

Senescence in the worker honey bee *Apis Mellifera*

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Abstract

Honey bees are social insects that exhibit striking caste-specific differences in longevity. Queen honey bees live on average 1–2 years, whereas workers live 2–6 weeks in the summer and about 20 weeks in the winter. It is not clear whether queen–worker differences in longevity are due to intrinsic physiological differences in the rate of senescence, to differential exposure to extrinsic factors such as predation and adverse environmental conditions, or both. To determine if the relatively short lifespan of worker bees involves senescence, we measured age-specific resistance to three different physiological stressors (starvation, thermal, and oxidative stress) while eliminating age-related differences in foraging activity and minimizing age-related differences in energy expenditure. Despite these manipulations, older worker bees were still significantly less resistant to all three stressors than were younger bees. These results indicate that the regulation of worker bee lifespan involves senescence, in addition to extrinsic factors.

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1. Introduction

Senescence is defined as an age-related decline in physiological function, performance, survival, or reproduction (Finch, 1990). Senescence (often referred to simply as “aging”) is a nearly universal feature of multicellular organisms, and appears to occur even in unicellular yeast and bacteria (Lithgow and Kirkwood, 1996; Kirkwood and Austad, 2000). Understanding the biological processes that lead to senescence, and why different organisms senesce at dramatically different rates, is a long-standing problem in both molecular and evolutionary biology (Lithgow and Kirkwood, 1996; Finch, 1990; Rose, 1991; Partridge, 1993; Kirkwood and Austad, 2000).

In some eusocial insects (ants, bees, wasps, and termites), queens and workers of the same species sometimes show a 100-fold difference in lifespan, with reproductive queens

having longer lifespans than the non-reproductive workers (Winston, 1987; Keller and Genoud, 1997; Page and Peng, 2001). Strikingly, the long life of social insect queens does not come at the cost of low reproduction: queens of many social insects lay hundreds or thousands of eggs per day throughout their adult life. Their ability to sustain both high reproductive effort and long life makes social insects particularly promising model systems for studies of aging (Parker et al., 2004; Seehuus et al., 2006; Corona et al., 2005; Corona et al., 2007).

In the honey bee, *Apis mellifera*, queens have an average lifespan of 1–2 years and workers have an average lifespan of 15–38 days in the summer and 140 days in the winter (Winston, 1987). Queens and workers are not genetically distinct, so biological differences between castes are due to gene expression differences that depend on social and dietary cues that individuals experience during development (Corona et al., 2005). Therefore, studying the comparative physiology and molecular biology of queens and workers is an attractive paradigm for investigating proximate mechanisms of lifespan differences (Parker et al., 2004; Corona et al., 2005). However, there is a potential

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serious flaw in this paradigm: it is currently not known whether caste-specific lifespan differences result from inherent physiological differences in the rate of senescence or, alternately, from caste-related differences in exposure to risk.

In nature, queen bees leave the protected environment of the hive only to take mating flights at 1–2 weeks of age, and possibly once more later in their life, during colony fission. In contrast, workers spend the first 2–3 weeks of adult life mostly in the hive performing tasks such as brood care (“nursing”) before shifting to foraging outside the hive for nectar and pollen, making over 10 trips a day, sometimes at distances of up to 2 km (Winston, 1987). Foragers thus experience risks from predation, thermal stress, and physical exhaustion; risks that queens (and pre-foragers, such as nurse bees) do not experience to the same extent. Thus, a plausible hypothesis for the difference in queen and worker lifespan is that workers, once they become foragers, experience high extrinsic mortality, and therefore have a much shorter mean lifespan than queens.

