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RESEARCH ARTICLE

Effects of flight activity and age on oxidative damage in the honey bee, *Apis mellifera*

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ABSTRACT

Frequent and highly aerobic behaviors likely contribute to naturally occurring stress, accelerate senescence and limit lifespan. To understand how the physiological and cellular mechanisms that determine the onset and duration of senescence are shaped by behavioral development and behavioral duration, we exploited the tractability of the honey bee (*Apis mellifera*) model system. First, we determined whether a cause–effect relationship exists between honey bee flight and oxidative stress by comparing oxidative damage accrued from intense flight bouts to damage accrued from D-galactose ingestion, which induces oxidative stress and limits lifespan in other insects. Second, we experimentally manipulated the duration of honey bee flight across a range of ages to determine the effects on reactive oxygen species (ROS) accumulation and associated enzymatic antioxidant protective mechanisms. In bees fed D-galactose, lipid peroxidation (assessed by measuring malondialdehyde levels) was higher than in bees fed sucrose and age-matched bees with a high and low number of flight experiences collected from a colony. Bees with high amounts of flight experience exhibited elevated 8-hydroxy-2′-deoxyguanosine, a marker of oxidative DNA damage, relative to bees with less flight experience. Bees with high amounts of flight experience also showed increased levels of pro-oxidants (superoxide and hydrogen peroxide) and decreased or unchanged levels of antioxidants (superoxide dismutase and catalase). These data implicate an imbalance of pro- to anti-oxidants in flight-associated oxidative stress, and reveal how behavior can damage a cell and consequently limit lifespan.

KEY WORDS: Honey bee, Oxidative stress, Flight, Senescence, Lifespan

INTRODUCTION

The exact role of oxidative damage in aging provokes great debate (Lapointe and Hekimi, 2010; Sanz and Stefanatos, 2008; Salmon et al., 2010; Costantini, 2008; see Parker, 2010 for social insects). Understanding how naturally occurring behaviors link lifespan and oxidative damage may reveal how activity levels influence senescence (Metcalfe and Alonso-Alvarez, 2010). While the oxidative stress model still elicits controversy in mammals, the theory appears to apply well to flying insects, especially hoverers (Yan and Sohal, 2000; Williams et al., 2008; Sohal et al., 1984; Lane et al., 2014). Behavioral manipulations and studies of longevity mutants show that age-related increases in oxidative damage and decreased protective mechanisms lead to senescence (Golden et al., 2002; 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, studies rarely connect behaviors occurring in an organism's natural environment to the accumulation of cellular damage. Furthermore, the mechanisms linking life history and behavioral traits to oxidative damage and functional senescence remain unclear (Metcalfe and Alonso-Alvarez, 2010). Understanding the relationship between age, behavioral duration, the accrual of cellular damage and senescence may provide mechanistic insight into how behavior may damage cells.

Honey bee (*Apis mellifera*) life history progression, known as temporal polyethism, is characterized by variation in flight frequency and a predictable senescence pattern, thus providing an ideal system to understand the connections between behavior and senescence. Senescence in solitary organisms typically occurs progressively over time, while honey bee senescence directly correlates to social task and behavioral development (Rueppell et al., 2007b), which are determined by both age and the social environment in the hive. During the first 2–3 weeks of life, honey bee workers perform tasks inside the hive such as hive maintenance and brood care or 'nursing' (Winston, 1991). Nurse bees fly infrequently and show none of the common characteristics of senescence during this period (Winston, 1991). Typically after the third week of life, bees transition from in-hive tasks to foraging behavior. Foraging bees leave the hive in search of pollen or nectar and may fly several hours per day over long distances (up to 8 km) (Winston, 1991). To meet the intense power requirements of hovering flight, the metabolic rate of forager bees reaches 100–120 ml O_2 g^{-1} h^{-1}, which is 10- to 100-times higher than non-flying behaviors (Suarez, 1996). Senescence (and mortality due to predation) accelerates after the transition to foraging, leading to nearly 100% mortality within 14 days (Visscher and Dukas, 1997). Delaying the nurse-to-forager transition increases longevity up to 8-fold (Rueppell et al., 2007a). Honey bee lifespan thus inversely correlates to the age at which the transition to foraging occurs (Rueppell et al., 2007b).

Increased metabolic rate corresponds to elevated production of several highly reactive chemical species, including superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) (Boveris and Chance, 1973; Hulbert et al., 2007). Upon production, ROS react with and damage lipids, proteins and nucleic acids. However, increased availability of antioxidants, such as superoxide dismutase (SOD) and catalase, neutralize the negative effects of ROS (Orr and Sohal, 1994; Cui et al., 2012). In worms, flies and mice, elevated oxidative stress resistance, or decreased ROS production, increases longevity (Arking et al., 2002; Hughes and Reynolds, 2005; Luckinbill et al., 1984; Partridge and Fowler,

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List of symbols and abbreviations

8-OHdG	8-hydroxydeoxyguanosine (measures DNA oxidation)
ELISA	enzyme-linked immunosorbent assay
GPDH	glycerophosphate dehydrogenase
H_2O_2	hydrogen peroxide
MDA	malondialdehyde (measures lipid peroxidation)
$O_2^{\bullet-}$	superoxide radical
$^{\bullet}OH$	hydroxyl radical
ROS	reactive oxygen species
SCC	single-cohort colony
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances – lipid peroxidation

1992). Overexpression of SOD1 and catalase increases lifespan in *Drosophila*, but increased antioxidant capacity does not universally extend longevity. ROS may still overwhelm defense systems, especially in organisms with high metabolic rates (Monaghan et al., 2008). Although ROS can be toxic, these molecules also play a vital role in cell signaling (D'Autréaux and Toledano, 2007). Most evidence suggests that oxidative cellular damage occurs through a disruption in ROS homeostasis. As of yet, no evidence linking this phenomenon to age and natural behavior has been established (Metcalfe and Alonso-Alvarez, 2010).

A well-established link exists between behavioral duration and senescence in honey bees. Accelerated behavioral progression leads to functional senescence, decreasing the overall performance of the entire colony (Visscher and Dukas, 1997; Dukas, 2008; Vance et al., 2009). Current data suggest that oxidative stress influences functional senescence in worker bees, but the precise series of cellular events leading to this phenomenon remain poorly understood. 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, an effect likely due to oxidative damage in flight muscle (Vance et al., 2009). In the flight muscle of nurse and forager bees, total antioxidant activity decreases with age. Furthermore, the environmental conditions that colonies are maintained in increase oxidative stress (Simone-Finstrom et al., 2016). In contrast, brain total antioxidant activity is independent of age (Williams et al., 2008), and the brain appears to mitigate damage more effectively than flight muscle (Margotta et al., 2013). Although all aerobic metabolism produces ROS, it is unclear whether the intense power requirements needed for prolonged honey bee flight increase ROS production and associated cellular damage.

