Behavioral Ecology

The official journal of the
ISBE
International Society for Behavioral Ecology

Behavioral Ecology (2013), 24(5), 1192–1198. doi:10.1093/beheco/art049

Original Article

# When every sperm counts: factors affecting male fertility in the honeybee *Apis mellifera*

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Received 18 January 2013; revised 29 April 2013; accepted 6 May 2013; Advance Access publication 18 June 2013.

Eusocial hymenopteran males have exceptionally high levels of ejaculate quality, which are assumed to result from extreme selection pressures for pre- and postcopulatory male–male competition and the necessity to retain viable sperm after years of storage in female (queen) spermathecae. We hypothesized that the production of high-quality sperm carries substantial costs so that fertility of males may be compromised by stress factors when they are operating at their physiological limits. To test this, we performed a series of experiments using honeybees as our model system, to establish possible effects of male age on sperm quality and to evaluate effects of elevated temperatures, food deprivation during sexual maturation, and immune challenges. We found that sperm viability decreases with male age but that males of some colonies were better able to delay ejaculate senescence than others. Exposure to elevated temperatures and wounding both significantly decreased male fecundity, but protein deficiency after hatching did not. This suggests that investment in drones is completed at pupation and that sexual maturation does not require additional protein feeding. The sensitivity of drone fitness to stress factors related to temperature and immune system activation illustrates that hygienic monitoring and active thermoregulation by workers are essential for colony-level reproductive success. These results underline that honeybee drones have been under strong selection for extreme specialization on reproductive performance and that this precludes any exposure to the stressful conditions that foraging workers normally experience.

*Key words*: **food restriction, heat stress, immunity, senescence, sperm viability.**

## INTRODUCTION

Males of eusocial bees, ants, and wasps live a very short and protected life inside their native colony where they are sheltered and cared for by their sisters until the time of their nuptial flight. They do not contribute to colony maintenance and often take a large toll on colony resources as they are abundantly present in colonies for prolonged periods of time before dispersing to mate (Hölldobler and Bartz 1985). Colony male reproductive investments of this kind are normal across the eusocial Hymenoptera (Boomsma et al. 2005) but are exceptional in swarm-founding honeybees (Page and Metcalf 1984) where sex ratios are extremely male biased (Baer 2005). Honeybee males (drones) are reared without being in direct competition with gynes, of which colonies produce only very few by supplying them with royal jelly (Wheeler 1986). Honeybee males are also produced in a separate category of drone cells enabling workers to accurately adjust the amount of larval provisioning to produce drones of maximal potential reproductive fitness (Seeley and Morse 1976).

Honeybee drones are suicidal maters (Page 1986; Baer 2005) and very few of them will realize any reproductive success. Their fitness is further challenged by the fact that virgin queens mate with 10–20 drones in quick succession, after which they discard more than 90% of the sperm received as only about 2.5% actively migrate into long-term sperm storage (Baer 2005). Sperm viability is crucial for eusocial hymenopteran male reproductive success, as only live sperm becomes stored in the spermatheca (Collins 2000). This selection process implies that the viability of newly transferred sperm in the lateral oviducts is lower than sperm that is stored in the spermatheca (Gencer and Kahya 2011). Average sperm viability (the proportion of live sperm) is, therefore, an important fitness determining trait in honeybee males, so that males are under strong selection for both flight stamina to achieve copulation and high sperm viability to maximize sperm storage in competition with other ejaculates (Berg et al. 1997; Collins and Donoghue 1999). A remarkable consequence of this extreme form of sexual selection has been that drones eclose from their pupae with all the sperm they will ever possess (Baer 2005), similar to ant males (Hölldobler and Bartz 1985), so the quality of their sperm cannot be restored if it has been compromised by any stress factors during development.

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It seems likely that honeybee workers optimize their reproductive efforts by rearing only those drone larvae until pupation that they have adequate resources to provision (Free and Williams 1975; Rowland and McLellan 1987) and that buffering systems might have evolved to ensure that reproductive returns from these larval investments are secured when stress factors might affect adult drones in the period between eclosion and dispersal. At this stage, however, different fitness components may also trade-off against each other (Stürup et al. 2011), so it remains unclear whether sperm quality will be prioritized under all circumstances. Here, we address questions of this kind by testing the robustness of honeybee sperm when drones face immunological, environmental, and nutritional challenges.

