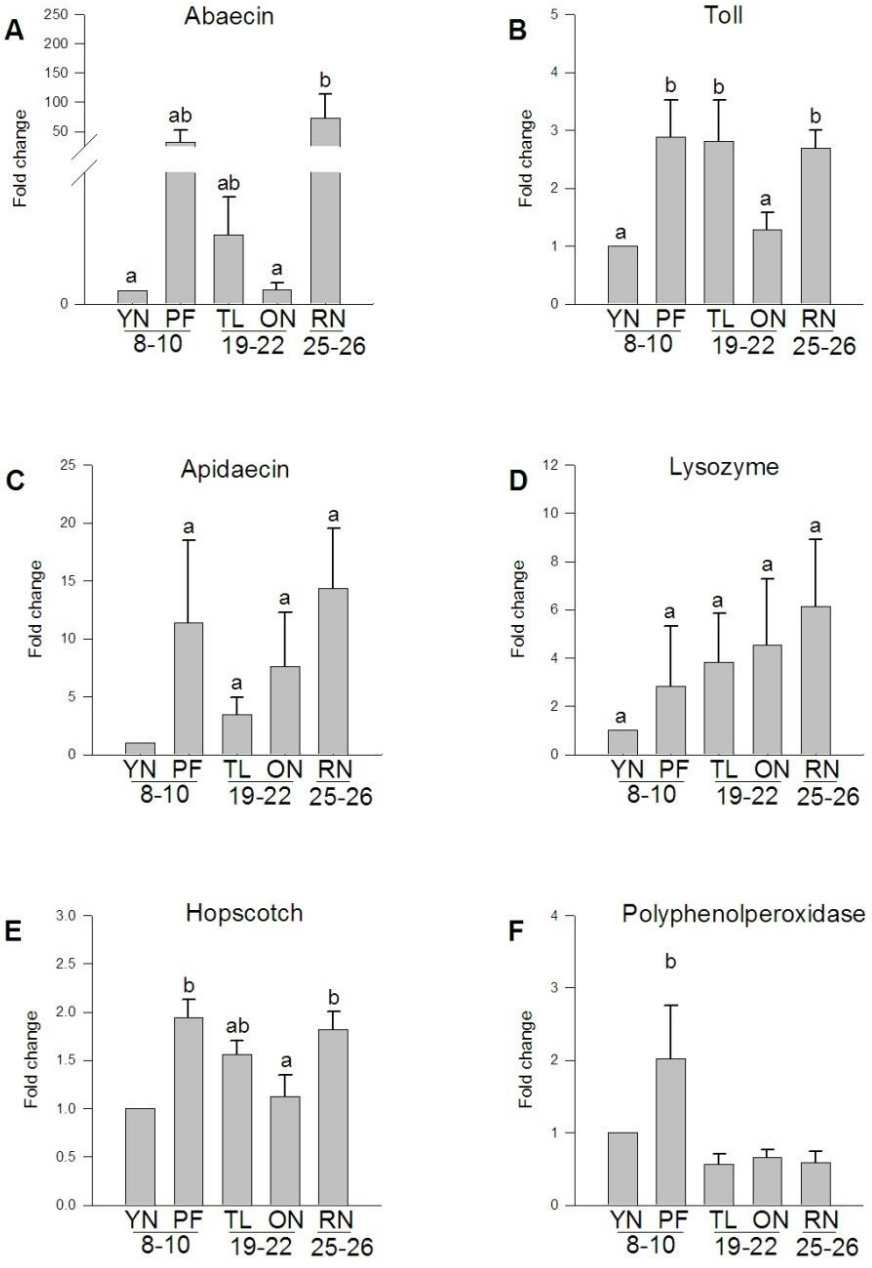


4.3.5: Expression of transcripts involved in the A. mellifera immune response

As nurse bees transition to foraging, systemic levels of JH increase (Huang and Robinson., 1992), and this increase in JH is correlated with a dramatic loss of hemocytes (Amdam et al., 2004; Rutz et al., 1974; Willie and Rutz, 1975). With the loss of functional hemocytes, the nodulation response, a key aspect of the insect infection response, is lost (Bedick et al., 2001; Franssens et al., 2005). Levels of both JH and hemocytes are restored during reversion of foraging bees to nurse bees (Amdam et al., 2005). These results suggest that loss of immune function may contribute to functional senescence in foraging honey bees. In a study measuring expression of a variety of immunity transcripts in adult worker bee abdomens Evans et al. (2006) found 6 immunity transcripts were highly up-regulated following injection of pathogens or wounding, and 25 immunity transcripts remained unchanged. A bee's status as a forager or nurse, therefore, could be more important in determining immune function than its age because as intensity of flight activity increases, proper immune responses may decrease.

We determined whether different amounts of flight and behavioral reversion have an effect on the expression of a subset of these immunity transcripts identified as differentially expressed in our flight muscle microarray data (Fig. 4.6). We measured mRNA levels in 2 bee-specific immunity transcripts: apidaecin (*apid1*) and abaecin (LOC406144), 2 immune signaling pathway transcripts: Toll-like receptor (*tlr1*) and hopscotch (*hop*), which is part of the Jak-Stat signaling pathway, and 2 transcripts involved in insect innate immunity: lysozyme (*lys1*) and polyphenoloxidase (*ppo*) using qRT-PCR. If the loss of immune function were primarily due to age, one would expect to see decreased expression of all of these immune function genes with aging in nurses as well as foragers.