Only a few studies have addressed the question of whether worker bee lifespan is determined by senescence or exposure to extrinsic risk. Neukirch (1982) compared lifespans of foragers with different amounts of flight experience and found that lifespan was inversely related to daily flight experience. She argued that foragers have fixed energy reserves, and, once the reserve is depleted, foragers cannot fly and fail to return to the hive. This idea does not require physiological senescence. In contrast, later studies found patterns consistent with senescence. Schmid-Hempel and Wolf (1988) found that workers had fixed lifespans regardless of energy expenditure, and Visscher and Dukas (1997) found that behavioral and foraging performance declined after 10 days of foraging (see also Tofilski, 2000). A limitation of all these studies is that age-specific survival data were collected on foragers, and so were possibly confounded by the cumulative effects of energy expenditure and foraging activity. Because of the lifestyle of the forager, age-related increases in mortality rates could be due to accumulation of injuries or exhaustion of energy reserves, which are not necessarily due to intrinsic physiological deterioration.

We exploited the honey bee’s strong plasticity for division of labor (Robinson, 1992) to remove the confounding effects of energy expenditure and risks associated with foraging. Worker bees respond to changing social conditions by accelerating, delaying, or reversing their typical pattern of behavioral maturation. For example, if there is a shortage of foragers or large numbers of young larvae in the hive, some bees delay their transition to foraging and become “overage” nurses (Robinson et al., 1989). We studied age-specific stress resistance in overage nurses that did not experience the extrinsic risk factors associated with foraging. We predicted that if there is worker senescence, then older bees should have lower survival under each stress treatment than younger bees.

2. Materials and methods

2.1. Experimental colonies

We set up five single-cohort colonies (Robinson et al., 1989), each initially composed of ca. 10,000 1-day-old bees. We obtained 1-day-old worker bees by removing frames of pupae from typical field colonies (headed by naturally mated queens) and placing them in an incubator (34 °C and 80% relative humidity). The bees were marked with a paint dot on the dorsal thorax, color coded according to day of emergence and source colony. This process was continued over a 5-day period for each colony to obtain the 10,000 bees. Each single-cohort colony was then given a (naturally mated) queen, 4 frames of honey and pollen, and 2 frames for the queen to lay eggs in. We encouraged the development of overage nurses by removing frames of brood prior to the emergence of new adult bees, and replacing them with frames of younger brood.

2.2. Collections of bees

At each collection date, we collected 300 bees from each age class (10, 30, and 50 days old) that was available at that date. We collected bees that were displaying typical nursing behavior (head in cell containing a larva; see Huang and Robinson (1996)). Collections were made when foragers were out of the hive during times of active foraging to minimize the chances of misidentification. The five single-cohort colonies were set up in a time-staggered design, so that bees of different age classes were available on the same day (Fig. 1). We were thus able to evaluate the effects of age on stress resistance, and decouple these effects from the effects of source colony and date of collection (seasonality). Bees were held individually in cages within a plexiglass tray, provided with 50% sucrose solution ad lib, and kept

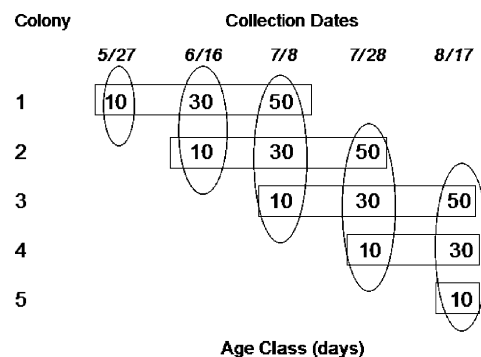


Fig. 1. Schematic of time-staggered experimental design, so that nurse bees of different age classes were available for treatment on the same day. Rows indicate each experimental (single-cohort) colony and columns indicate the collection dates for bees of the different age classes used in the three stress resistance tests. This experimental design enabled us to evaluate the effects of age on stress resistance, taking into account influences of both source colony and date of collection (seasonality). We collected 10-, 30-, and 50 day-old bees from all colonies with the exception of colony 4 (10- and 30-day-old bees only) and colony 5 (10-day-old bees).