We performed a series of experiments to quantify the effects of environmental stress factors on drone fertility. We first quantified the effect of male age on sperm viability to assess the extent to which drones are able to maintain the high quality of their fixed number of sperm throughout their lives. The relevant time span is 20–40 days after eclosing (the average longevity of drones; Page and Peng 2001) and particularly the last 4 weeks of this period as drones cannot start their mating flights until they are around 8 days of age (Tofilski and Kopel 1996). We then used age-controlled males to experimentally investigate the effects of adult protein deprivation and high-temperature stress on sperm viability, and we challenged the immune system of drones to see whether stress by sterile wounding of drones elicits an immune response that would reduce sperm viability.

## MATERIALS AND METHODS

All honeybee males used for experimental work originated from hives that we kept in an apiary on campus at the University of Western Australia. To breed males of known age, we collected male brood shortly before they eclosed and placed them in an incubator at 32 °C and 55% humidity. Drones hatched within 2 days and were collected and relocated into individual cages that we placed back into their host colonies to allow males to sexually mature. As drones matured, we collected them from the colonies and assessed sperm viability at 3- to 5-day intervals for drones between 9 (mean 9.8 ± 0.18 standard error [SE]) and 36 (mean 29.1 ± 1.34 SE) days after eclosion. Because the number of hatching drones differed between days and colonies, the number of drones per cage ranged between 25 and 50, which is well below the maximum number of drones that can be kept in such cages (Stürup M, unpublished data). Males used for the experiment were randomly chosen from different cages.

Semen collection was done according to a previously developed protocol used to collect sperm for artificial insemination of honeybees (Collins and Donoghue 1999; Baer et al. 2009). In short, males were killed using chloroform, which initiates male ejaculation as they partially evert their endophallus. Ejaculation was further enhanced by manually squeezing the male's abdomen between 2 fingers until semen eventually appeared at the tip of the male's endophallus. We collected 2 μL of ejaculate using a pipette and transferred it to a 1.5-mL Eppendorf tube containing 200 μL of semen diluent (11 g NaCl, 1 g glucose, 0.1 g L-arginine, 0.1 g L-lysine, 6.08 g Tris in 1000 mL dd$H_2O$, pH 8.72). Sperm was mixed by gently shaking the tube, and each sample was incubated for an hour at room temperature before further processing.

To quantify sperm viability, we used a method used previously by Collins and Donoghue (1999), den Boer et al. (2008), and Simmons and Beveridge (2011). In brief, we used 5 μL of the diluted semen, added 5 μL of SYBR14 (staining all sperm cells green), and incubated the sample in the dark for 10 min. In a next step, we added 2 μL of propidium iodide (staining all dead sperm cells red) and incubated the sample for 7 min in the dark. The number of live and dead sperm cells was finally determined using a fluorescence microscope (Leica DM 1000, at ×400 magnifications). For each sample, we estimated sperm viability twice and counted a minimum of 300 sperm cells per sample. All samples were collected and handled in the same way, and all estimates of sperm viability were made blind to treatment. Because the collecting procedure is likely to affect sperm viability, we always compared relative sperm viabilities with a direct control treatment (cf. Holman 2009b).

To test for effects of food protein deprivation on sperm viability, we reared drones as described above. A minimum of 200 emerging drones were collected from 3 different colonies and they were randomly allocated to 1 of 2 treatment groups, where access to pollen was either restricted or ad libitum. To do this, we placed drones in small miniature Styrofoam colonies and provided them with ad libitum access to a 50:50 sugar:water solution. Colonies were separated according to treatment group and placed in flight cages that allowed bees to leave the nest to forage for water but prevented them from collecting pollen.