In this study, we establish a connection between honey bee age, flight behavior, oxidative damage and senescence. We tested the effects of age, behavioral development and behavioral duration on the accumulation of oxidative damage to lipids and DNA along with levels of ROS activity and antioxidant activity. Because previous experiments suggest that brain tissue and flight muscle respond differently to cellular stress, we examined both tissues. In these experiments, we assessed ROS accumulation and antioxidant activity in bees with varying amounts of flight experience while controlling for age. We found that flight activity significantly affects oxidative damage, ROS accumulation and antioxidant activity.

MATERIALS AND METHODS

Honey bee rearing, collection and dissection

Honey bees (*Apis mellifera* Linnaeus 1758) for this study were reared at the University of Nevada, Las Vegas (UNLV), NV, USA apiary. 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 carrying nectar. Bees used to determine production of ROS and malondialdehyde (MDA) levels were collected off the comb (nurses) or at the entrance of the colony (foragers), and their intact heads and thoraces were assayed immediately after collection. 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 dissected from the head capsule to include the optic lobes, antennal lobes and mushroom bodies, but exclude the hypopharyngeal glands and subesophogeal glands. Thoraces were dissected to remove only flight muscle and avoid the esophagus and wings. Dissected tissues were kept frozen until assayed.

Experiment 1: D-galactose treatment, lifespan analysis and MDA measurement

To understand the connection between oxidative insults, decreased lifespan and increased oxidative damage in bees from a typical hive environment, we measured lifespan and estimated MDA levels in bees fed D-galactose. Insects fed D-galactose show increased oxidative damage (Cui et al., 2004).

We first associated an oxidative insult (through D-galactose consumption) to decreased lifespan in foragers collected from a typical hive environment. A total of 60 foragers (carrying pollen) were randomly collected (unknown age) at the entrance of a typical honey bee colony. Foragers were placed in Lebenfelder cages (6×10 cm) inside incubators in the laboratory (37°C and 75% relative humidity) and fed D-galactose [sucrose solution (50% w/v) supplemented with D-galactose (10% w/v)]. Control bees were fed sucrose only. Owing to the dry environment of the Mojave Desert region, water was also available *ad libitum*. Dead honey bees were counted daily until all bees fed D-galactose had died.

Next, we associated oxidative insults to increased oxidative damage. We compared foragers and nurses that were captured and fed D-galactose or sucrose for 6 days inside of the laboratory. These bees were assayed at 15–17 days of age and compared with 15- to 17-day-old bees that were collected directly from the hive and foragers that were restricted from flight (see Flight restriction, below). This analysis was completed by body segment (head/thorax) to understand the dynamics of how oxidative insults increase oxidative damage. Lipid peroxidation was used as a measure of oxidative damage and estimated by measuring MDA levels using a commercial kit for thiobarbituricacid reactive substances (TBARS; Cayman Chemical, Ann Arbor, MI, USA). Briefly, fresh unfrozen whole heads or thoraces were homogenized in 250 µl RIPA buffer. These homogenates were added to a reaction mixture and boiled at 60°C for 1 h. Samples were then centrifuged at 1600 ***g*** for 10 min and the absorbance was read at 530 nm in a plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA).

Experiment 2: flight-induced oxidative damage using single-cohort colonies

After determining a connection between oxidative insults, decreased lifespan and increased oxidative damage, we used single-cohort colonies (SCCs) to determine whether flight (known to be an oxidative insult) increases ROS generation and oxidative damage. We utilized SCCs to decouple the effects of age and behavior, allowing comparison of same-aged bees with

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drastically different flight histories and bees of different ages with the same behavioral activity. Forager bees with varying amounts of flight experience and age-matched nurse bees were obtained with SCCs. This method can both hasten and delay foraging (producing precocious foragers or over-aged nurse bees, respectively) by altering colony demography (Giray and Robinson, 1994; Huang and Robinson, 1992). In this study, 10 SCCs were created using bees from multiple source colonies headed by naturally mated queens. SCCs were used to obtain age-matched nurses and foragers or foragers with known amounts of flight experience (caged versus unrestricted flight).

To generate the SCCs, frames from source colonies were placed in an incubator (35°C, 75% relative humidity, 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 a nucleus colony containing one frame each of pollen and honey and 3 frames with empty comb 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. 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.

Flight experience

Once a SCC was placed outdoors, the colony was observed until the onset of first foraging, which occurred between 8 and 11 days of age in this study. Once a bee was identified as a forager, an additional dot of paint was placed dorsally 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 24th and 25th days after eclosion. Only marked foragers were collected in this study.

Flight restriction

On the 10th day after eclosion, 25–35 marked foragers, which by this time had <3 days of flight experience, were collected from each SCC and restricted from taking additional flights by placing them in a wire mesh cage pushed into a wax comb. Care was taken to place foragers in an area that contained no eggs, larvae or pupae to prevent the possibility of them reverting to nursing behaviors. To ensure that 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 that, in the event of their food stores being depleted, the caged foragers would have been fed by their uncaged nest mates.

Measures of oxidative damage, ROS and antioxidants

Oxidative damage measurements

8-Hydroxy-2-deoxyguanosine

To approximate DNA damage in bees with varying amounts of flight experience, levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) were measured in groups of free-flying foragers and flight-restricted foragers. DNA was extracted from dissected brains and flight muscle using a DNeasy extraction kit (Qiagen, Valencia, CA, USA). Levels of 8-OHdG were measured using a commercially available kit (Cayman Chemical). Briefly, DNA samples, an antiserum to 8-OHdG, and 8-OHdG linked to an acetylcholinesterase were added to a 96-well binding plate and incubated at 4°C for 18 h. Unbound reagents were removed from the plate by washing and the plate was developed for 90 min using Ellman's reagent then read at 410 nm. The amount of 8-OHdG in each sample was calculated from an 8-OHdG standard curve.

MDA

Age and flight experience normally covary in bees. Because we saw no statistically significant effect of flight restriction, we designed another experiment to assess whether lipid damage varied 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, frozen dissected brains and flight muscle were used instead of whole heads and thoraces. Levels of MDA in this experiment were measured using an ELISA (commercial kit, Cell Biolabs, San Diego, CA, USA). To determine MDA levels, samples containing 10 µg ml^{-1} of protein was added to a 96-well binding plate and incubated at 37°C for 2 h, washed, and blocked with assay diluent. The plate was then washed and incubated with primary and secondary antibodies separately. Following a set of washes, the plate was incubated with a color development reagent and the absorbance was read at 450 nm. The MDA in each sample was calculated in reference to a standard MDA curve.