Table 1
Effects of starvation, heat, and hydrogen peroxide (oxidative stress) on lifespan for nurse honey bees 10, 30, and 50 days of age

Treatment	Age class	Mean (SE)	Median	Sample size
Starvation	10	91 (5.0)	42	478
	30	46 (1.4)	36	375
	50	31 (0.9)	24	287
Heat	10	120 (2.2)	120	484
	30	107 (2.5)	102	371
	50	106 (2.6)	102	288
Hydrogen Peroxide	10	82 (2.5)	75	479
	30	81 (2.8)	66	371
	50	75 (2.5)	66	287

Mean and median lifespan (h).

at constant temperature (34 °C) for 24 h, prior to the start of the stress tests. After 24 h, the surviving bees were randomly assigned to the three treatment groups. Total sample sizes for each age class and treatment group are given in Table 1.

2.3. Stress tests

To detect senescence, we measured the effects of oxidative stress, heat stress, and starvation on bees from each age class. Resistance to these stressors typically declines in senescing insects, causing increased mortality (Luckinbill et al., 1984; Rose, 1984; Nghiem et al., 2000). After the collections were made, during the next 24 h, bees were housed in an incubator at 34 °C and were provided a 50% sucrose solution so that they could feed freely. After that, bees of the same age class that were still alive were randomly assigned to three different trays and one of the three trays was assigned to each one of the treatments (starvation, heat stress, or hydrogen peroxide). Treatment details are as follows. *Hydrogen peroxide*: Bees were given a 50% sucrose solution that contained 20% hydrogen peroxide. This dose was based on results from *Drosophila melanogaster* that showed that a dose of 5% hydrogen peroxide produced high mortality (Sun and Tower, 1999), adjusting for differences in body mass between honey bees and fruit flies. *Heat stress*: We exposed bees to 42 °C in an incubator; colonies typically maintain their hives at approximately 34 °C by behavioral thermoregulation, and it has been reported (Mardan and Kevan, 2002) that bees kept at 42 °C showed decreased longevity. Bees were kept at 42 °C until they died. *Starvation*: Bees were maintained in an incubator without any food at 34 °C, and were provided with water to prevent desiccation. In all treatments, bees were housed in individual cages within a plexiglass tray. With the exception of the starvation treatment, food was provided in the tray, and bees were allowed to feed freely. Food in the trays was replaced every 6 h, and water was replenished for the bees in the starvation treatment. Food replacement was of special importance for the hydrogen peroxide treatment, since hydrogen peroxide

degrades in water. Bees in all incubators were maintained in a 24-hour dark cycle; the hive is naturally dark, except for whatever light penetrates from the hive entrance.

2.4. Censusing mortality

Bees were censused at 0:00, 6:00, 12:00, and 18:00 h until all were dead. Information on age and source colony was obtained from the thorax markings. Six bees escaped during the experiment (3 in the heat stress and 3 in the starvation treatment) the escape time of these bees was treated as a right-censored observation in the data analysis.

2.5. Lipid analysis

Because the most striking differences in age-specific stress resistance were observed in the starvation test (see Results), we explored whether the results could be explained by differences in lipid reserves. We measured the abdominal lipid levels of young and overage nurses, using foragers as a comparison group, since foragers have the lowest lipid levels among worker bees (Toth and Robinson, 2005). We used young nurses less than 7 days of age ($n = 23$), 50-day-old nurses ($n = 23$) and 50-day-old foragers ($n = 22$). Each abdomen was dissected and the digestive tract and sting apparatus removed; abdomens were then freeze-dried, homogenized in a 2:1 chloroform:methanol solution, and dried down to a constant volume of 2 ml. The lipid assay was performed using 100 µl of each sample, following the procedures in Toth et al. (2005). We measured the absorbance of each sample using a SpectraMax 190 spectrophotometer (Molecular Devices, CA), with readings at 525 nm. Absorbance readings were converted to milligrams of lipid using a cholesterol standard. The lipid assay was performed twice on each sample.

