Honey bee (*Apis mellifera*) drones survive oxidative stress due to increased tolerance instead of avoidance or repair of oxidative damage

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**Abstract**

Oxidative stress can lead to premature aging symptoms and cause acute mortality at higher doses in a range of organisms. Oxidative stress resistance and longevity are mechanistically and phenotypically linked; considerable variation in oxidative stress resistance exists among and within species and typically covaries with life expectancy. However, it is unclear whether stress-resistant, long-lived individuals avoid, repair, or tolerate molecular damage to survive longer than others. The honey bee (*Apis mellifera* L.) is an emerging model system that is well-suited to address this question. Furthermore, this species is the most economically important pollinator, whose health may be compromised by pesticide exposure, including oxidative stressors. Here, we develop a protocol for inducing oxidative stress in honey bee males (drones) via Paraquat injection. After injection, individuals from different colony sources were kept in common social conditions to monitor their survival compared to saline-injected controls. Oxidative stress was measured in susceptible and resistant individuals. Paraquat drastically reduced survival but individuals varied in their resistance to treatment within and among colony sources. Longer-lived individuals exhibited higher levels of lipid peroxidation than individuals dying early. In contrast, the level of protein carbonylation was not significantly different between the two groups. This first study of oxidative stress in male honey bees suggests that survival of an acute oxidative stressor is due to tolerance, not prevention or repair, of oxidative damage to lipids. It also demonstrates colony differences in oxidative stress resistance that might be useful for breeding stress-resistant honey bees.

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1. Introduction

Molecular damage due to oxidative stress may be one of the primary causes of organismal aging (Finkel and Holbrook, 2000; Harman, 1956). Oxidative stress occurs when reactive oxygen species (ROS) are accumulating in a living system faster than they can be detoxified. Oxidative stress has been linked empirically to aging (Finkel and Holbrook, 2000) and age-related diseases such as diabetes, cancer, cardiovascular diseases, Parkinson disease, and Alzheimer disease (Beckman and Ames, 1992; Harman, 2006; Markesbery, 1997; Pandey et al., 2010). For example, selection for stress resistance results in long-lived flies (Rose et al., 1992), experimental up-regulation of enzymes that defend against ROS in *Drosophila* increases lifespan (Arking et al., 2000), and elevated concentrations of antioxidants contribute to longevity and longer life span (Radyuk et al., 2010; Svensson and Larsson, 2007). ROS include (but are not limited to) peroxyl radicals, hydroxyl radicals, hydrogen peroxides, and superoxide anions (Farooqui, 2012) and may derive from endogenous and exogenous sources (Monaghan et al., 2009). ROS levels that exceed the capacity of cellular antioxidant defenses, such as detoxifying enzymes and radical scavenging molecules, cause lipid peroxidation of cell membranes, modification of proteins, DNA mutations or fragmentation, and potential cell death (Hughes and Reynolds, 2005). Oxidative stress causes dramatic changes in gene expression and cellular functions (Li et al., 2008; Zou et al., 2000). Individual cell components can be affected differently by oxidative stress, but surprisingly little is known on the overall relation between different classes of oxidative damage (Sohal, 2002).

Considerable natural variation in ROS susceptibility and longevity exists among and within species. The relatively long life of birds
compared to similarly-sized mammals has been explained by lower ROS production or biomolecules that are less susceptible to ROS damage (Costantini, 2008). Overexpression of antioxidant enzymes decrease oxidative damage and increase life expectancy in Drosophila melanogaster (Sohal and Weindruch, 1996). However, the mechanisms of natural intraspecific variation have not been sufficiently elucidated. Surviving oxidative stress could be due to prevention, repair, or tolerance of molecular damage. This distinction has not been sufficiently investigated although exceptional longevity in humans has been characterized in terms of these three categories (Evert et al., 2003).