ROS generation and measurements

H_2O_2 and $O_2^{\bullet-}$ generation was measured in heads and thoraces using 2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Molecular Probes, Eugene, OR, USA), a chemically reduced form of fluorescein. Upon oxidation by H_2O_2 and $^{\bullet}OH$ (but not $O_2^{\bullet-}$), CM-H_2DCFDA is converted into highly fluorescent 2′,7′-dichlorofluorescein (DCF). Superoxide production was accessed using MitoSOX (Molecular Probes), 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 min. Head or thorax (100 µl) homogenate was added to each well (96-well microtiter plate) with 5 µmol l^{-1} CM-H_2DCFDA to measure H_2O_2 and $^{\bullet}OH$ or MitoSOX to measure superoxide. To compare samples from multiple microplates, positive controls for each microplate were used. For H_2DCFDA, 100 µmol l^{-1} hypoxanthine, 5 mU ml^{-1} xanthine oxidase and 0.2 U ml^{-1} horseradish peroxidase were used. For MitoSOX, 100 µmol l^{-1} hypoxanthine and 5 mU ml^{-1} 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 min before adding the positive control, which was incubated for an additional 5 min at room temperature. MitoSOX samples and controls were incubated at 37°C for 30 min. Plates were then read at 485 excitation (Ex)/535 emission (Em) for CM-H_2DCFDA or at 510 Ex/580 Em for MitoSOX on a SpectraMax M2 plate reader (Molecular Devices).

Glycerol-3-phosphate dehydrogenase activity

Glycerol-3-phosphate dehydrogenase (GPDH), an enzyme that maintains redox potential across the inner mitochondrial membrane during glycolysis, is a major source of ROS in insects (Sohal, 1993; Drahota et al., 2002; Miwa and Brand, 2005). GPDH activity was determined by using the method described by Wise and Green (1979). Samples were homogenized in a buffer containing 10 mmol l^{-1} Tris-HCL (pH 7.4), 0.15 mol l^{-1} NaCl and a protease inhibitor cocktail. The assay buffer contained 100 mmol l^{-1} triethanolamine, 2.6 mmol l^{-1} EDTA, 0.12 mmol l^{-1} NADH, 0.1 mmol l^{-1} 2-mercaptoethanol, 0.120 mmol l^{-1} dihydroxyacetone phosphate. The

change in absorbance at 340 nm was monitored at 37°C with a spectrophotometer. One unit of enzyme activity corresponds to the oxidation of 1 nmol NADH min^{-1}.

Measurement of antioxidant activity

SOD and catalase activity were measured to assay antioxidant activity. SOD activity was determined using a commercial assay kit (product 706002, Cayman Chemical). This enzyme activity assay measures the copper/zinc, iron and manganese forms of SOD by utilizing a water-soluble tertrazolium 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 mmol l^{-1} HEPES buffer, pH 7.2 containing 1 mmol l^{-1} EGTA, 210 mmol l^{-1} mannitol and 70 mmol l^{-1} sucrose. Homogenates were spun at 1500 ***g*** for 5 min at 4°C. Formazan dye absorbance was measured at 450 nm.

Catalase activity was measured using a commercially available kit (product 707002, Cayman Chemical). This assay utilizes the peroxidatic function of catalase to react with methanol in the presence of an optimal concentration of H_2O_2 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 mmol l^{-1} potassium phosphate buffer, pH 7.2, with 1 mmol l^{-1} EDTA. Homogenates were spun at 1000 ***g*** for 10 min at 4°C. Absorbance was measured at 540 nm.

Statistical analysis

Statistical analyses were performed in JMP (SAS, Cary, NC, USA) and graphs were prepared in GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Differences in survival were determined using Mantel–Cox test for survivorship. After determining a normal distribution, mixed-model ANOVAs with Tukey's HSD *post hoc* tests were used to compare the means of all experimental groups except the lifespan analysis. *P*-values less than 0.05 were considered statistically significant. Data are represented as means±s.e.m.

RESULTS

D-galactose treatment, lifespan analysis and MDA measurement

The mean lifespan of laboratory-caged foragers decreased ($P<0.05$, Mantel–Cox test) when their food was supplemented with 10% D-galactose (Fig. 1A). The median survival time for control bees and bees fed D-galactose was 12 and 7 days, respectively. The experiment was discontinued after the death of all bees fed D-galactose. At this point, 50% of the control bees were still alive. Survivorship between the two groups was similar during the first 5 days of the experiment, but sharply declined in bees fed D-galactose after this point.

Comparing lab-held versus hive-collected bees suggests that the caging procedure increases stress as evidenced by increased MDA. Feeding D-galactose to laboratory-housed bees increased MDA in all tissues, and sucrose-fed bees showed a moderate increase in MDA relative to bees housed in a hive (Fig. 1B–E). This pattern suggests an effect of caging alone that increases MDA, which is then further increased by D-galactose feeding. Precocious foragers captured and fed D-galactose in the laboratory showed higher MDA levels in the head than counterparts fed only sucrose. These bees fed D-galactose also displayed higher MDA levels than foragers caged at 10 days of age (i.e. flight restricted) or allowed to forage freely since the initiation of foraging.

DNA oxidative damage and lipid peroxidation

In general, larger differences in 8-OHdG accumulation were observed when comparing high amounts of flight (>14 days) to low amounts of flight (<3 days), whereas incremental increases in flight showed little difference (Fig. 2A,B). In flight muscle, 8-OHdG increased in foragers with the most flight experience compared with all other groups, suggesting that intense flight increases DNA oxidative damage in this tissue.

When comparing hive-collected foragers and nurse bees, the pattern of lipid peroxidation is both tissue- and behavior-specific. In foragers, brain MDA levels were independent of age, but flight muscle MDA levels significantly increased in 40-day-old individuals (Fig. 2C,D). Alternatively, in nurses, brain MDA increased with age, but flight muscle MDA did not (Fig. 2C,D).

ROS: levels of H_2O_2, $^{\bullet}OH$ and SO^{-}

Patterns of H_2O_2 and $^{\bullet}OH$ accumulation were tissue- and behavior-specific. Levels of H_2O_2 and $^{\bullet}OH$ increased in the thoraces, and additional flights led to further accumulation. In forager heads and thoraces, levels of H_2O_2 and $^{\bullet}OH$ increased with the number of flights taken (Fig. 3). Restricting forager flight increased H_2O_2 and $^{\bullet}OH$ levels in heads (Fig. 3A) but decreased H_2O_2 and $^{\bullet}OH$ levels in thoraces (Fig. 3B). The presence or absence of flight caused the largest effect in superoxide (SO^{-}) accumulation. In forager brain tissue, superoxide levels were lowest in middle-aged (15–17 days old) flight-restricted foragers (Fig. 4A). In forager thoraces, levels of superoxide were independent of flight activity (Fig. 4B).