2.6. Data analysis

We calculated Kaplan–Meier (product-limit) survival estimates for the 10-, 30-, and 50-day-old workers for each stress treatment. We tested for differences in survival among age classes within a treatment using the log-rank and Wilcoxon tests produced by SAS Proc Lifetest (SAS System v.9.1). Wilcoxon tests are more sensitive to differences in survival occurring earlier in the trials, while log-rank tests are more sensitive to differences that occur later (Allison, 1995). Results of both tests were consistent in every case, so we report only the log-rank test results. We also tested for significant differences between age classes using Cox proportional hazards models as implemented in SAS Proc Phreg. This test allowed direct comparison of the hazard rate (risk of death per unit time) for each age class within a treatment group, and formal statistical tests for pairwise differences in hazard rates between age classes (Allison, 1995). In this analysis, a hazard ratio >1 indicates a higher hazard for the older

bees, and a value <1 indicates a lower hazard for the older bees. We repeated the pairwise contrast analysis after removing data for colonies 4 and 5; because these colonies are represented by two (or one) age classes, there is a possibility of confounding age and colony effects. For the analysis of lipid data, we treated the replicate measures for each sample as repeated measures in a general linear model (repeated measures ANOVA) using SAS Proc Mixed (Littell et al., 2002).

3. Results

Mean survival times for 10-day-old bees were longer than for older bees in all three stress tests (Table 1). Survival curves show that 10-day-old bees had higher survival at each time point than did 50-day-old bees (Fig. 2).

Log-rank tests of survival times indicated that age classes differed significantly for the starvation ($\chi^2 = 202.6$, $p < 0.0001$) and heat stress treatments ($\chi^2 = 20.9$, $p < 0.0001$), but not for the hydrogen peroxide treatment ($\chi^2 = 2.6$, $p = 0.27$). However, the semi-parametric tests of the proportional hazards model indicated that differences in hazard rates between age classes were significant for all three treatments: starvation ($\chi^2 = 102.2$, $p < 0.0001$), heat stress ($\chi^2 = 50.82$, $p < 0.0001$), and hydrogen peroxide ($\chi^2 = 7.8$, $p = 0.0205$).

Similarly, pairwise contrasts of the hazard rates within treatments indicated that 10-day-old bees had significantly lower mortality per unit time than did 50-day olds in each treatment (Table 2). All hazard ratio estimates were >1 , indicating higher mortality rates for older bees in each comparison; comparisons were significant in 6 out of 9 pairwise tests, and marginally non-significant at $P = 0.05$ in one additional comparison (Table 2). Limiting the analysis to colonies 1–3 produced qualitatively identical results. In this analysis, 10-day-old bees had significantly lower mortality than 50-day-old bees in all three stress treatments (Appendix 1).

There were no significant differences in stored lipid in young and old nurses ($F_{[1,64]} = 2.6$, $P = 0.12$, Fig. 3). Both young and old nurses had significantly higher lipid content than foragers (young nurses vs. foragers, $F_{[1,64]} = 52.3$, $P < 0.0001$; old nurses vs. foragers, $F_{[1,64]} = 31.9$, $P < 0.0001$). These results indicate that results of the starvation test are not attributable to differences in stored lipids between young and old nurse bees; overage nurses have lipid levels characteristic of nurses, and not of foragers. These results are consistent with findings from Toth et al. (2005).

4. Discussion

Our results provide the first clear demonstration of worker honey bee senescence. In our experiments, this physiological decline began between 10 and 30 days of age and continued through 50 days of age. These results

indicate that honey bee workers experience an intrinsic physiological decline at an age that is consistent with their observed maximal lifespan in the summer and their longevity does not depend solely on extrinsic mortality factors.

Our results are unlikely to be due to differences in physical activity because we used overage nurses rather than foragers. It is unlikely that our results, especially for the starvation treatment, can be attributed to older nurses having lower nutritional reserves than younger nurses. Our lipid analysis showed no difference between lipid stores in young and overage nurses, but other nutritional indicators such as glycogen content were not measured. We conclude that the marked decline in stress resistance in 30- and 50-day-old bees strongly suggests physiological senescence.

Results from the heat stress assay indicated that 30- and 50-day-old bees were more likely to die than 10-day-old bees. Although the differences were highly significant, they were less extreme than in the starvation assay. Perhaps, this is because the treatment was relatively less extreme. Honey bees can tolerate temperatures up to 45 °C for at least 2 h, and humidity is an important factor in their ability to tolerate high temperatures (Free and Spencer-Booth, 1962). Perhaps our treatment was not as stressful as it could have been, because bees were provided with an unlimited source of sugar syrup and full water containers were kept in the incubator at all times.