Figure 4.6. Expression of immune transcripts in *A. mellifera* flight muscle. Quantification of mRNA levels of bee specific immune transcripts (**A**, **C**), transcripts involved in immune signaling pathways (**B**, **E**), and innate immune response transcripts (**D**, **F**) revealed differential expression between ages and behaviors. Fold change represents the relative difference compared to one day old bees.



Expression of apidaecin mRNA (Fig. 4.6A) was similar between bees performing different behaviors regardless of age and no statistically different expression patterns by age or behavior alone were apparent (Fig. 4.6C). Expression of both transcripts involved in immune signaling pathways (toll-like receptor and hopscotch) was up-regulated with the transition to foraging and reverted nurse bee expression levels were more similar to those of forager bees than to nurse bees (Fig. 4.6B,E). Expression of lysozyme did not statistically differ with age or behavior (Fig, 4.6D). As foraging flights increased, mRNA levels of polyphenoloxidase decreased and remained lower when reverted bees returned to in-hive nursing tasks (Fig. 4.6F).

These data suggest that for nurse bees, age is not the primary influence on the expression of these transcripts in flight muscle. Although the expression of most immune transcripts did not change with age, some transcripts in flight muscle were behaviorally up-regulated as nurses transitioned to foragers. This transition increases levels of ROS in the flight muscle as both metabolic rate and metabolic capacity increase (Harrison, 1986). In mammals, reactive oxygen species stimulate innate immunity signal transduction pathways (Kim et al., 2002), such as the toll-like receptor signaling pathway (Kohchi et al 2009). Because honey bees have activity rates and levels of endothermy more similar to mammals than most insects, (Stokes, 1987; Harrison et al., 1996) this immune pathway upregulation may also occur in bees and other active, strongly endothermic insects. In all of the immune transcripts we measured, reverted nurse bees had mRNA levels similar to that of foragers, suggesting transcripts involved in the immune response may not revert during transition from forager to nurse. Another explanation for absence of detectable changes in reverted nurse bees may that reverted nurse bees were collected 3 days post-

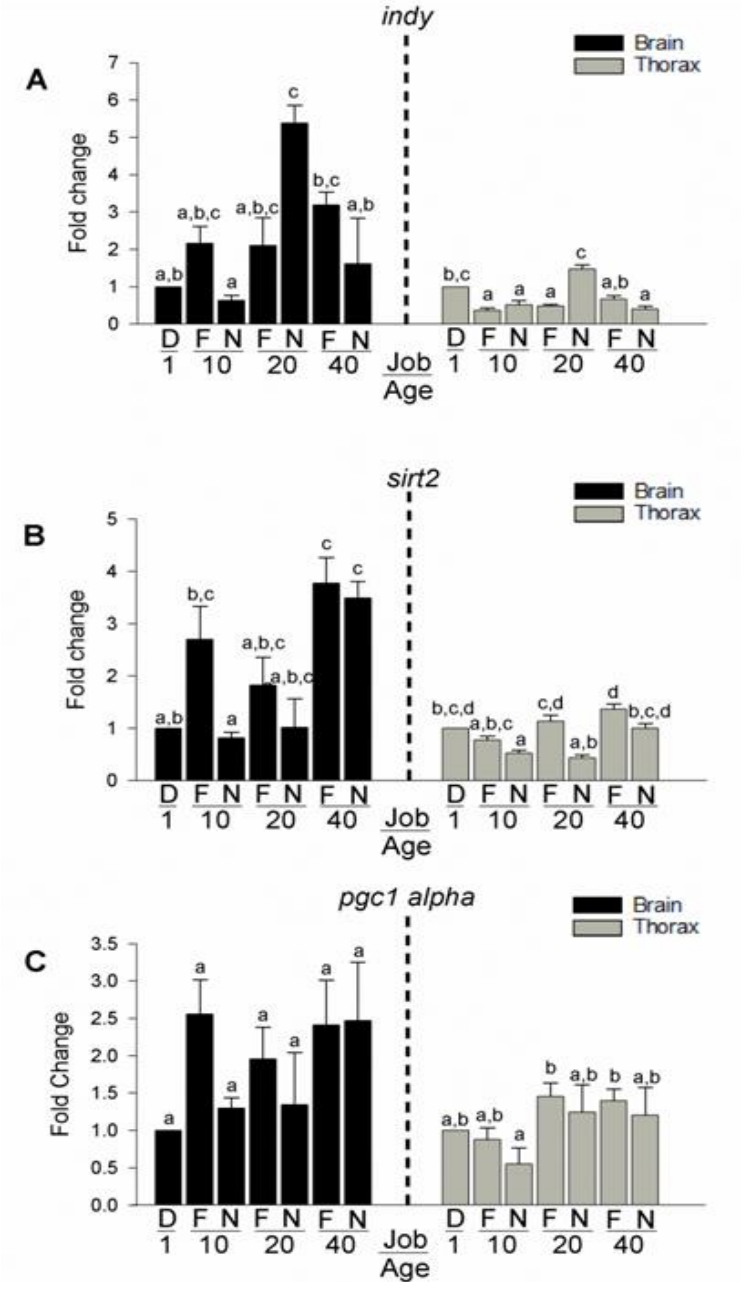
reversion and the transcriptional control mechanisms of immune transcripts in honey bee flight muscle may require greater than 3 days to revert. In *Drosophila*, expression of immunity genes increase with age (Landis et al., 2004), but honey bee immune gene transcription appears to be more tied to typical behavioral trajectories rather than age. Although reverted nurses experience a physiological reversion (enough to care for and feed young larvae), this was accompanied only by a partial genomic reversion. The linked pressures of intense flight metabolism and ROS production in foragers may lead to a loss of immune function that is delayed, but not prevented, by the reversion process. Therefore, the longevity conferred upon older nurses restricted to the hive (Rueppell et al., 2007) may be more generous than that accorded to reverted nurses that once foraged.