Although oxidative stress is studied in a variety of insects (Holmstrup et al., 2011; Kodrik et al., 2007; Krishnan et al., 2007), studies that link oxidative stress with aging and survival are largely restricted to D. melanogaster and a few other dipterans (Sohal et al., 1995). However, a broad comparative data basis, including relatively long-lived species, is needed for a comprehensive understanding of the links between oxidative stress and aging (Costantini, 2008). Social insects in the order Hymenoptera are exceptionally long-lived and display a very high degree of intraspecific plasticity in life expectancy (Carey, 2001; Rueppell et al., 2004). The best studied social insect is the honey bee, Apis mellifera (L). This species is a long-standing research model with a completely annotated genome (Weinstock et al., 2006) and it is the most important pollinator in a variety of natural and agricultural ecosystems (Calderone, 2012). Thus, the continued decline of honey bee health is particularly concerning. While no single cause for honey bee decline has been identified, a combination of factors may be responsible, including various pesticides (Goulson et al., 2015).

In honey bee females, the reproductive protein vitellogenin plays an important role as an antioxidant that may explain the aging plasticity between female castes and individual variation (Amdam et al., 2009; Amdam and Omholt, 2002; Corona et al., 2007; Seehuus et al., 2006). Specifically, the hemolymph titer of vitellogenin is directly linked to survival of acute oxidative stress (Seehuus et al., 2006) and classic antioxidant defenses may be less important for explaining differences in life expectancy between honey bee castes (Corona et al., 2005). In contrast, male honey bees (= drones) have much lower levels of vitellogenin (Piulachs et al., 2003) and may be more susceptible to oxidative stress due to their haploidy (Stürup et al., 2013). However, drones display mortality dynamics under natural conditions that are similar to workers (Rueppell et al., 2005).

Oxidative stress in honey bee drones has not been studied even though they are essential to the honey bee life cycle. Drones mature inside the colony and most initiate flights to mating arenas, called drone congregation areas, when they are 8–10 days old (Rueppell et al., 2005). Staying airborne in the drone congregation areas, when they are 8–10 days old (Rueppell et al., 2005). Staying airborne in the drone congregation areas, drones were placed into separate mesh-wire cages that trapped adults as they flew. Oviposition in the host colony is the most important pollinator in a variety of natural and agricultural ecosystems (Calderone, 2012). Thus, the continued decline of honey bee health is particularly concerning. While no single cause for honey bee decline has been identified, a combination of factors may be responsible, including various pesticides (Goulson et al., 2015).

In this study, we characterize the variation in oxidative stress resistance in drone honey bees after paraquat injection by determining the mortality dynamics of treatment and control groups. Additionally, we quantify the levels of oxidative damage to proteins and lipids in different tissues of drones that exhibited low or high resistance to paraquat injection. Resistant drones displayed elevated levels of lipid damage, suggesting that tolerance of molecular damage is primarily responsible for survival of an acute oxidative stress in honey bee drones. This pattern may be compatible with previous studies in honey bee workers and support a similar model of mortality between the drone and worker honey bees, despite their stark differences in life history. Moreover, our results represent another example of a dissociation between functional decline and mortality risk in honey bees (Rueppell et al., 2007) because the individuals with higher levels of oxidative damage survived longer.

2. Materials & methods

2.1. Drone sources

All honey bee (Apis mellifera) drones used in this study were reared from colonies near the North Carolina State University Lake Wheeler Honey Bee Research Facility (Raleigh, NC, (GPS coordinates: 35.725°N, 79.831°W)) and the University of North Carolina at Greensboro Honey Bee Research Facility (Greensboro, NC, GPS coordinates: 36.063°N, 79.831°W). In order to maximize the genetic diversity represented in our samples, we included drones from nine different colony sources, including a colony from a breeding program for hygienic behavior (Spivak and Downey, 1998). The majority of drones were from four colonies: the hygienic colony “Dhyg”, and colonies “D52”, “D57”, and “D65”. Overall, the individuals used were representative of the commercial honey bee population in the U.S.