Differences between age classes in the hydrogen peroxide treatment were relatively small (though statistically significant) compared with the starvation and heat treatments. It seems unlikely that bees in the hydrogen peroxide treatment were not feeding, since the median lifespan of bees of all age classes surpassed that of bees in the starvation treatment. It is possible that the concentration of hydrogen peroxide we used was too weak to induce much oxidative stress or stress-related mortality in our bees. This speculation is supported by the observation that paraquat (another free radical-inducing agent) caused greater mortality in a comparable experiment (Corona et al., 2007). In that experiment, the median lifespan for worker bees 30 days of age was 33 h, compared with 66 h in our experiment. This observation is further supported by another experiment comparing paraquat-induced oxidative stress resistance in worker bees where complete mortality was reached within 60 h of paraquat injection (Seehuus et al., 2006).

Hydrogen peroxide is an oxidizing agent that slowly decomposes into water and oxygen at room temperature. The decomposition of hydrogen peroxide can be accelerated in the presence of light and at high temperatures, increasing by a factor of 2.2 for every 10 °C rise in temperature. Such decomposition is also catalyzed by dissolved ions of metals, and suspended oxides and hydroxides (Goor et al., 1992). Even though we replaced the hydrogen peroxide and sugar solution in the trays every 6 h, there is a possibility that the decomposition of