4.3.6: Expression of longevity mediating transcripts

A large body of the research on honey bee longevity and senescence has focused on a few main factors such as vitellogenin expression and insulin signaling (Munch and Amdam, 2010). These data from our microarray experiment suggests additional factors for investigation. To further explore the role of aging-related genes identified in the GO analysis relative to behavior and age, we set up another SCC experiment and obtained age-matched nurse bees and forager bees at days 10, 20 and 40 post eclosion. We quantified brain tissue and flight muscle mRNA levels of 2 transcripts differentially expressed in our array analysis and known to mediate longevity in other model organisms such as flies and nematodes, I'm not dead yet (*indy*), and silent mating-type information regulation 2 (*sirt2*) (Fig. 4.7). We chose an additional transcript not on the array but identified in other model organisms involved in regulating mitochondrial biogenesis,

peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*pgc1alpha*) which may be intimately connected with honey bee metabolism and senescence (Fig. 4.7).

Figure 4.7. Expression of longevity mediating transcripts in *A. mellifera* flight muscle and brain. mRNA levels of putative genes involved in aging/senescence were measured in brain tissue and flight muscle. Expression of (**A**) *indy*, (**B**) *sirt2*, and (**C**) *pgc1alpha* revealed distinct expression patterns both between tissues and within tissues. D = single day old bee; N = nurse; F = forager. Fold change represents the relative difference compared to one day old bees.



Although controversial, decreased activity in the *indy* gene, a transporter of Kreb's cycle intermediates, extends lifespan in *Drosophila* (Rogina et al., 2000). Expression of *indy* (Fig. 4.7A), in both the brain and flight muscle, significantly increased in nurse bee mRNA between 10 days of age and 20 days, and then decreased to levels seen at 10 days by 40 days of age. However, single day old bees had mRNA levels similar to 20 day old nurse bees. These data are consistent with studies that show delaying the onset of foraging and remaining a nurse bee extends lifespan (Rueppell et al., 2007). We did not see an effect of the transition to foraging on expression of *indy* in either brain tissue or flight muscle. This result suggests that the transition to foraging does not affect levels of *indy*, negating the potential protective effects of its decreased activity.

In *Drosophila* increased levels of the NAD dependent histone deacetylase, *sirt2*, increased lifespan (Imai et al., 2000). In our study, expression of *sirt2* (Fig. 4.7B) levels in the brain revealed precocious foragers had higher levels of *sirt2* compared to their age-matched nurse-bee counterpart. There was also an age-related increase in nurse bees. In flight muscle, we saw an age-related increase in *sirt2* mRNA in both foragers and hive bees. Earlier up-regulation of *sirt2* in brain tissue may contribute to the brain's ability to mitigate flight-associated cellular stress better than flight muscle, which up-regulates *sirt2* later.

Because honey bee flight is metabolically intensive (Suarez et al., 1996), high levels of ATP are needed during this behavior. However, mitochondrial activity has been shown to decrease with age in *Drosophila* (Ferguson et al., 2005). In contrast, honey bee flight occurs towards the later part of the honey bee life cycle, thus a mechanism likely exists to maintain levels of ATP and mitochondrial activity. Hence, we measured levels of

pgc1alpha, a gene involved in regulating mitochondrial biogenesis (Wu et al., 1999). Expression of *pgc1alpha* (Fig. 4.7C) in the brain did not significantly differ between age or behavioral groups. In flight muscle, however, young nurse bees (10 days old) had lower *pgc1alpha* mRNA levels than that of 20 or 40 day old nurses. Expression of *pgc1alpha* in forager flight muscle did not significantly differ between age or behavioral groups. These results may imply functional senescence is delayed by maintaining steady levels of *pgc1alpha* expression in honey bee brain tissue and flight muscle. This is consistent with the findings of Williams *et al.* (in prep), which shows foragers and nurses, regardless of age, have similar activity levels of citrate synthase, an extremely robust enzyme that is routinely used to estimate mitochondrial number (Lyons et al., 2006; Schippers et al., 2010). The impaired metabolic and flight capacity of aged honey bees may instead be due to structural lesions in the mitochondria and the sliding filament apparatus of flight muscle (Correa-Fernandez and Cruz-Landim, 2010; Fernandez-Winckler and Cruz-Landim, 2008); a possibility we are currently investigating.