2.2. Drone rearing

From each drone source, we collected up to two frames of capped drone brood that were close to adult emergence. Entire drone frames were placed into separate mesh-wire cages that trapped adults as they emerged. These cages were housed in a temperature-controlled incubator set at 33 °C. All adults that emerged during a 2-day period were collected and placed into drone cages (Laidlaw and Page, 1997) that were labeled with a unique source identification code. Each cage was 10 × 10 × 2.5 cm (L × W × H) and consisted of a wood frame, a sheet of metal screen mesh on one side, and a sheet of plastic slotted queen-excluder material on the opposite side. These cages were placed inside of full-sized queen-right host colonies. This setup allowed workers to walk into and out of the cages to care for the adult drones and provided a common maturation environment. All drones were selected randomly and the drone cages were randomly placed into the host colonies. A maximum of 50 drones was placed into each cage and up to nine cages were placed into a single host colony. All collected drones were of approximately the same age (within 2 days) and all were allowed to mature in the host colonies for an additional 10 days before experimental treatment.
2.3. Oxidative stress treatment

Oxidative stress was induced by injecting paraquat, a common herbicide that has been shown to cause oxidative stress in honey bees (Seehuus et al., 2006). Paraquat (Sigma-Aldrich) was dissolved at a concentration of 1 μg/μL into a solution of standard insect saline (135 mM NaCl, 5 mM KCl, 10 mM MgCl₂, 1.6 mM CaCl₂, 65 mM Tris–HCl buffer at pH 7). After preliminary testing, 1 μL of this paraquat solution was injected manually into each drone with a micro-syringe. The syringe was composed of a dispenser with a rubber bulb and a glass capillary tube that was pulled to a sharp needle point. The exact amount that was injected into each drone was first measured using a micro-pipet and dispensed as a single droplet onto a glass slide. This droplet was immediately drawn up into the micro-syringe and injected into a drone. The injection site for each drone was the inter-segmental membrane between the second and third segment from the distal end of the abdomen. Drones were manually immobilized by applying gentle pressure against their thorax during the injection process. Control drones were injected with 1 μL of the standard insect saline.

Each injected drone was marked with a specific color to identify its colony source and treatment before they were randomly put into drone cages and returned to their host colonies. Each host colony contained up to five paraquat treatment cages and one control cage with a mix of drones from the different sources. An equal number of individuals from each drone source was included in each cage whenever possible, with a maximum of 80 injected drones per paraquat treatment cage and 20 saline-injected drones per control cage.

2.4. Sample collection

In total, 537 drones were collected in this study, 489 paraquat-injected and 48 control individuals. Sample collection of the experimental drones began 2 h after injection. This initial delay reduced the likelihood of including drones that may have died solely as a result of physical damage incurred during the injection process and allows for paraquat detoxification. After this starting point, the cages were checked regularly for dying bees (every 2 to 4 h during the day with a longer overnight break) and numbers of deaths from each source were calculated according to standard life table procedures (Carey, 2001). Drones collected directly after the initial 24-hour waiting period (between 10% and 70% mortality within each cage) were frozen and designated as ‘low-resistant’ individuals. The longest-lived drones that survived >80% of their cage-mates were also collected, frozen, and designated as ‘high-resistant’ individuals.

2.5. Quantifying oxidative stress damage

ROS-mediated molecular damage was quantified by measuring protein carbonylation and lipid peroxidation. Lipid peroxidation was quantified by measuring a mixture of thiobarbituric acid reactive substances, including lipid hydroperoxides and aldehydes. This assay was carried out using the OXItek™ Thioibarbituric Acid Reactive Substances (TBARS) Assay Kit (ZeptoMetrix Corp.), which measures the concentration of malondialdehyde (MDA). We used the whole head of individual drones because this tissue has the highest content of phospholipids (Haddad et al., 2007), the thorax was used for protein carbonyl assay, and the abdomen as the direct site of paraquat injection showed a low repeatability in preliminary tests of the TBARS assay. Each head was separated from the frozen drone and immediately submerged in liquid nitrogen. The frozen tissue was then ground in a 1.5 mL micro-centrifuge tube with an autoclaved disposable plastic pestle. The tissue powder was mixed with 280 μL of PBS, vortexed to homogenize the cellular suspension, and centrifuged briefly to precipitate large pieces of tissue and cuticle. Subsequently, one half of the supernatant was used in the TBARS assay and the other half to quantify total protein concentration with a Pierce™ bicinechinic acid (BCA) Protein Assay Kit (Thermo Scientific) to standardize the MDA concentration. Both, TBARS and BCA kits were used strictly according to the manufacturer’s recommendations.