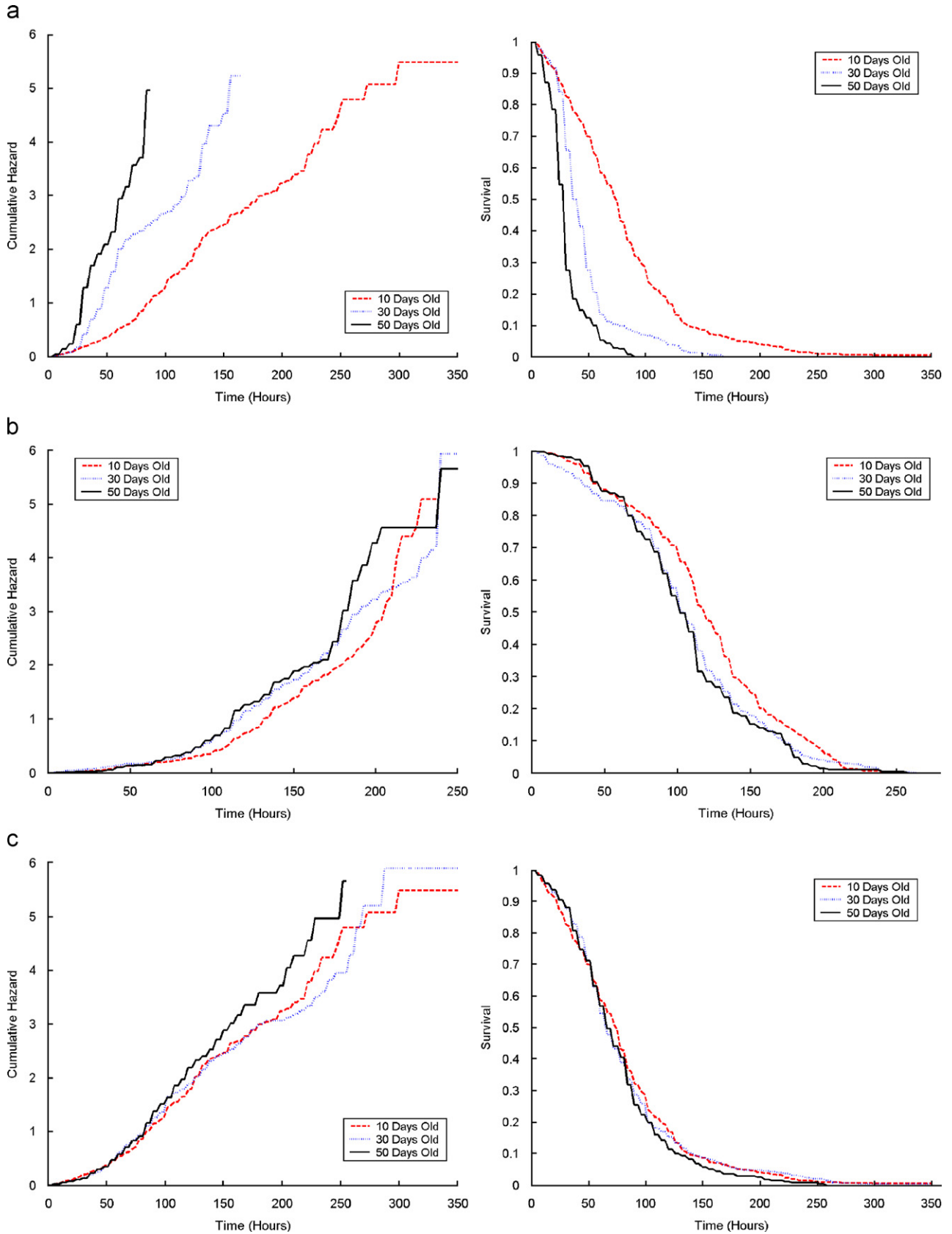


Fig. 2. Age-related differences in resistance to: (a) starvation; (b) heat, and (c) hydrogen peroxide in nurse honey bees. Cumulative hazard function (-Log(Survival), left) and Survival distribution function (right) of 10- (red) 30- (blue) and 50-day-old (black) nurse bees. Bees were censused every 6 h. Note difference in scale for treatments. Eighteen 10-day-old bees were alive in the starvation treatment; one 10-day-old bee and one 30-day-old bee were alive in the hydrogen peroxide treatment after 350 h of exposure to stress.

Table 2
Effects of starvation, heat, and hydrogen peroxide (peroxide) on lifespan for nurse honey bees 10, 30, and 50 days of age

Treatment	Contrast	Hazard ratio	Confidence intervals	χ^2	P
Starvation	10 vs. 30	1.514	(1.280, 1.791)	23.4	<.0001
Starvation	10 vs. 50	2.637	(2.183, 3.184)	101.4	<.0001
Starvation	30 vs. 50	1.741	(1.469, 2.064)	40.9	<.0001
Heat	10 vs. 30	1.506	(1.3, 1.744)	29.7	<.0001
Heat	10 vs. 50	1.739	(1.475, 2.049)	43.5	<.0001
Heat	30 vs. 50	1.155	(0.982, 1.358)	3.0	0.08
Peroxide	10 vs. 30	1.073	(0.927, 1.241)	0.9	0.34
Peroxide	10 vs. 50	1.257	(1.069, 1.478)	7.6	0.006
Peroxide	30 vs. 50	1.172	(0.998, 1.3376)	3.7	0.05

Hazard ratios for each pairwise comparison between age classes (ratio of older to younger bees); degrees of freedom = 1 in every case. Results in bold indicate significant differences.

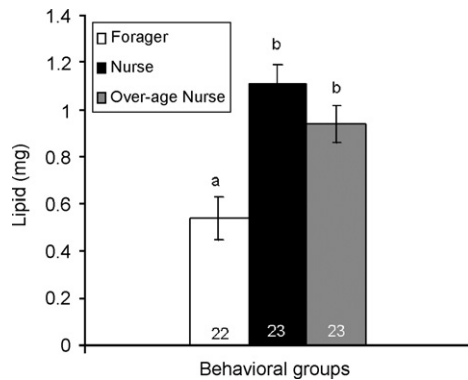


Fig. 3. Abdominal lipid content for 7-day-old nurses, 50-day-old nurses, and 50-day-old foragers. Letters indicate groups that differ significantly in mean lipid content by pair-wise contrasts. Numbers at bottom of bars indicate sample size.

hydrogen peroxide into water and oxygen may have caused failure to induce mortality in our bees.