Brain tissue and flight muscle responded differently to the cellular stress induced by flight. Our data suggest that intense flight (forager) *vs.* little-to-no flight (nurse) has a major effect on gene expression, rather than smaller-scale variation flight activity. The reversion from forager to nurse was also accompanied by a partial genomic reversion revealed by our microarray data, but the cellular and genomic mechanisms of how this reversion occurs remain unclear. Upregulation of *sirt2* in the brain during the transition from nurse to forager suggests a possible epigenetic mechanism of regulation and role for energy balance processes in honey bee senescence. Therefore, the behavioral and ecological contexts of bee flight should also be taken into account when considering the

progression of honey bee senescence. Although honey bee flight likely produces high levels of ROS (Williams et al., 2008), which may be a contributing factor to senescence, this has not been demonstrated experimentally and the ultimate cellular cost of foraging has yet to be determined. Understanding how variation in the timing and duration of foraging leads to whole-organism functional senescence and what mechanisms are employed to prevent senescence in old and reverted nurse bees will be important in understanding honey bee aging and control of aging in general.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Despite conflicting literature, the free radical theory of aging originally proposed by Harman (1957) is the most widely accepted theory of aging. This theory explains the mechanism by which organisms age over time and provides for inter- and intra-species variation in aging rates. According to this theory, superoxide radicals, which are normal byproducts of metabolism in the mitochondria, provoke cellular damage resulting in the accumulation of oxidatively damaged macromolecules inside of cells. Accumulation of damage perturbs cellular homeostasis leading to organismal senescence. Despite the amount of evidence supporting the free radical theory of aging, much criticism has arisen (Sanz and Stefanos, 2008; Perez et al., 2009). ROS are a part of cell signaling and stress defense systems (de Magalhaes and Church, 2006), indicating their relationship with aging is both more complex and less well understood than believed.

The free radical theory of aging makes 3 basic predictions: 1) short-lived individuals should produce more ROS leading to increased oxidative damage relative to long-lived; 2) decreased ROS production should increase lifespan; 3) increased ROS production should decrease lifespan. Most evidence that supports the free radical theory comes from studies evaluating prediction 1. Studies in various systems demonstrate mitochondria isolated from long-lived individuals produce less ROS compared to short-lived individuals (reviewed in Sanz et al., 2006). However, numerous studies provide evidence contradicting the free radical theory. For example, the mitochondria of Ames dwarf mice, the longest living mouse strain, produce more ROS compared to normal controls (Csiszar et al., 2008). Also, genetic mutations of the antioxidant system in mice

largely failed to decrease lifespan (Jang et al., 2009). Consequently, most data supporting the free radical theory indirectly show negative correlations between ROS production and lifespan, and positive correlations between ROS production and oxidative damage. Data from this dissertation provide a causal relationship between flight behavior, and oxidative stress while other studies indicate flight behavior decreases lifespan. All together, these studies provide the most direct evidence to date that honey bee flight decreases lifespan through increased oxidative damage. Establishing a connection between metabolically intense behavior and increased oxidative stress is important because this relationship indicates natural behavior can decrease lifespan.

Data from this dissertation support a partial subset of the predictions generated from the ROS theory of aging, while others are not supported. In chapter 3, we provide evidence supporting the prediction stating increased ROS production correlates with increased markers of oxidative stress and decreased lifespan. In honey bees with high flight time, levels of ROS increased and DNA oxidation rose relative to foragers restricted from flight. Additionally, markers of oxidative damage increased in bees fed D-galactose, a known oxidative stressor. Along with several lines of evidence demonstrating foraging is negatively correlated with survivorship and leads to functional senescence, these data support the prediction that increased ROS generation leads to increased oxidative stress and decreased lifespan.

The link between flight activity and decreased lifespan is well established in foraging honey bees, and my data demonstrate that high amounts of honey bee flight leads to increased ROS generation supporting the prediction that increased ROS production decreases lifespan. Restricting forager flight decreased ROS levels relative to

foragers allowed free access to flight. In contrast, the link between decreased flight activity and increased lifespan is less clear. Previous studies show foragers with high daily flight activity have decreased longevity compared to foragers with low daily flight activity (Neukirch, 1982); suggesting decreased flight activity actually increases lifespan. In houseflies, suppression of flight activity slows oxidative damage, extends lifespan, and decreases ROS levels (Yan and Sohal, 2000; Magwere et al., 2006). Similarly, ROS generation is lower in flight restricted bees, suggesting the prediction that decreased ROS generation increases lifespan is true in foraging honey bees. Neukirch (1982) showed small-scale variation in flight over the course of a day effects lifespan, but my data demonstrate flight compared to negligible flight, rather small-scale variation in flight, has a major effect on ROS generation. This result is similar to data in houseflies, which show flight restriction lowers ROS levels (Yan and Sohal, 2000), but ROS levels are similar in high flight activity flies compared to flies with normal flight activity (Magwere, 2006). So to fully understand if data supports this prediction in honey bees, it is critical to determine if complete flight restriction, rather than small-scale variations in flight, extends forager lifespan. It is important to consider the changing environment foragers encounter when leaving the hive as a potential source of ROS. After the transition to outside tasks, forager bees encounter challenges not faced inside of the colony. For example, forager bees face UV radiation, which may trigger ROS production, and pathogens, which ROS may be used to defend against. From my experiments, I cannot exclude these effects as possible sources of ROS. However, it is unlikely these scenarios produce additional ROS, because during transition from nurse to forager, gene expression increases in immune and UV stress defense genes (Whitfield et al., 2000; Margotta et al.,

2012). Fruthermore, it is unlikely UV light can penetrate the honey bee exoskeleton.