Colonies of the pollen-deprived treatment were provided solely with sugar syrup, whereas control colonies were also provided with redgum (*Corymbia calophylla*) pollen ad libitum. Colonies were left undisturbed until males were 12 days old, after which the drones were collected and sperm viability was estimated as described above. For one of the colonies, we extended the experiment and collected males at 5-day intervals until the age of 22 days. Statistics were performed including drone age as a continuous variable for the colony where these data were available.

To measure the effect of wounding on male fertility, sexually mature males were caught daily between 2 and 4 PM at the hive entrance before or after mating or orientation flights. Males were taken back to the lab and randomly allocated to 1 of 2 treatments. We wounded half of the males using a hypodermic needle (0.5 mm) to puncture a hole in their intersegmental membrane between the 3rd and 4th tergite. This treatment has previously been shown to induce an immune response in drones, and our treatment, therefore, resulted in a stimulation of the immune system (Laughton et al. 2011). Males of the control treatment were handled the same way but without performing the actual puncturing. All drones were color marked based on treatment and placed together on a honey frame in an incubator overnight at 32 °C and 55% humidity. Twenty-four hours after the treatment, the male ejaculates were collected and sperm viability analyzed using the same protocol as above. We analyzed sperm from a minimum of 5 males per treatment group and replicated the experiment 9 times.

To measure the effect of temperature on male fertility, we collected newly hatched drones and randomly allocated them to 2 different treatments, being heat-treated or nonheat-treated (control). When males reached sexual maturity at an age of 10–16 days, we exposed them for 4 h to a temperature of either 39 °C (treatment) or 25 °C (control). Drones were afterward returned to their original host colonies for a day before we collected their ejaculates as described above. To test for possible effects of temperature on ejaculates, we exposed half of the semen samples to 39 °C (treatment) and kept the other samples at ambient temperature of 25 °C

(control). Consequently, we ended up with 4 treatments, where we obtained semen samples from heat-treated males, whose semen samples were either exposed to heat or not, as well as nonheat-treated males whose ejaculates were exposed to heat or not. Males used for experiments were 10, 11, 15, and 16 days old, and all 4 age groups participated in all 4 treatments. To estimate sperm viability, we counted a minimum of 400 sperm cells per sample using the same protocol as described above.

All data analyses were carried out using JMP software version 10.02. As we found that all of our sperm viability data were highly overdispersed, we performed logit transformations to normalize data and used the transformed data for all the analyses. Colony and replicate were added as random factors in the analyses.

## RESULTS

We found that senescence negatively affected sperm viability, but only in some colonies (Figure 1). The overall decrease with increasing drone age was highly significant ($P = 0.0035$; Table 1) with average sperm survival being reduced by almost 50% in older drones compared with young ones. However, there was also a significant interaction between age and colony ($P = 0.025$, Table 1), indicating that mean sperm viability remained high throughout the life of drones in some colonies but decreased more dramatically in other colonies. There was a negative correlation between the number of drones per cage at the beginning of the experiment and the rate of change in sperm viability for each colony ($r = -0.79$), but the power of this test was low ($\beta = 0.34$) and the relationship did not reach statistical significance ($P = 0.11$).

When we tested for effects of an immune challenge on male fertility, we found that males pricked with a needle had significantly lower sperm viabilities 24 h after the treatment compared with males of the control group ($P = 0.0021$; Figure 2 and Table 1), and this was consistent throughout all replication rounds, even though we found significant differences in male fertility between rounds. Depriving males from access to pollen had no significant effect on sperm viability ($P = 0.43$; Table 1), and this was consistent across colonies ($P = 0.77$; Figure 3 and Table 1). We found no confounding effect of drone age in the single colony where drones could be sampled continuously between age 13 and 22 days ($P = 0.87$; Table 1), so we pooled all males in Figure 3. The lack of an age effect is not surprising, as senescence tends to only become visible when drones are older than 22 days (Figure 1).