GPDH activity

Generally, GPDH activity was dependent on flight activity. The lowest GPDH levels occurred in bees with the least flights taken (nurses and flight-restricted foragers) (Fig. 5). GPDH levels increased in aged foragers compared with aged nurse bees (Fig. 5C,D).

Catalase and SOD activity

Although levels of H_2O_2 and $^{\bullet}OH$ increased with flights taken, a similar increase in catalase activity was absent. In forager brains, catalase activity increased in middle-aged foragers (15–17 days old) relative to flight-restricted foragers of the same age (Fig. 6A). In flight muscle, catalase activity was independent of age and flight experience (Fig. 6B).

SOD activity was both tissue and behavior dependent. In forager brains, SOD activity was independent of flight experience (Fig. 7A). In forager flight muscle, SOD activity decreased in experienced foragers (<14 days) relative to their caged counterparts restricted from flight (Fig. 7B).

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 consumption presumably accelerates ROS production. Transitioning from nurse to forager induces upregulation of oxidative stress defense and repair pathways (Margotta et al., 2013; Harrison, 1986; Williams et al., 2008;

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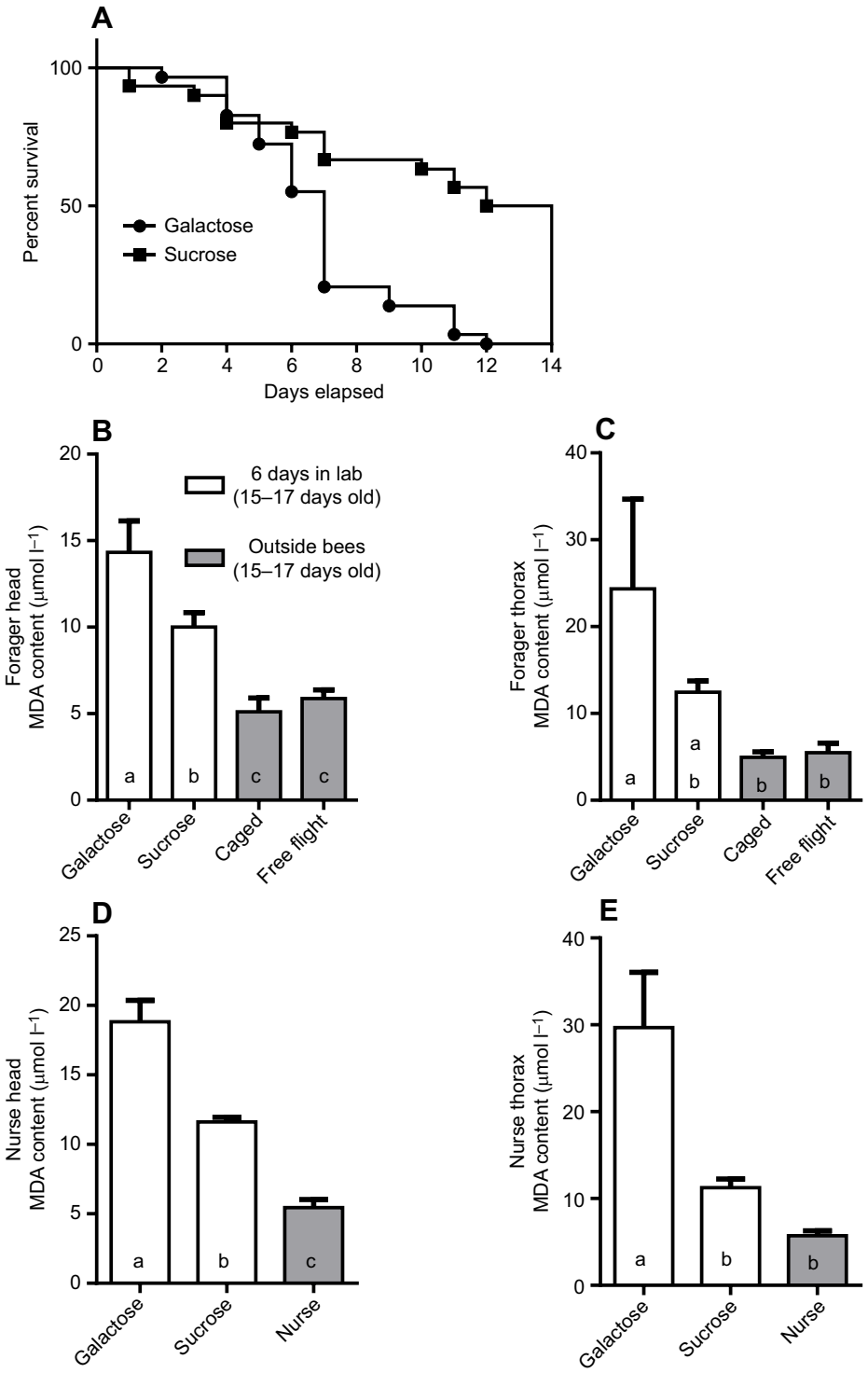

Fig. 1. Galactose treatment decreases lifespan and increases malondialdehyde (MDA) content. (A) Circles represent forager bees fed 50% sucrose (w/v) supplemented with 10% D-galactose. Squares represent forager bees fed only 50% sucrose (w/v). MDA was quantified by using the thiobarbituricacid reactive substances (TBARS) method in (B) forager heads, (C) forager thoraces, (D) nurse heads and (E) nurse thoraces. White bars represent 15- to 17-day-old bees fed 10% D-galactose+50% sucrose or 50% sucrose for 5 days post capture. Gray bars represent 15- to 17-day-old nurse bees (D,E) or forager bees that were either given free flight or were restricted from taking flights (B,C). Bars (means±s.e.m., n=5–7 per bar) not connected by the same letters are statistically different from one another.

Wolschin and Amdam, 2007). After flight bouts, antioxidant mechanisms likely clear ROS, but this ability declines in aged nurses and foragers (Williams et al., 2008). Defense systems other than enzymatic antioxidants, such as glutathione, may also help to clear ROS after metabolically intense foraging bouts. Once defense systems are overwhelmed (Finkel and Holbrook, 2000) foraging bees likely incur oxidative damage. Our data indicate that ROS levels increase with incremental increases in flight activity. However, antioxidant and cellular damage data demonstrate active flight, whereas no flight or little flight increases oxidative damage, suggesting that accumulation rather than ROS production alone influences senescence.