Measuring the lifespan of foragers restricted from flight is challenging because restricting bees to the hive may be a stress in itself that may decrease lifespan. In my study, I physically restricted foragers to the hive by placing them in wire mesh cages pushed into wax comb, but other studies have restricted flight by using an artificial rain source. Thwarting a natural drive to forage with an artificial rain source may eliminate stress arising from confining foragers to the hive. It is unclear if a stimulus-response relationship where flight ceases with rainfall or a natural cue to fly controls foraging. The latter suggests restricting foragers to the hive using any manipulation is inherently stressful, but because this phenomenon is not yet understood we chose to use the most logistically feasible restriction procedure. Using diutinus, which naturally do not fly, may alleviate stress from flight restriction as a confounding factor. However, the lifespan of diutinus bees sharply increases, suggesting that either stress-resistance is profound in these bees or they do not experience the same levels of stress as other bees. A broader understanding of stress mitigation in diutinus bees and the dynamics of winter hives will help to understand how these bees achieve maximum lifespan.

The last prediction of the ROS theory of aging states short-lived individuals should produce more ROS compared to long-lived individuals. In honey bees this translates into foragers, which are short-lived, should produce more ROS compared to nurses, which are long-lived in comparison, or workers should produce more ROS compared to long-lived (up to 8 years) queens. Data from queen bees demonstrate the relatively long lifespan in this caste is associated with peroxidation-resistant membranes (Haddad et al., 2007). However, extended lifespan has evolved independent of increased

antioxidant protective mechanisms (Corona et al., 2005). This suggests queens do not produce less ROS, but mitigate stress well. Therefore, ROS levels in queens do not support this prediction of the ROS theory. Furthermore, my worker data do not support this prediction either because nurse bees generally had ROS levels similar to age-matched foragers. But, the manipulation (SCC) used to obtain aged nurses may confound these results. In a single-cohort colony, the altered demography of the colony forces a subset of nurse bees to forgo the transition to foraging, becoming over-aged nurses. Although nurse bees used in my study are considered long-lived (25-40 days as nurses) compared to foragers (typically 14 days before senescence), in a natural colony setting nurses would begin foraging before 25 days of age. The ideal comparison to test this prediction is to compare diutinus, bees to forager bees. Diutinus bees develop near the end of summer, when the colony is absent of brood. The natural lifespan of diutinus bees approaches 300 days, compared to foragers which reach senescence after approximately 14 days of flight experience. If ROS levels in diutinus bees are lower compared to forager bees, then this prediction is true for foraging honey bees. If ROS levels in diutinus bees, like in aged nurse bees, are similar to forager bees, then nurse bees may employ unique mechanisms to delay aging when high levels of ROS are present.

Taken together, my data support the ROS theory of aging in forager bees, but the predictions generated from the ROS theory are less clear in nurse bees. However, nurses eventually transition to foragers, so the ROS theory may be life history stage specific. My data may actually suggest polyphenic aging in workers where nurses are in a protected phase but "typical" aging begins with the transition to foraging. It is possible that daily defecation flight bouts by nurse bees upregulate stress defense mechanisms through a

hormetic response, but the long and intense flight of foragers overwhelm defense systems leading to oxidative damage (homesis reviewed in Gems and Partridge, 2006). Polyphenisms in aging are also found in *C. elegans*, which has a larval "dauer" stage that exhibits stress resistance and negligible aging similar to honey bee nurses (reviewed in Van fleteren and Braekman, 1999). Additionally, *Drosophila* that over-winter in reproductive diapause are stress resistant and age more slowly (Tatar et al., 2001). Though polyphenic aging is a common phenomenon, the ability for honey bee workers to make bidirectional shifts between phenotypes (nurse to forager and forager to nurse) is an ability unsurpassed in other organisms. This phenotypic plasticity also supports plasticity in aging. For example, workers that preciously transitions to foraging senescence at an earlier age compared to workers that transition at the normal point. Similarly, delaying the transition to foraging increases lifespan.

The differences in metabolic rates between nurse bees and foragers may explain this discrepancy in support for the ROS theory as well as provide a mechanism of protection in nurses. Foraging honey bees have metabolic rates 10-100 times higher than nurse bees (Suarez et al., 1996), which likely leads to increased protective mechanisms, but data suggest high amounts of flight overwhelm these mechanisms (Williams et al., 2008). Consequently, flight-restriction may increase lifespan through decreased ROS generation and increased protective mechanisms. However, the relationship between ROS and aging is less clear in nurse bees. Higher vitellogenin in nurse bees may have a positive influence on nurse longevity by promoting increased immunity and stress resistance (Munch et al., 2008). Additionally, in, nurse bees have larger nutrient stores, survive longer under starvation stress, and have upregulated energy metabolism genes,

relative to foragers (Ament et al., 2008). This suggests nurse bees may upregulate cell signaling pathways associated with nutrient sensing and longevity. As with other model organisms, diet may confound data that support the ROS theory of aging because nurses have a protein-based diet, while foragers consume mostly carbohydrates. Additionally, the unusually high metabolic rate in foragers may lead to more pronounced changes in oxidative stress and the associated protective mechanisms.