Finally, we found that drones are surprisingly heat sensitive. When we exposed males to a temperature of 40 °C for 24 h, they all died (unpublished data). Furthermore, an exposure of males to 42 °C for 4 h still resulted in substantial mortality of 77%. In these pilot experiments, all drones of the control group survived. Consequently, we ended up exposing males in our formal experiment to rather moderate levels of heat (39 °C), which nevertheless resulted in a significant decrease in sperm viability (Figure 4, see Table 1 for statistical details). Heat treatment of either the male ($F_{1,39} = 6.00$, $P = 0.019$) or the ejaculate ($F_{1,39} = 11.08$, $P = 0.0019$) caused a decrease in viability of the male's sperm, but there was no significant interaction between the two ($F_{1,39} = 1.74$, $P = 0.19$), suggesting that these effects were additive. There was also a strong effect of male age ($F_{1,39} = 10.94$, $P = 0.0020$), a difference that was largely driven by the 10-day-old drones, which had significantly lower sperm viability than the 15- and 16-day-old drones, suggesting that sperm of fully mature drones is more robust to heat shock.

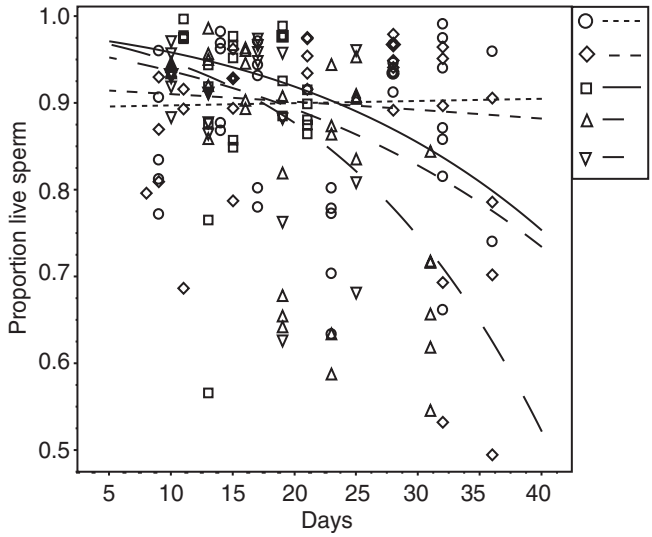


**Figure 1**
Sperm viability of honeybee males as a function of drone age (see Table 1 for statistical details). Each data point represents a measurement of sperm viability in a single male, and colonies are depicted by different symbols. Regression lines are based on the back-transformed logit-predicted sperm viabilities obtained from a standard least squares model.

## DISCUSSION

Eusocial hymenopteran males have exceptionally high levels of sperm viability (Hunter and Birkhead 2002), which can be expected from their life histories, with extremely high levels of male–male competition for mating opportunities and high demands of queens for viable sperm, both maintaining strong selection for high sperm quality. Our experiments are consistent with honeybee drones operating at their physiological limits and thus being very vulnerable to stress factors that are normally controlled by their social environment. We found that even relatively mild stressors, such as a slight increase in temperature and a mild immune challenge, immediately compromise sperm quality. Some of these effects interacted with drone age but access to protein did not appear to make a difference. In the sections below, we will discuss these findings and relate them to previous studies.

### Do colonies differ in the rate of drone senescence?

Sperm viability decreased significantly with age even though our oldest males only lived to the age of 36 days, which is within the estimated average age of *Apis mellifera* drones (20–40 days, Page and Peng 2001), but well below the maximum reported age of 59 days (Howell and Usinger 1933). Figure 1 indicates that the decline in sperm quality starts when drones are only circa 20–25 days old. This is surprisingly early and would likely have severe reproductive fitness consequences if drones would not be able to obtain matings at a relatively young age. A study by Schlüns et al. (2004) showed that larger ejaculate size translates into higher levels of paternity—at least in worker offspring and irrespective of insemination order. As honeybee queens only store live sperm (Ruttner and Koeniger 1971; Collins 2000; Gencer and Kahya 2011—see Introduction), a reduction in sperm viability would, therefore, negatively affect