Flight induces oxidative damage

Flight activity may contribute to senescence when foragers accumulate more than 10 days of flight experience. In fruit flies with free access to flight in vials, mitochondrial respiration and electron transport chain activity declines with age (Ferguson et al., 2005), and forcing flight induces early metabolic and behavioral senescence (Lane et al., 2014). As foragers age, glycogen

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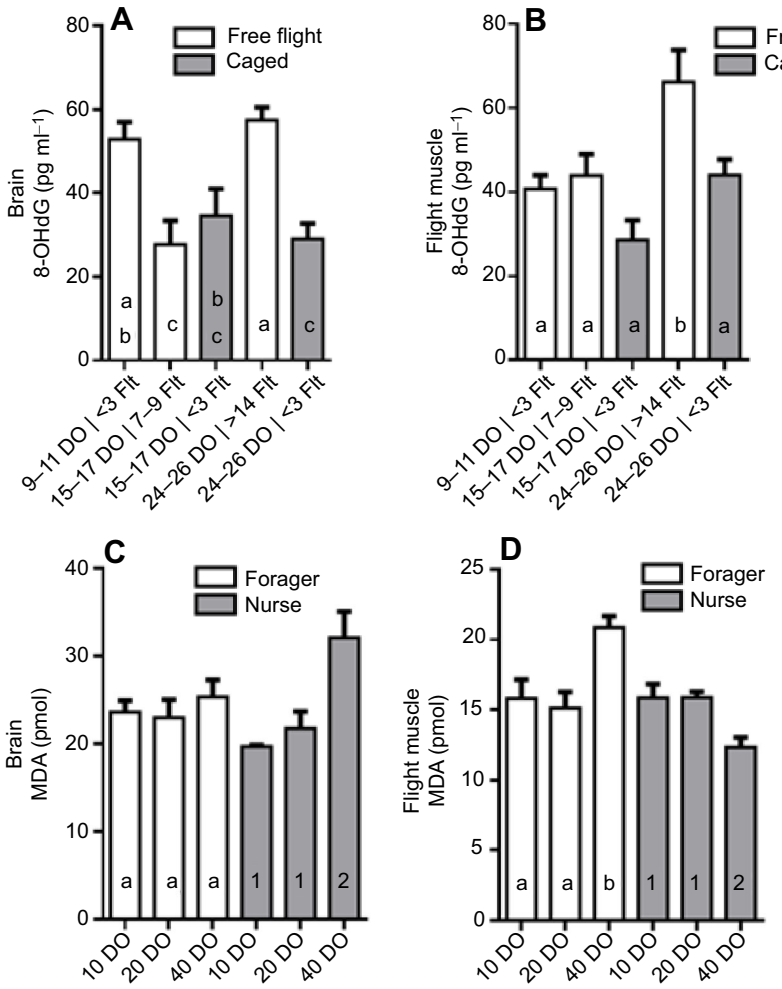

Fig. 2. Flight activity and age are associated with increased oxidative damage. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) was quantified using ELISA in forager (A) brain tissue and (B) flight muscle. In A and B, white bars represent foragers that had unrestricted access to flight and gray bars represent foragers that were restricted from taking flights after 3 days. The *x*-axis describes ages (DO: days old) and flight experience [<3, 7–9 or >14 days flight (Flt) experiences]. The *x*-axis represents each group's respective age at the time of collection. MDA was quantified by ELISA in the (C) brain tissue and (D) flight muscle of 10-, 20- and 40-day-old foragers (white bars) and nurses (gray bars). Bars (means±s.e.m., *n*=5–7 per bar) not connected by the same letter are statistically different from one another.

synthesis slows and eventually ceases (Neukirch, 1982), suggesting that foraging damages carbohydrate metabolism. Accordingly, high levels of ROS have been shown to damage enzymes in the glycogen synthesis pathway (England et al., 2004; Shanmuganathan et al., 2004; Kashiwagi et al., 1996). Experienced foragers (>14 days) perform poorly in olfactory learning tests compared with bees with up to 13 days of foraging time (Behrends et al., 2007), suggesting that cognitive function also declines as flight experience increases. Furthermore, oxidative carbonylation and nitration increases independently of age in the brains of forager bees (Seehuus et al., 2006). These prior studies collectively implicate oxidative damage as being

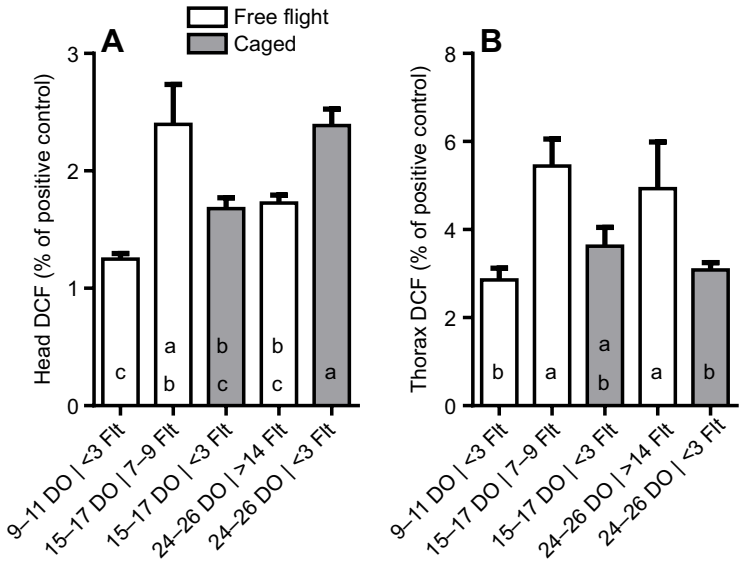

Fig. 3. Reactive oxygen species (ROS) accumulate with flight activity. Using a probe [2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA)] that reacts with hydroxyl radicals ($^{\bullet}$OH) and hydrogen peroxide (H_2O_2), ROS accumulation associated with flight was measured in the heads (A) and thoraces (B) of 9- to 11-, 15- to 17-, or 24- to 26-day-old (DO) foragers that were allowed free access to flight [white bars: <3, 7–9 or >14 days flight (Flt) experience] or restricted to the hive (gray bars: <3 days flight experience). Values are represented as percentages of positive controls (100 μmol l^{-1} hypoxanthine, 5 mU ml^{-1} xanthine oxidase and 0.2 U ml^{-1} horseradish peroxidase). Bars (means±s.e.m., *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).