Protein carbonylation quantification, relative to total protein content, provides an accurate measure of oxidative stress (Dalle-Donne et al., 2003). We used the thorax, the most proteinaceous part of the honey bee. The relatively small size of honey bees did not allow for a simultaneous assessment of lipid and protein oxidation in the head. The abdomen containing the digestive tract was considered unsuitable due to variable diet content. Protein carboxylation was quantified using the Oxiselect™ Protein Carbonyl Fluorometric Assay kit (Cell Biolabs). The frozen thorax tissue was ground in a 1.5 mL micro-centrifuge tube with an autoclaved disposable plastic pestle. The pulverized thorax tissue was thoroughly mixed with 200 μL diluent solution from the assay kit. One half of this solution was used for the actual protein carbonyl assay, while the other half was used for quantifying the amount of total soluble protein in the sampled tissue with a Pierce BCA™ Protein Assay kit (Thermo Scientific). Both assays were performed strictly according to the manufacturer’s recommendations. The Bovine Serum albumin (BSA) standard serial dilutions from (0–1500 μg/mL) served as a standard curve, and protein concentrations of 3 replicates per sample were calculated based on this standard curve. Protein concentrations were subsequently used to normalize the corresponding values obtained in the protein carbonyl assay.

2.6. Statistical analysis

Survival of treatment and control drones was compared by Mantel Cox log-rank test, pooling data from eight treatment cages and two control cages, after preliminary tests revealed no significant cage effects. Drone survival was also compared among the nine different source colonies by log-rank test. The levels of protein carboxylation and lipid peroxidation were separately compared among untreated controls, and drones that were categorized as low-resistant or high-resistant to paraquat exposure with one-way ANOVAs. Tukey-Kramer post-hoc tests were used to perform pair-wise comparisons among the three experimental groups. Oxidative damage measures of all paraquat-exposed drones were compared among the four main drone sources, following the same statistical procedures.

3. Results

Control drones did not experience any noteworthy mortality over the experimental period, while paraquat-injected drones exhibited a sharp decline in survival (Fig. 1). The survival of control and paraquat-injected drones was significantly different across all cage replicates (Log-Rank Test, \( \chi^2 = 117.6, df = 1, p < 0.0001 \)). The 12-hour mortality rate of the paraquat-injected drones between 12 and 84 h post-injection varied from 32 to 57% but did not show a consistent increase or decrease over time.

Lipid peroxidation in the head was significantly different among control, low-resistant, and high-resistant drones (\( F_2, 113 = 7.43, p = 0.0099; \) Fig. 2). High-resistant drones displayed the highest level of oxidative damage (\( N = 43, 37.72 \pm 3.84 \text{ nmol/mg}, \) this was significantly higher than the levels in low-resistant drones (\( N = 55, 21.13 \pm 3.39 \text{ nmol/mg}, \) \( p = 0.0044 \)) and controls (\( N = 22, 16.02 \pm 5.36 \text{ nmol/mg}, \) \( p = 0.0038 \)). No significant difference between low-resistant drones and controls was detected (\( p = 0.7008 \)). Protein carboxylation levels in the thoracic tissue were similar in control (\( N = 9, 27.50 \pm 8.64 \text{ nmol/mg}, \) low resistant (\( N = 24, 39.86 \pm 5.29 \text{ nmol/mg}, \) high resistant (\( N = 24, 47.39 \pm 5.29 \text{ nmol/mg} \)) and groups were not significantly different (\( F_2, 54 = 1.97, p = 0.1500; \) Fig. 3).