**Table 1**

**Results of Anova in sperm viability (logit-transformed proportions live sperm) with wound healing, protein supplementation, and heat shock as treatments and age as covariates, combining data from the 4 consecutive experiments**

| Experiment | Number of colonies used | Drone age in days | Treatments | *P* Shapiro | *N* | Factor | df | *F* | *P* |
|---|---|---|---|---|---|---|---|---|---|
| Age | 5 | 9–36 | Senescence | 0.994 | 144 | Age | 1, 135 | 8.68 | 0.0035 |
| | | | | | | Colony* | 4, 135 | 0.58 | 0.68 |
| | | | | | | Age × Colony | 4, 135 | 2.90 | 0.025 |
| Wound healing | n/a | n/a | Membrane puncture | 0.082 | 101 | Treatment | 1, 8.7 | 5.18 | 0.0021 |
| | | | | | | Replicates* | 8, 8 | 4.72 | 0.021 |
| | | | | | | Treatment × replicates* | 8, 83 | 0.22 | 0.99 |
| | | | Treated drones | | 53 | | | | |
| | | | Control | | 48 | | | | |
| Protein starvation | 3 | 12–22 | With/without protein | 0.765 | 53 | Treatment | 1, 4.8 | 0.75 | 0.43 |
| | | | | | | Colony* | 2, 12.6 | 1.39 | 0.29 |
| | | | | | | Treatment × colony* | 2, 46 | 0.27 | 0.77 |
| | | | | | | Age | 1, 46 | 0.027 | 0.87 |
| | | | Without protein | | 28 | | | | |
| | | | With protein | | 25 | | | | |
| Heat shock | 1 | 10–16 | | 0.631 | 44 | Drones heated | 1, 39 | 6.00 | 0.019 |
| | | | | | | Ejaculate heated | 1, 39 | 11.08 | 0.0019 |
| | | | | | | Drones heated × ejaculate heated | 1, 39 | 1.74 | 0.19 |
| | | | | | | Age | 1, 39 | 10.94 | 0.0020 |
| | | | Drones and ejaculate heated | | 12 | | | | |
| | | | Drones heated | | 11 | | | | |
| | | | Ejaculate heated | | 10 | | | | |
| | | | Control | | 11 | | | | |

Factors marked with an asterisk were treated as random factors, which mean that some numerator degrees of freedom are not whole numbers. The first column of *P* values refers to Shapiro–Wilk test for normality of residuals of the transformed data. Drones for the wound healing experiment were caught while flying, so the number of colonies providing these drones was unknown.

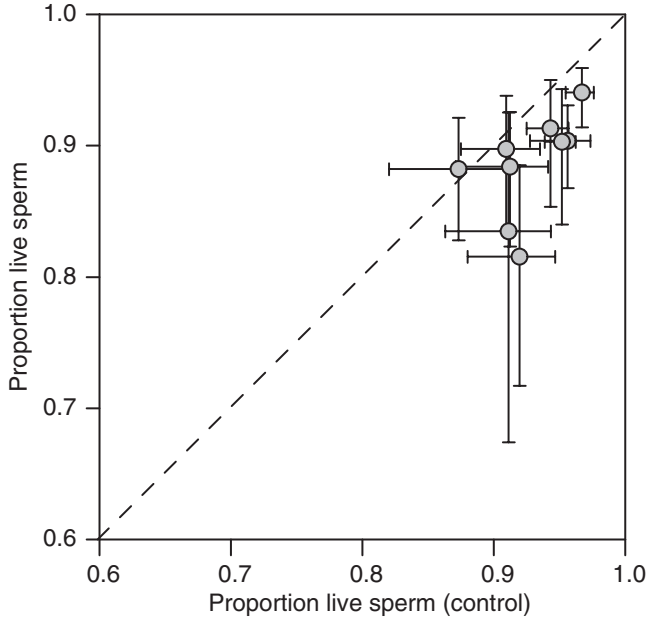


**Figure 2**

Sperm viability of males punctured with a needle was significantly lower than sperm viability of control males. Each point represents the average back-transformed logit viability (±SE) of an experimental replicate. The $y = x$ diagonal is given for comparison.