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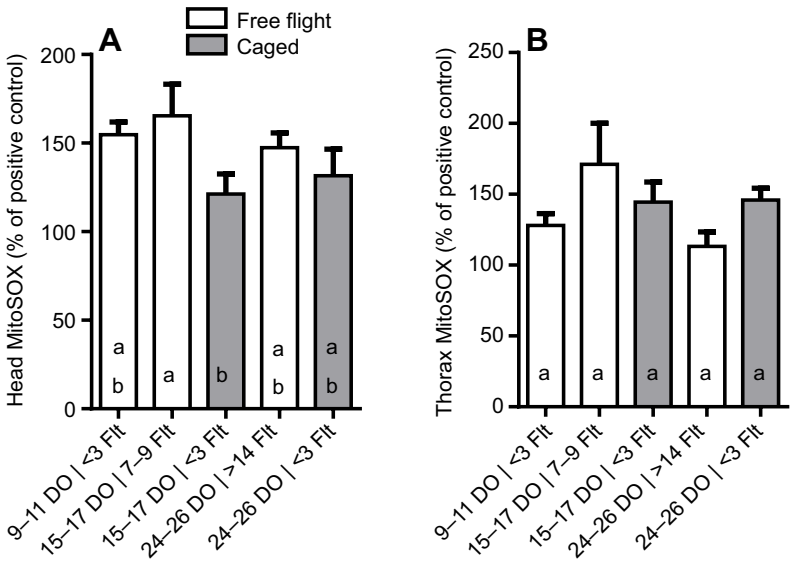

Fig. 4. Superoxide accumulates in the brain and flight muscle. Superoxide accumulation (measured with MitoSOX) associated with flight was measured in the heads (A) and thoraces (B) of 9- to 11-, 15- to 17-, or 24- to 26-day-old (DO) foragers that were allowed free access to flight [white bars: <3, 7–9 or >14 days flight (Flt) experience] or restricted to the hive (gray bars: <3 days flight experience). Values are represented as percentages of positive controls (100 μmol l^{-1} hypoxanthine, 5 mU ml^{-1} xanthine oxidase). Bars (means±s.e.m., *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).

detrimental to flight performance and foraging ability. Our data corroborate these studies and provide evidence showing flight activity directly leads to increased 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 this

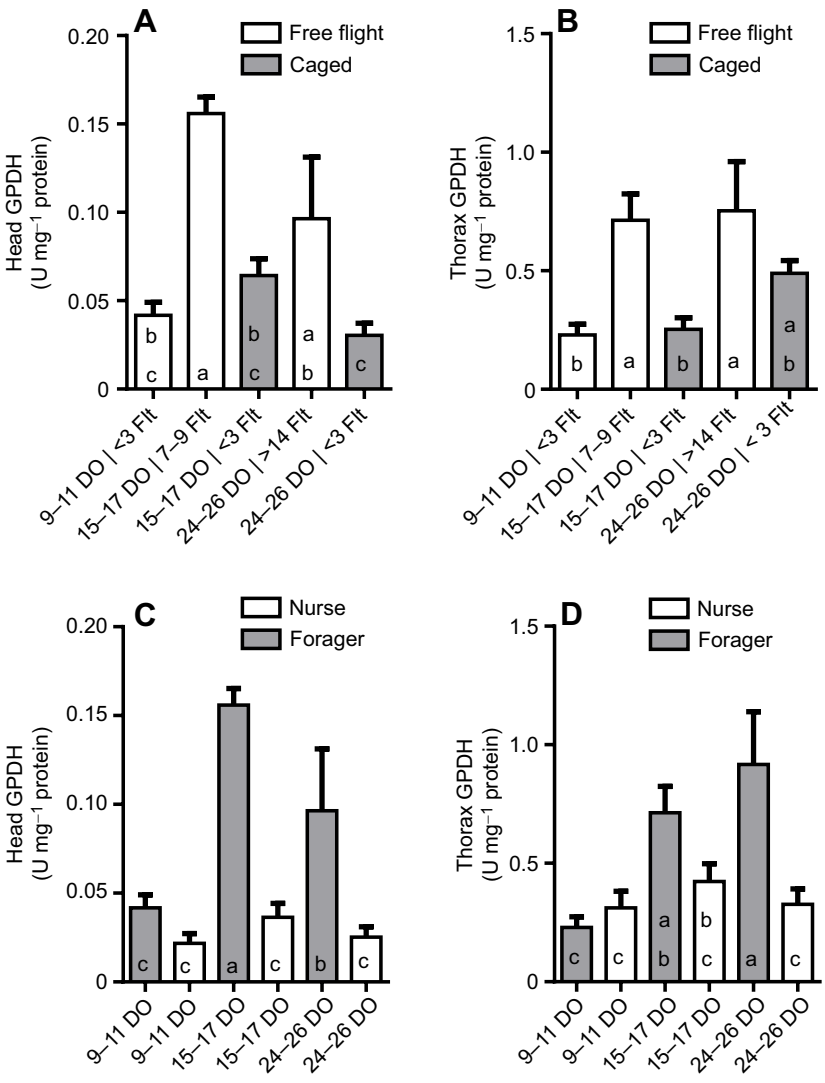

Fig. 5. Glycerol-3-phosphate dehydrogenase (GPDH) activity is dependent on flight activity. To assess GPDH levels associated with flight, GPDH enzyme activity was measured in the heads (A) and thoraces (B) of 9- to 11-, 15- to 17-, or 24- to 26-day-old (DO) foragers that were allowed free access to flight [white bars: <3, 7–9, or >14 days flight (Flt) experience] or restricted to the hive (gray bars: <3 days flight experience). GPDH activity associated with age and behavioral differences in heads (C) and thoraces (D) was measured in forager bees (gray bars: 9–11, 15–17 or 24–26 days old) and nurse bees (white bars: 9–11, 15–17 or 24–26 days old). Values are represented as GPDH activity in U mg^{-1}. Bars (means±s.e.m., *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).

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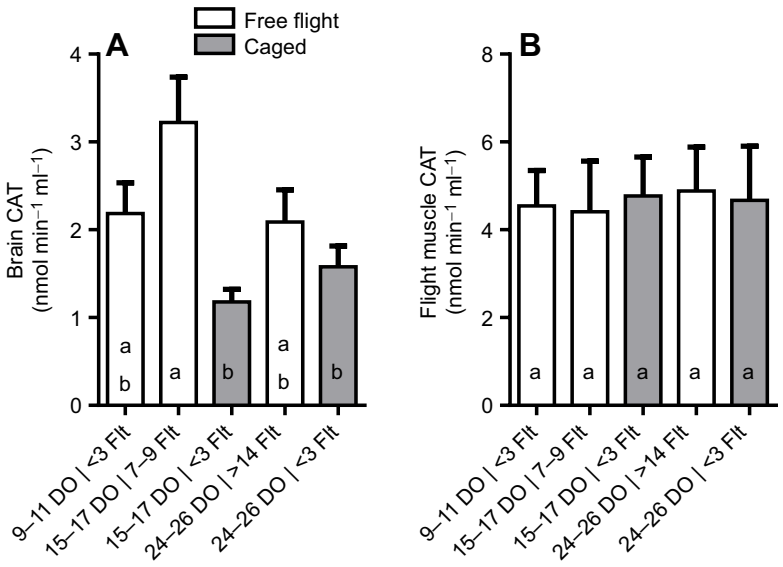