Although my data suggest the source of ROS in honey bee foragers is GPDH dependent, and not oxygen dependent as suggested by the Harman (1956) theory, my data supports the fundamental idea behind the ROS theory that increased ROS causes oxidative damage and decreases lifespan. However, there is little evidence to suggest metabolic rates typical of mammals and non-flying nurse bees lead to oxidative damage and limit lifespan. Therefore, my data suggests the foremost cause of aging in animals with lower metabolic rates than flying honey bees is not oxidative damage. However, oxidative stress in foraging honey bees may be analogous to late-life oxidative damage in other animals, including mammals. Oxidative stress may not induce the aging process in other animals but could significantly hasten aging later in life because of chronic low-level damage that progressively increases with age. However, we are unable to determine from this work if oxidative stress leads to chronic sub-lethal damage in nurses that is associated with secondary damage, which may lead to senescence. Nonetheless, understanding aging in honey bees may yield insight into the interactions between sub-lethal stress early in life and the more extreme effects of aging that occur later in life.

Many models currently used to study aging, such as flies, worms, and mice may be confounded by living in the laboratory. For example, *C. elegans* spends its entire

laboratory life on an agar plate eating *E. coli*, but in nature needs to mitigate stress from starvation, predation, and disease and hence evolved a dauer stage. Although the laboratory lifestyle is unavoidable in these organisms, they did not evolve living in protected, well-fed environments (Reviewed in Lapointe and Hekimi, 2010). Exploring model systems outside of flies, worms, and rodents will prove crucial in the future to dissect both the data that support and contradict the free radical theory. Alternatively, studying current model systems, such as nematodes, in their natural habitats may yield insight into how laboratory life shapes the conclusions that describe the mechanisms involved in aging. Unfortunately, knowledge of the natural ecology and life history of *C. elegans* is sparse compared to the extensive data as an experimental system (Hodgkin and Doniach, 1997; Caswell-Chen et al., 2005). Understanding the natural history and ecology of model organisms in aging is important because it provides a context for the evolution of life history traits (Caswell-Chen et al., 2005).

Transfer theory (Lee, 2003) might explain patterns of senescence observed in worker bees and provide insight into the ultimate causes of aging in honey bees. The hypothesis that behaviorally related senescence is the outcome for selection on transfers explains senescence in worker bees well. However, it does not provide an explanation for senescence in queen or drone bees, and furthermore does not provide a mechanistic, or ultimate basis for honey bee aging (Amdam and Page, 2005). Yet, vitellogenin, a storage protein, provides a link between the proximate and ultimate causes of aging in honey bees. Transfers for proteinacious jelly are also accompanied by changes in hemolymph vitellogenin levels. Because vitellogenin is used for jelly production, levels increase when workers are net donors of jelly, and declines in older nurses, which are receivers of

jelly (Amdam and Omholt, 2002). After bees start to forage, vitellogenin production ceases (Pinto et al., 2000). Several pieces of data indicate vitellogenin promotes survival in honey bees through increased immune function (Amdam et al., 2004b) and increased stress resistance (Amdam and Omholt, 2002). This mechanistic theory is also compatible with queen and drone senescence, because long-lived queens have high vitellogenin levels throughout their lives, including the seasons when they don't lay eggs (Engels and Fahrenhorst, 1974). In contrast, short-lived drones only synthesize vitellogenin at basal levels until 7-10 days of age (Tranczek et al., 1989). Understanding resource transfers in other social insects, such as ants, will help to understand if resource transfers regulate investment into the soma in social insects (Amdam and Page, 2005).

Other theories of aging, such as the mutation accumulation and antagonistic pleiotrophy theories of senescence account for situations where replicating genotypes have many life-shortening or life-extending alleles relative to levels of extrinsic mortality. However, using these theories to explain senescence in functionally sterile eusocial worker bees, which show temporal patterns of parental functions, is beyond the theoretical framework of these theories. The fact that nurse bees and diutinus bees experience comparable levels of extrinsic mortality, but have different lifespan is consistent difficulties for these theories to explain honey bee senescence (Amdam and Omholt, 2002; Omholt and Amdam, 2004).

In honey bees, the provisioning of food between sisters regulates physiological plasticity, behavioral ontogeny, and longevity. This dissertation provides data explaining how ontogenetic transitions from nurse to forager effect levels of biological complexity, including gene expression and biochemistry to physiology and behavior, interact to

influence senescence and longevity. Patterns of survival in the hive are thus affected by changes in social environment and social task diversity. This dissertation provides a complete view of biological complexity to help understand how an organism's social environment affects lifespan, and thus is applicable to broad aspects of aging and life-history research. Exploring aging in honey bees has the advantage of studying an organism in its natural environment, where consideration is given to environmental stressors. Additionally, the plasticity of the honey bee system to naturally revert to earlier behaviors with a reversal of some aspects of senescence permits studies examining aging reversal in a natural system. Because honey bees represent a model to understand how variation in the onset of senescence can develop through a tightly controlled and highly plastic set of processes based on social relationships that rival human societies they will likely continue to be an important model to study aging in the future.