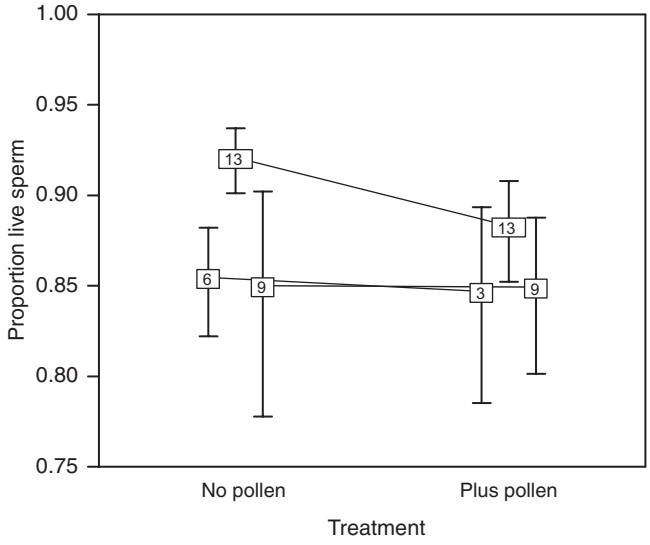


**Figure 3**

Back-transformed mean logit sperm viabilities ± SE from drones that were fed with a protein-free diet or had access to pollen. Drones were from 3 different colonies and between 10 and 22 days old. Numbers within squares are sample sizes.

male reproductive success in honeybees. However, it is important to note that the decline in sperm viability that we observed with increasing age only occurred in 3 of the 5 colonies, suggesting that there may be variation in male life histories across colonies. Further experimental work will thus be needed to establish the consistency of this variation.

Social insects are unique in their caste-specific variation in life span, with queens being able to outlive workers by several orders of magnitude (Page and Peng 2001; Keller and Jemielity 2006). In honeybees, worker life span is closely related to task

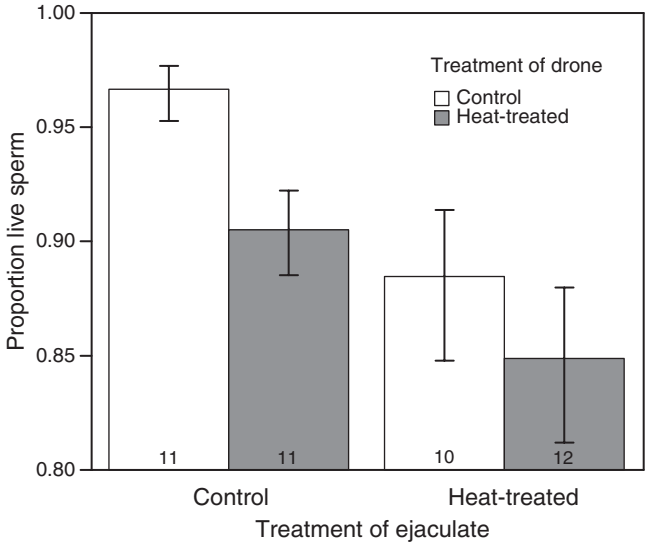


**Figure 4**
Sperm viability after single or double exposure to heat shock treatment. White bars represent drones kept at 25 °C for 4 h and shaded bars represent drones exposed to heat shock (39 °C) for 4 h. Collected ejaculates were kept at either 25 or 39 °C. Bars represent back-transformed mean logit sperm viabilities (±SE). Numbers in bars are sample sizes.

performance, not just due to increased extrinsic mortality risks following the shift from nurse bees to foragers but also due to the changes in gene expression accompanying this behavioral switch (Amdam and Page 2005). Although drones do not participate in colony tasks, their life history is similar to that of workers in that they spend their first days inside the colony and start venturing outside the hive on orientation or mating flights from 8 days of age (Tofilski and Kopel 1996) and continue until they succeed or die. Drones are suicidal maters and might, therefore, not be expected to invest energy resources into late-life somatic maintenance. Drone quality could, therefore, deteriorate after the drones start leaving the hive, as energy expenditure and increased exposure to disease will likely augment their rate of senescence, something that could be tested in the future. Furthermore, Rueppell et al. (2005) found evidence of ageing before drones leave the hives because delayed onset of first flight did not increase drone longevity. This is supported by our findings, as all of the drones in our experiment were locked inside their colonies, so the variation observed in the present study reflects either genetic variation in drone quality or colony variation in worker provisioning of drone larvae. We did not replicate the experiment on identical lineages and therefore cannot assess which of the above factors contributes most to the variation in late-life ejaculate quality, but a pilot study where a cohort of brothers was fostered either in their mother colony or in a nonrelated hive did not give increased variation in sperm viability of drones (Baer-Imhoof B, unpublished data). This suggests that our findings are due to genetic differences between different lineages, and it would thus be interesting to test whether some hives might specifically aim for early and other hives for late dispersing drones.