Fig. 6. Catalase (CAT) activity is independent of age and behavior. To assess CAT levels associated with flight, catalase enzyme activity was measured in the heads (A) and thoraces (B) of 9- to 11-, 15- to 17-, or 24- to 26-day-old (DO) foragers that were allowed free access to flight [white bars: <3, 7–9 or >14 days flight (Flt) experience] or restricted to the hive (gray bars: <3 days flight experience). Values are represented as nmol formaldehyde produced min^{-1} ml^{-1}. Bars (means±s.e.m., 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).

effect. Houseflies prevented from flying have lower mitochondrial damage and live 3× longer than flies permitted to fly (Yan and Sohal, 2000), demonstrating that flight activity accelerates senescence, which is consistent with our finding. During acute bouts of activity or long-term exercise, many organisms upregulate heat shock proteins and antioxidants to reduce exercise-induced damage (Ji, 1993; Hernando and Manso, 1997; Higuchi et al., 1985; Jenkins et al., 1984; Salo et al., 1991). However, this ability declines with increased age in many organisms (Banerjee et al., 2003; Ebbeling and Clarkson, 1989; Ji, 2002; Liu et al., 2004), suggesting that activity may have contributed to aging in these organisms, similarly to our findings. In this study, MDA levels increased in bees treated with D-galactose, establishing a cause–effect relationship between an oxidative insult and increased oxidative damage. Levels of 8-OHdG increased in experienced foragers compared with their same-aged counterparts restricted from flight. Similarly, in houseflies, 8-OHdG levels declined 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 2 weeks of flight experience, the time point at which senescence reaches nearly 100% in foraging bees, these data suggest that increased oxidative damage contributes to decreased longevity. Collectively, our work and these studies demonstrate that highly metabolic behaviors, such as flight, can increase oxidative damage and decrease lifespan.

Contrary to our result showing no difference in MDA between flight-restricted foragers and free-flying foragers, Tolfsen and colleagues (2011) observed increased MDA levels in the brains of flight-restricted bees. The authors suggest that flight activity increases fatty acid saturation levels in the brain. This phenomenon may provide a level of neural protection due to the decreased susceptibility of saturated fatty acids to oxidation (Haddad et al., 2007). Because our flight-restriction procedure involved caging bees inside of the hive, foragers likely still received a signal to forage. However, being caged physically constrained their ability to fly. This caging procedure inherently inhibits natural behavior and therefore likely induces stress. In contrast, Tolfsen et al. (2011) used an artificial rain source to restrict foraging,

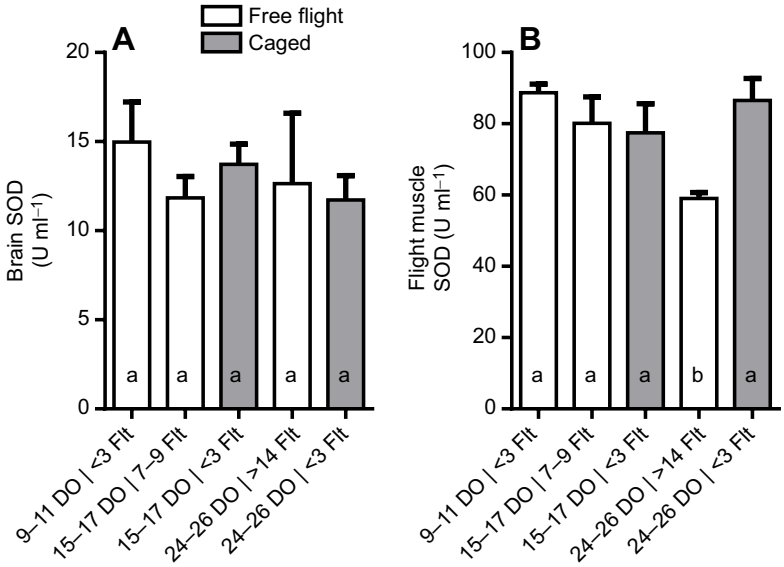

Fig. 7. Superoxide dismutase (SOD) activity is independent of age and behavior. To access SOD levels associated with flight, SOD enzyme activity was measured in the heads (A) and thoraces (B) of 9- to 11-, 15- to 17-, or 24- to 26-day-old (DO) foragers that were allowed free access to flight [white bars: <3, 7–9 or >14 days flight (Flt) experience] or restricted to the hive (gray bars: <3 days flight experience). Values are represented as SOD activity in U ml^{-1}. Bars (means±s.e.m., 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).

possibly eliminating the entire hive's signal to forage, thus altering fatty acid saturation levels. In both studies, MDA levels did not increase in free-flying foragers, indicating that MDA does not accumulate with flight activity. We found no difference in MDA levels between flight-restricted foragers and free-flying foragers, suggesting that lipid damage functions independently of flight. Conversely, MDA levels increased in aged nurse brain tissue, which has a similar fatty acid saturation level compared with forager bees (Haddad et al., 2007). Based on these results, it appears that repair mechanisms decline in older nurse bees. Surprisingly, MDA levels increased in the brains of foragers and nurses caged inside the laboratory compared with their counterparts kept inside of a regular hive. The social context of the hive may modulate fatty acid saturation levels in the brain or even confer neural protection. The presence of foragers and their pheromones control the transition from in-hive behaviors to foraging (Huang and Robinson, 1992; Pankiw et al., 1998), while pheromones released from developing larvae control the number of nurses inside the hive (Pankiw et al., 1998). Removing bees from these physical and chemical cues may alter their ability to mitigate oxidative damage.

ROS levels conflictingly decreased in the heads of experienced foragers but increased in the heads of old flight-restricted foragers and thoraces of free-flying foragers. These data suggest that brain tissue may become damaged after increased flight, resulting in slowed metabolism and less ROS production. If neurons become oxidatively damaged, then ROS production declines due to lowered metabolism. ROS differentially affect glial cells and neurons because of their terminal differentiation (Gilgun-Sherki et al., 2001). Although oxidatively damaged proteins accumulate in forager brains, it is unlikely that this accumulation leads to whole-organism decline because these bees still retain the ability to learn (Seehuus et al., 2006).

Paradoxically, intracellular ROS [H_2O_2, ^{-}OH and peroxynitrite ($ONOO^{-}$)] levels increased in thoraces, but superoxide levels and SOD activity remained unchanged. This result suggests that there is likely 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 DCF-DA used to measure ROS reacts with H_2O_2, ^{-}OH and $ONOO^{-}$, we are unable to determine which specific ROS increased. Using fluorescent probes as a direct measurement of ROS is problematic because of the dynamics of these molecules (Kalyanaraman et al., 2012). Additionally, our homogenization procedures disrupted membranes and released a complex mixture of oxidizing and antioxidant molecules into solution. Because of this, we used these probes as indirect indicators of oxidants between experimental groups, rather than a quantitative measurement.