Survival of paraquat treated drones was significantly different among the colony sources (Log-Rank test: \( \chi^2 = 69.9, df = 8, p < 0.001, \) Fig. 4). “D65” had the highest survival rate (46.76 ± 2.80 h),
and “D29” the lowest survival rate (17.63 ± 1.05 h) for the duration of the experiment. Measurements of lipid peroxidation were significantly different among the four major drone sources ($F_{3, 116} = 4.06$, $P = 0.0087$; Fig. 5). “D52” ($N = 28$, 16.49 ± 4.82 nmol/mg) and “D57” ($N = 29$, 19.82 ± 4.74 nmol/mg) exhibited statistically indistinguishable oxidative damage levels, which both were significantly lower than the values of “D65” ($N = 45$, 35.73 ± 3.80 nmol/mg). Drones from “Dhyg” ($N = 18$, 27.35 ± 6.01 nmol/mg) exhibited damage levels that were intermediate and not significantly different from any other drone source.

**4. Discussion**

This study linked survival of an acute oxidative stress to measures of molecular damage in honey bee drones to test whether survival is due to avoidance, repair, or tolerance of oxidative damage. While the hypothesis that survival is due to avoidance or repair of molecular damage predicted lower levels of oxidative damage in the paraquat resistant individuals, the tolerance hypothesis does not. The paraquat injections caused a significant increase of mortality over the experimental observation period. Our data showed that protein carbonylation was not significantly different among treatment groups and lipid peroxidation was higher in the high-resistant drones compared to the low-resistant groups. Thus our experiment provided support of the tolerance hypothesis and was not consistent with the avoidance or repair hypotheses.
Many experimental manipulations of defense mechanisms highlight the importance of avoidance of oxidative damage (Bowler et al., 1992) but complementary stress resistance and longevity-assurance mechanisms may be important for natural variation in these traits (Evert et al., 2003). Tolerance of damage may be an underappreciated stress resistance mechanism related to life expectancy (Gavrilov and Gavrilova, 2004). Higher tolerance of oxidative damage could be achieved by a higher quantity of critical cellular components, such as the mitochondria that also differ between long-lived honey bee queens and short-lived workers (Osanai and Rembold, 1968).

Individual variation in stress resistance and lifespan has been addressed in honey bee workers, where it is linked to variable titers of the yolk protein vitellogenin that is preferentially oxidized (Seehuus et al., 2006). Drones have very low levels of vitellogenin (Trenczek et al., 1989) and our general measures of protein carbonylation in the thorax did not reveal any differences among our experimental groups. Thus, it is unlikely that variation in oxidative stress resistance has a similar mechanistic basis in worker and drone honey bees. A variety of defenses against oxidative stress exist (Pamplona and Costantini, 2011) and the short-lived drones might lack extensive anti-oxidant defenses, but empirical data are lacking. Flight muscles make up the majority of the thorax but measures of protein carbonylation in the thorax may not be sensitive to oxidative stress in honey bees (Williams et al., 2008). This finding is confirmed by our results that protein carbonylation levels were not significantly different between paraquat-injected and non-injected drones. Our results suggest that the injected paraquat does not penetrate into muscle cells well or muscles cells are well-defended against paraquat damage. Repair and/or turn-over of damaged muscle proteins during the experiment seem less likely explanations for our observations.

In contrast, lipid peroxidation measures were significantly affected by treatment. Opposite to our initial prediction, the drones dying early in response to paraquat injection did not exhibit significantly higher levels of lipid peroxidation in the brain than non-injected controls. This result suggests that either very low levels of lipid peroxidation kill the low-resistant individuals or the quantified MDA accumulation is not killing honey bee drones but merely represents an indicator of oxidative stress. The low-resistant drones may have died of other oxidative damage before MDA in the brain could accumulate. High-resistant drones survived despite high levels of lipid peroxidation, indicating that they did not effectively detoxify the injected paraquat to avoid molecular damage but instead tolerated the damage. Thus, our results corroborate the view that lipid peroxidation may not be strictly coupled to survival (Halliwell and Chirico, 1993). To the extent that oxidative stress resistance and longevity are linked (Finkel and Holbrook, 2000; Lithgow and Walker, 2002), these findings weaken the proposition that...