## The cost of upregulating immune defense

When we challenged the immune system of drones, we registered an immediate decline in their sperm quality. This reduction occurred even though the challenge was relatively mild, as sterile wound healing should have been less damaging than wound healing accompanied by a pathogen infection. It is somewhat surprising that drone fertility is so sensitive to the stress factors that we applied, as natural selection would not necessarily be expected to have favored drones that reduce sperm quality to survive longer. This suggests that drones may actually have reduced their investments to cope with injury or pathogen infection because they are either staying in the very protective hive environment while enjoying the grooming services of their sisters or they are on the wing where they are unlikely to become infected. Indeed, social insect males have repeatedly been reported to have lower immunity compared with their sisters. Baer et al. (2005) found that immunocompetence of leaf-cutter ant males was significantly lower compared with workers, something that has also been shown in bumblebees (Gerloff et al. 2003) and in wood ants where males were shown to have reduced immune responses compared with queens (Vainio et al. 2004).

In honeybees where final sperm storage only happens after ejaculates have been in a queen's reproductive tract, the bursa copulatrix, for up to 40 h (Baer 2005), sperm viability is likely to be a crucial predictor of drone reproductive success. This raises interesting proximate questions about the mechanisms that mediate declines in sperm quality after immune challenge. Our study cannot provide final answers on this matter but 2 scenarios seem possible. First, sperm cells could be directly damaged by the actual immune challenge. This could happen when challenges occur via natural infections but seems less likely in our experimental design where the challenge was intersegmental puncture with a sterile needle. Second, the reduction in sperm viability in response to wounding could result from immune activation affecting the sperm cells themselves. Such "autoimmune" self-harm has been observed in beetles (Sadd and Siva-Jothy 2006). However, as sperm cells are not in direct contact with the hemolymph where the potentially harmful cytotoxins are circulating, this seems less likely in honeybees. Finally, the sperm viability decline that we observed after wounding could result from a trade-off between investment into the immune system and sperm viability. Sperm maintenance is likely to be metabolically costly, as has previously been shown for queens of *Atta* leaf-cutting ants (Baer et al. 2006). This would imply that even in short-lived males there may be a trade-off between resource allocation to sperm maintenance in the accessory testes and other vital functions. We tested the viability of sperm cells in a full ejaculate that consists of both sperm cells and seminal fluid, a substance that contains secretions, which in insects are mainly produced by the accessory glands (Chapman and Davies 2004; den Boer et al. 2010) and are crucial to sperm survival (den Boer et al. 2009). The reduction in sperm viability could, therefore, also have been caused by an altered seminal fluid composition as a result of the wounding procedure.

Honeybee (*Apis*) males have exceptionally large accessory glands compared with other genera of bees (Colonello and Hartfelder 2005) and it is thus possible that the wound healing challenge we inflicted on the drones resulted in a metabolic trade-off preventing them from allocating resources to the production of seminal fluid, thereby causing a reduction in fertility because of suboptimal sperm maintenance before ejaculation. However, it is important to bear in mind that honeybee males in well-functioning hives are unlikely to suffer from significant parasite/pathogen exposure, so our results may also reflect that drones have been under consistent natural selection to downregulate investment into immune defenses

in order to allocate more to sperm production and maintenance, because their highly protected lifestyle implies that such physiological adjustments normally have no fitness costs.