APPENDIX A: All known genes only expressed in forager young vs. forager oldest.

Gene name	Description
LOC552406	similar to thyroid hormone receptor interactor 4
LOC550922	similar to mab-2 CG4746-PA
LOC724511	similar to zinc finger protein 239
LOC725732	similar to CG30492-PB, isoform B
LOC409141	similar to CG7272-PA
LOC410775	similar to wing blister CG15288-PB, isoform B
trxr-1	thioredoxin reductase
csnk1a1	similar to casein kinase 1, alpha 1
LOC726679	similar to CG5913-PA
LOC727045	similar to yellow-e2 CG17044-PA
LOC724136	similar to CG6305-PA
DWVgp1	DWVgp1
jhamt	similar to juvenile hormone acid methyltransferase CG17330-PA
LOC411021	similar to Ugt86Dc CG4739-PA
LOC725700	similar to vacuolar protein sorting 29 isoform 2
LOC412767	similar to CG2016-PB
LOC725149	similar to GPI7 protein
LOC551537	similar to enoyl Coenzyme A hydratase domain containing 3
Hydr2	similar to CG3488-PA
LOC411927	similar to CG15111-PB, isoform B
rpl21	similar to Ribosomal protein L21 CG12775-PA
LOC410487	similar to CG2446-PC, isoform C
LOC409573	similar to CG1832-PA, isoform A
Cpap3-c	similar to Gasp CG10287-PA
LOC411269	similar to CG14621-PA
LOC724457	hypothetical protein LOC724457
LOC409336	similar to CG4330-PA
GMCOX4	similar to CG9518-PA
LOC726400	similar to Fork head domain protein FD2
LOC410600	similar to Neu3 CG7649-PB, isoform B
LOC413274	similar to Guanylyl cyclase at 89Db CG14886-PA
rho-5	similar to rhomboid-5 CG33304-PA
CAP-D2	similar to Condensin complex subunit 1 (Chromosome condensation-related SMC-associated protein 1) (Chromosome-associated protein D2) (hCAP-D2) (XCAP-D2 homolog)
Dap160	similar to intersectin 2 isoform 3
LOC725547	similar to CG6131-PA
LOC551732	similar to Protein farnesyltransferase/geranylgeranyltransferase type I alpha subunit (CAAX farnesyltransferase alpha subunit) (Ras proteins prenyltransferase alpha) (FTase-alpha) (Type I protein geranyl-geranyltransferase alpha subunit) (GGTase-I-...
Mxp	similar to proboscipedia CG31481-PD, isoform D

LOC724611	similar to CG11323-PA
ras	similar to Inosine-5-monophosphate dehydrogenase (IMP dehydrogenase) (IMPDH) (IMPD) (Protein raspberry)
LOC725421	similar to CG7759-PB, isoform B
rtet	similar to tetracycline resistance CG5760-PA
LOC411412	similar to CG11665-PA
APP-BP1	similar to amyloid beta precursor protein binding protein 1
NT-12	similar to CG8291-PA, isoform A
LOC412815	similar to CG3523-PA
Mvl	malvolio
LOC724627	hypothetical protein LOC724627
LOC552061	similar to lemur tyrosine kinase 3
MRPL20	similar to mitochondrial ribosomal protein L20 CG11258-PA
KEL	similar to Ring canal kelch protein
LOC411564	similar to CG32645-PB
LOC552717	similar to CG5590-PA
LOC727381	similar to tRNA phosphotransferase 1 isoform 1
LOC552029	hypothetical LOC552029
LOC727201	similar to Carbamoyl-phosphate synthase (ammonia), mitochondrial precursor (Carbamoyl-phosphate synthetase I) (CPSase I)
LOC409207	similar to Synaptic vesicle membrane protein VAT-1 homolog

APPENDIX B: All known genes expressed only in forager old vs. forager oldest.

Gene name	Description
Sirt2	similar to Sirt2 CG5085-PA
jim	similar to jim CG11352-PC, isoform C
LOC409063	similar to black CG7811-PA
LOC408396	similar to rhea CG6831-PA
LOC725475	similar to COMM domain containing 8
LOC551270	similar to CG5222-PA
Mrjp1	major royal jelly protein 1
LOC411777	similar to CG17255-PA, isoform A
mGlutR2	similar to Metabotropic glutamate receptor 7 precursor (mGluR7)
Mrjp2	major royal jelly protein 2
LOC411476	similar to CG11505-PB, isoform B
LOC725607	similar to CG18292-PA
Mrjp5	major royal jelly protein 5
bib	similar to big brain CG4722-PA
Tpx-4	similar to Peroxiredoxin 2540 CG11765-PA
Med15	similar to Mediator complex subunit 15 CG4184-PA
LOC412235	similar to Caspase precursor (drICE)
LOC552787	similar to DNA topoisomerase 3-beta-1 (DNA topoisomerase III beta-1)
LOC726826	similar to Moca-cyp CG1866-PA, isoform A
LOC552065	similar to CG4785-PA

APPENDIX C: All known genes expressed only in nurse old vs. nurse oldest.