Although the exact mechanisms of ROS generation by mitochondria remain incompletely understood, most studies suggest that generation occurs in 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 also produces ROS because of the reducing equivalents present (Sohal, 1993; Drahota et al., 2002; Miwa and Brand, 2005). In insects, GPDH 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 all flight ability, demonstrating the necessity of GPDH for flight activity (O'Brien and McIntyre, 1972). We found that GPDH activity was dependent on flight activity in forager brain tissue and flight muscle. Because oxidant production was flight dependent as well, this result suggests GPDH activity as a site of ROS production in foraging honey bees. Measuring ROS production of foragers flown in hypoxic and hyperoxic conditions should reveal additional insight into this phenomenon. Metabolic rates for bees flown in levels of oxygen varying between 10 and 21% remain unchanged but slightly decrease in bees flown in 5% oxygen (Harrison et al., 2001). Although most evidence shows hypoxia-induced decreases in ROS production, some recent evidence suggests increased ROS generation in hypoxic conditions in mammals (Waypa et al., 2016). Understanding the dynamics of oxygen concentration and ROS generation may uncover the ultimate source of ROS generation during bee flight.

Antioxidants decrease or remain unchanged

Catalase is an antioxidant found mainly in peroxisomes and mitochondria (Halliwell and Gutteridge, 1985). Catalase activity occurs in conjunction with high concentrations of mitochondria in metabolically active tissues, providing protection in highly metabolic organisms (Powers et al., 1994). In this study, flight restriction led to decreased catalase activity in forager brain tissue. If foragers forgo flight for an extended period, their ability to maintain catalase activity in the brain may decrease, possibly due to a reduction in metabolic rate or mitochondrial activity. In contrast, honey bee flight muscle contains 39–42% mitochondria per muscle volume (Block, 1994). So, it is unsurprising that active flying did not increase catalase activity, because catalase upregulation likely occurs at maximum levels during flight. Thus, our data suggest that forager brains likely exhibit elevated sensitivity to oxidative stress, while high constituent levels of catalase functions as a crucial defense mechanism in flight muscle.

During acute and chronic exercise, many organisms, including honey bees, increase other antioxidant levels, such as SOD, in muscle and other body parts (Powers et al., 1999; Vollaard et al., 2005; Williams et al., 2008). Exercise capacity decreases in mice heterozygous for manganese SOD (Kinugawa et al., 2005), indicating that SOD contributes to an organism's ability to perform highly aerobic behaviors. Additionally, endurance training in rats increases manganese SOD activity in fast-twitch oxidative muscle (type IIa), the same muscle type that drives honey bee flight (Hollander et al., 1999). In our experiments, flight muscle SOD activity decreased in the oldest foragers with the most time spent flying, indicating an association between intense flight and lowered SOD activity. An organism's ability to perform intense locomotor activity likely depends on SOD activity. As honey bees spend more time flying, 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 unequivocally demonstrates whole-organism senescence. Therefore, we suggest that oxidative damage due to decreased SOD activity in flight muscle contributes directly to whole-organism senescence.

Future directions

Most studies relate the onset and duration of flight to senescence in honey bees, and an accelerated transition to foraging leads to functional senescence (Visscher and Dukas, 1997; Dukas, 2008; Vance et al., 2009). Measuring and manipulating the frequency, time, distance and actual intensity of each individual flight in addition to days spent flying will yield a far more comprehensive view of flight-associated oxidative damage. However, the effects of these variables on senescence and oxidative damage in a natural environment have not been well studied. In this study, we varied the onset and duration of flight because the effects of these

manipulations are well understood and relate to natural events in the hive that cause precocious foraging (Huang and Robinson, 1992). Nonetheless, this design gives an incomplete picture of how all aspects of bee foraging affect oxidative damage.

Using methods that increase flight intensity in natural environments, controlled laboratory settings or flight tunnels can give a clearer picture of how flight damages cells. In a laboratory setting, adding weights to flying bees increases flight intensity by making lift more difficult to generate (Schmid-Hempel, 1986; Buchwald and Dudley, 2010). Because aerodynamic power inversely relates to atmospheric density, bees flying in variable density gasses can also increase flight intensity (Dudley, 1995; Vance and Roberts, 2014). In a natural setting, manipulating foraging distance to floral resources or controlling artificial winds in a resource area can increase flight intensity. Combining data from experiments that manipulate flight intensity with the data from this study will give a detailed picture of the cellular events associated with flight and senescence.

Future studies using diutinus bees (overwintered bees that do not initiate flight until a later age) and post-mating queens, who do not fly but have longer lifespans than foragers (Winston, 1991; Remolina et al., 2007), will be important to unravel the effects of long-term natural flight restriction on antioxidant activity and ROS production. Diutinus bees exhibit the maximum lifespan of worker bees and show decreased oxidative damage in brains (Seehuus et al., 2006). Diutinus bees show negligible senescence during winter conditions (Münch et al., 2013). Understanding antioxidant activity and ROS production in these bees may show how ROS accumulation and mitigation ultimately affects lifespan. The mechanisms of delayed senescence in winter bees and queens will provide further insight into the relationship between flight activity and senescence. Showing how vitellogenin localization in the brain (Münch et al., 2015) changes with age and behavior might also yield insight into how the bee brain tolerates flight stress in the brain. Furthermore, understanding how male drone bees tolerate oxidative damage (Li-Byarlay et al., 2016) may lend insight into oxidative stress in different life histories. In this study, we measured only two antioxidants (SOD and catalase) in a complex system of many others. In future studies, measuring additional important antioxidants, such as glutathione and glutathione peroxidase, will give a more complete picture of how the honey bee antioxidant system responds to increased oxidative stress.

Conclusions

Numerous studies suggest that flight activity in insects decreases longevity and leads to functional senescence (Behrends et al., 2007; Neukirch, 1982; Seehuus et al., 2006; Williams et al., 2008). This study provides new information on the mechanisms underlying this effect and identifies a causal relationship between oxidative insults and oxidative damage. We established that behavioral state, flight experience and chronological age influence oxidative damage in a tissue-dependent manner in honey bee foragers. Although the fundamental aspects of Harman's (1956) theory hold true, the actual source of ROS in honey bee foragers may not fit predictions of the ROS theory. Because foraging honey bees exhibit metabolic rates higher than most animals, determining whether oxidative damage leads to senescence in animals with lower metabolic rates remains difficult. While oxidative damage may not induce biological aging, 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 whether negligible flight in nurses produces sub-lethal damage that affects their lifespan later as foragers. 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.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.W.M., S.P.R., M.M.E.; Methodology: J.W.M., S.P.R., M.M.E.; Formal analysis: J.W.M.; Resources: S.P.R., M.M.E.; Writing - original draft: J.W.M.; Writing - review & editing: J.W.M., S.P.R., M.M.E.; Supervision: S.P.R.; Funding acquisition: S.P.R., M.M.E.

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