### The effect of heat shock on sperm viability

Both drones as well as ejaculated sperm appeared to be highly sensitive to temperature increases. The optimal temperature in *A. mellifera* hives is 35 °C, and the temperature around the brood cells is maintained at 32–36 °C (Seeley 1985; Jones et al. 2004), yet extended exposure to a temperature of 40 °C caused 100% mortality in the drones, whereas all the escorting workers survived. This was surprising as thorax temperature of drones just before leaving the hive for a mating/orientation flight on average is above 39 °C and has been reported to reach 48 °C in very warm weather conditions (which frequently occur during the summer season in Western Australia) (Coelho 1991). On the other hand, drones do not seem able to cool themselves down when ambient temperatures increase (Coelho 1991), which might explain the lowered lethal temperature compared with workers that we observed.

In addition to the lethal effect of heat on the drones, their fertility was also compromised after heat exposure. We found a negative effect of elevated temperature exposure on sperm viability, and this was true both for exposure of males and semen. As in the previous experiments, we tested the viability of the ejaculated sperm, as we were interested in determining the reproductive potential of males, so we did not partial out whether the reduction in sperm viability was due to the death of sperm cells, a reduction in seminal fluid quality or a combination of the two.

### Why protein food does not influence sperm viability?

Our results show that food protein deprivation does not affect the sperm viability of the drones. Sperm production in social insect males ceases on eclosion (Hölldobler and Bartz 1985), after which the sperm cells mature and are subsequently stored in the accessory testes until mating. Hence, it might be possible that nutritional manipulations after eclosion have little effect on the sperm cells themselves as they are already produced and are protected inside the accessory testes. However, deterioration in seminal fluid quality as opposed to direct sperm death would also result in reduced sperm viability. It is well known from studies in both social and nonsocial insects that seminal fluid not only protects the male's own sperm cells but also affects other males' sperm, female behavior and physiology, and even offspring fitness (Simmons 2001; Poiani 2006; den Boer et al. 2008, 2010; Priest et al. 2008). We also know that social insect seminal fluid consists of a large array of different proteins (Chapman 2001; Chapman and Davies 2004; Baer et al. 2009; Holman 2009a), which might need to be continuously produced to ensure that sperm has the best possible survival conditions at the rather unpredictable time of mating. Manipulating resource access in mature males might, therefore, not affect the innate quality of their sperm, but rather decrease a male's ability to maintain optimal functional seminal fluid and thus competitiveness of the final ejaculate. Our observation that sperm quality was not significantly affected by food protein manipulation then suggests protein ingested after eclosion cannot be metabolized to favor sperm quality. This might be because enough essential seminal fluid proteins are already synthesized during the pupal stage, and remain functional in mature drones, or because protein supply is not a limiting factor in seminal fluid. Szolderits and Crailsheim (1993) found that drones consume pollen after eclosure and that the highest intake happens 3 days after hatching. However, at all times, the pollen intake as well as the proteolytic activity of drones is less than that of workers, and by the time they start leaving the hive, drone protein intake is negligible, supporting our above notion that mature drones might not be protein limited. However, we did observe larger variation in the viability counts in the nonprotein group compared with the control (see Figure 3). The change related to this was either positive or negative, and although it seems obvious to argue that the lower sperm viabilities was due to protein deficiency, the results here are not consistent across colonies and thus need further investigation.

We were interested in exploring whether sperm quality in hatched drones could be affected by external factors, and with protein provisioning it seems likely that the critical point is prepupation, during the larval stages where the testes are being formed and spermatogenesis happens. Manipulating nutrition during this period is difficult though as drone eviction is tightly linked to food availability (Free and Williams 1975; Rowland and McLellan 1987; Boes 2010), so workers tend to kill drones and drone brood when there is food shortage. Nevertheless, it would be interesting to test the effect of food protein restriction in drone larvae, especially as a recent study on a nonsocial insect revealed a trade-off between immune investment, sperm quality, and food restriction (Simmons 2012). Additionally, there might be significant differences in the way food-deprived drones vary in senescence rates, heat sensitivity, or immune responses.

## FUNDING

This work was supported by the Centre for Social Evolution, a grant from the Danish National Research Foundation to J.J.B. (DNRF57), and a Future Fellowship and Linkage Grant from the Australian Research Council to B.B.

**Handling editor:** Anna Dornhaus

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