Gene name	Description
1790120	hypothetical LOC551946
1794082	similar to CG32645-PB
1790046	similar to Fork head domain protein FD2

APPENDIX D: All known genes expressed only in nurse young vs. nurse oldest.

Gene name	Description
LOC724457	hypothetical protein LOC724457
Mvl	malvolio
LOC725441	similar to DEAD (Asp-Glu-Ala-Asp) box polypeptide 49
LOC406097	integrin betaPS
LOC550655	similar to CG32066-PB, isoform B
LOC410775	similar to wing blister CG15288-PB, isoform B
LOC410272	similar to CG5535-PA, isoform A
nAChRa5	neuronal nicotinic acetylcholine Apisa7-2 subunit
CPR5	similar to CG1259-PB
LOC725547	similar to CG6131-PA
LOC724958	similar to Putative odorant receptor 13a
LOC724320	similar to transcription factor INI
LOC411908	similar to HLA-B associated transcript 5
LOC726127	similar to JTBR CG1935-PA

APPENDIX E: All known genes expressed only in nurse young vs. nurse old.

Gene name	Description
LOC725783	similar to CG11160-PA, isoform A
LOC409336	similar to CG4330-PA
LOC410600	similar to Neu3 CG7649-PB, isoform B
LOC412598	similar to CG18507-PA, isoform A
LOC725732	similar to CG30492-PB, isoform B
LOC411777	similar to CG17255-PA, isoform A
LOC552662	similar to CG11265-PA, isoform A
Tango10	similar to CG1841-PA, isoform A
Dap160	similar to intersectin 2 isoform 3
stnB	similar to Protein stoned-B (StonedB) (Stn-B)
LOC552333	similar to Organic anion transporting polypeptide 26F CG31634-PA
LOC726118	similar to CG30415-PA, isoform A
LOC412476	similar to CG7102-PA
LOC727045	similar to yellow-e2 CG17044-PA
rtet	similar to tetracycline resistance CG5760-PA
LOC411163	similar to Nat1 CG12202-PA
NT-12	similar to CG8291-PA, isoform A
LOC412065	similar to CG13646-PA
LOC411021	similar to Ugt86Dc CG4739-PA
LOC409969	similar to dihydrouridine synthase 3-like
5-HT1	similar to SERotonin/octopamine receptor family member (ser-4)
LOC551537	similar to enoyl Coenzyme A hydratase domain containing 3
Y-g	yellow-g
LOC410554	similar to methylcrotonoyl-Coenzyme A carboxylase 2 (beta)
LOC409395	similar to chromodomain Y-like protein 2
Lys-2	similar to CG11159-PA
LOC725759	similar to Peritrophin A CG17058-PA, isoform A
LOC726801	similar to CG8032-PA
LOC409573	similar to CG1832-PA, isoform A
LOC724776	hypothetical protein LOC724776

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Education

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M.S. – Biology, Bloomsburg University of Pennsylvania, 2009
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Publications

Margotta, J.W.; Elekonich, M.M. Mechanisms of flight-induced senescence in the honey bee, *Apis mellifera.* In preparation

Margotta, J. W., Mancinelli, G. E., Benito, A. A., Ammons, A., Roberts, S. P., & Elekonich, M. M. (2012). Effects of Flight on Gene Expression and Aging in the Honey Bee Brain and Flight Muscle. *Insects*, *4*(1), 9-30.

Presentations

Poster - Margotta, J; Hranitz, J; Barthell, J; Brubaker, K. (2010) Evolutionary genetics of heat shock cognate 70 in the leafcutting bee, *Megachile apicalis*. *Society for Integrative and Comparative Physiology*. Seattle, WA.

Poster - Margotta, J; Mancinelli, G; Benito, A; Ammons, A; Roberts, S, Elekonich, M. (2011) Life history differences between two social bees affect expression of genes involved in senescence: *Apis* vs. *Bombus* *Arthropod Genomics*. Kansas City, MO.

Poster – Margotta, J; Roberts, S; Elekonich, M. (2013) Understand how oxidative stress and senescence are connected through honey bee flight. *Society for Integrative and Comparative Biology.* Austin, TX.

Oral Presentation – UNLV SOLS biology colloquium. 2012. Mechanisms of behaviorally-induced senescence in the honey bee, *Apis mellifera.*

Awards and Honors

Beta Beta Beta, National biological honor society, 2009
Hermsen Fellowship, 2013, $20,000
UNLV Graduate and Professional Student Association, travel grant, 2013, $550

Teaching Experience

BIOL 223: Anatomy and Physiology Laboratory, Spring semester and summer session 2010, Summer session 2013.

Community Outreach

3rd Grade classroom oral presentation on the importance of honey bees and science, Tartan Elementary School, North Las Vegas, NV, 2012

4th Grade classroom oral presentation on the importance of honey bees and science, Tartan Elementary School, North Las Vegas, NV, 2012

5th Grade classroom oral presentation on the importance of honey bees and science, Green Valley Christian Academy, Henderson, NV, 2012

Oral Presentation for adults focusing on the importance of honey bees and science. Whole Foods, Las Vegas Store, NV, 2012

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Introductory beekeeping class assistance and honey bee research presentation. UNLV extension Orchard, North Las Vegas, NV, 2011

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