Rueppell et al. (2005) found that age-specific mortality increased exponentially in drones after about the 10th day of flying activity, consistent with either senescence, non-replenishment of resources, or 'wear and tear'. They also reported that lifespan after the initiation of flying activity was negatively correlated with age at first flight, and suggested that this pattern was due to the onset of senescence even before the initiation of flight. This suggestion is consistent with our experimental results for workers.

Senescence of honey bee hemocytic cells has been reported by Amdam et al. (2004, 2005). Amdam et al. (2004) found that foragers had low zinc concentrations compared with nurses, which in turn resulted in decreased hemocyte counts in the hemolymph; foragers also possessed a higher number of pycnotic cells than nurses. Working with reverted nurses, Amdam et al. (2005) showed that these changes were related to both age and behavioral role; reverted nurses had a higher hemocyte count relative

to similarly aged bees that continued to forage, but reverted nurses had lower counts relative to normal-age (young) nurses. The authors assumed that hemocyte count and cell pycnosis are measures of senescence at the cellular level. There are no data on the relationship between hemocyte count and immune response or mortality rate, so it is not clear in this case that cellular senescence leads to organismal senescence.

Our results show that worker bees show senescence. In contrast, in a recent study Rueppell et al. (2007) assessed age-dependent behavioral performance of foragers using a battery of behavioral tests that included light sensitivity, sucrose responsiveness, learning of olfactory cues, and walking velocity. In that study, the authors concluded that worker bees did not exhibit an age-dependent decline in performance but showed an increase in mortality with chronological age. The discrepancies between our results and those of Rueppell et al. may be attributed to the nature of the behavioral tests employed. Although the behavioral tests employed are related to foraging activity they may not prove demanding to the bees, and thus not allow the possibility for a decline to be manifest. Previous studies in *D. melanogaster* show that age-related declines in behavior differ, depending on the nature of the behavior being tested, the genotype, and the gender of the flies (Fernandez et al., 1999; Martin and Grotewiel, 2006; Simon et al., 2006).

We have shown here that limited worker lifespan is due at least in part to intrinsic senescence and not solely to extrinsic mortality factors. Of interest would be to determine if honey bee queens also show senescence. Studying senescence in queens is a more difficult question to address than in workers, given their extended lifespan. In addition, conducting such tests in a eusocial species presents special challenges since queens are fed and groomed by workers. Although we did not directly study queen senescence, queens are known to lay up to 2000 eggs per day and the laying rate does not appear to decline at least through the first year of life (Winston, 1987), suggesting negligible senescence during this period. In contrast, we have shown that senescence in workers begins before 50 days of age. This comparison suggests that the extended lifespan of queens is due to slower senescence, and not just to lower extrinsic mortality.

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Table A1

Treatment	Contrast	Hazard ratio	Confidence intervals	χ^2	<i>P</i>
Starvation	10 vs. 30	1.109	(1.821 2.651)	67.4	0.27
Starvation	10 vs. 50	2.197	(0.921 1.334)	1.2	<0001
Starvation	30 vs. 50	1.982	(1.658 2.368)	56.5	<0001
Heat	10 vs. 30	1.524	(1.286 1.806)	23.6	<0001
Heat	10 vs. 50	1.702	(1.434 2.020)	37.1	<0001
Heat	30 vs. 50	1.117	(0.945 1.321)	1.68	0.19
Peroxide	10 vs. 30	1.025	(0.869 1.209)	0.0839	0.7721
Peroxide	10 vs. 50	1.213	(1.027 1.433)	5.1783	0.0229
Peroxide	30 vs. 50	1.184	(1.002 1.399)	3.9418	0.0471

Results in bold indicate significant differences.

Appendix

Effects of starvation, heat, and hydrogen peroxide (peroxide) on lifespan for nurse honey bees 10, 30, and 50 days of age (restricted to colonies 1, 2, and 3). Hazard ratios for each pairwise comparison between age classes (ratio of older to younger bees), degrees of freedom = 1 in every case (Table A1).

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