Fig. 4. Survival of paraquat injected drones differed significantly among source colonies but not according to cage replicates (not shown). Sample size for each source colony is listed next to the respective cumulative survival curve. Post-injection time was defined as the time between the injection and the periodic surveys of experimental cohorts that were performed at different times for different replicates.

Fig. 5. Comparison of lipid peroxidation levels among the four major drone sources used in the study. Lipid peroxidation, measured as the concentration of malondialdehyde (MDA), was normalized by the total concentration of soluble proteins (BCA) in each sample. Overall, colony source affected lipid peroxidation significantly. In particular, we measured higher levels in the D65 than in the D52 and D57 colony. The black dot represents the mean, the line shows the median with the 1st and 3rd quartiles making up the rest of the rectangle and small open circle indicate statistical outliers.
membrane composition may afford the extraordinary longevity of honey bee queens (Haddad et al., 2007).

Our result is the first report to reveal the difference of lipid peroxidation from the head tissues of drones under oxidative stress. Previous studies of worker bees showed the inhibition of mitochondria complex I and oxidative stress was associated with aggregation in the brain (Li-Byarlay et al., 2014, Chandrasekaran et al., 2015). Future work can be conducted to test whether there is difference between drone and worker brain metabolism and oxidative stress, as well as potential behavioral changes of drones under oxidative stress.

The differences between our measurement of protein carbonylation and lipid peroxidation may be due tissue-specificity because methodological limitations prevented us from performing the two assays on the same tissue. Furthermore, paraquat toxicity has been linked to protein carbonylation in honey bees (Seehuus et al., 2006). However, our results suggest that the paraquat injection leads to systemic damage because lipid peroxidation was increased in the head, furthest away from the site of injection. Consequently, paraquat must have traveled to the head after injection into the abdomen without significant carbonylation of the proteins in the thorax. The insect flight muscles are a central place for naturally occurring oxidative stress because of the intense flight metabolism which might make them intrinsically more resistant to oxidative stress (Williams et al., 2008). The drone’s flight muscles are absolutely critical to fitness, so that they have evolved to be particularly resistant to oxidative stress and hence explain why they do not show damage as seen in the lips. Future studies will have to distinguish the relationship between oxidative damage in different body compartments and among different classes of biomolecules to gain a more comprehensive understanding of oxidative stress at the organismal level in honey bees and other species. Future studies will also have to clarify whether variation in oxidative stress resistance in honey bee drones is linked to their considerable variation in life expectancy under normal circumstances of free flight (Rueppell et al., 2005), which seems to be mediated in female honey bees through the titer of the antioxidant vitellogenin (Seehuus et al., 2006; Corona et al., 2007). In addition to antioxidant defenses, tolerance of molecular damage may be an important contributor to ensure survival in the longer-lived workers and queens in honey bees and more generally (Evert et al., 2003).

Different drone sources exhibited significant differences in survival and levels of lipid peroxidation. The longest-lived drone source (D6S) also experienced the highest levels of lipid peroxidation, and the general relation of survival and lipid peroxidation across different colonies confirmed the overall relationship between these two variables at the colony level. The significant differences among drone sources but not among replicate cages suggest that genetic and/or environmental factors during development, such as nutrition (Andersen et al., 2010; Huang, 2012), are more important for the survival of an acute oxidative stress than the adult social environment during exposure to that stress. Our experimental design did not enable us to distinguish between genetic and developmental causes of the influences of the colony source and more detailed studies are required to distinguish between genotype and developmental effects. Genetic variability in stress resistance would encourage future efforts in honey bee breeding to select for tolerance to oxidative damage as a general stress resistance mechanism (Monaghan et al., 2009). Regardless of the mechanism, the selectively bred hygienic bees (Dhyg) did not show a particularly low survival, indicating that this form of disease resistance and stress tolerance may be independently selected for in the same breeding program (Rueppell, 2014).

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