LETTERS

Sperm storage induces an immunity cost in ants

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Ant queens are among the most long-lived insects known[1,2]. They mate early in adult life and maintain millions of viable sperm in their sperm storage organ until they die many years later[3,4]. Because they never re-mate, the reproductive success of queens is ultimately sperm-limited, but it is not known what selective forces determine the upper limit to sperm storage. Here we show that sperm storage carries a significant cost of reduced immunity during colony founding. Newly mated queens of the leaf-cutting ant *Atta colombica* upregulate their immune response shortly after completing their nest burrow, probably as an adaptive response to a greater exposure to pathogens in the absence of grooming workers. However, the immune response nine days after colony founding is negatively correlated with the amount of sperm in the sperm storage organ, indicating that short-term survival is traded off against long-term reproductive success. The immune response was lower when more males contributed to the stored sperm, indicating that there might be an additional cost of mating or storing genetically different ejaculates.

Delayed internal fertilization has evolved multiple times[5] and allows sperm competition and cryptic female choice between ejaculates[6–8]. Sperm storage imposes selection on males to increase sperm number and viability, directly or indirectly by means of the seminal fluids, and selection on females to maintain ejaculates[9]. However, the selective forces that have shaped sperm storage traits in ants are not primarily related to sexual selection, as in most other animals, but to iteroparous perennial reproduction and extreme reproductive division of labour[10]. Ant queens obtain their lifetime supply of sperm on a single mating flight. When they succeed in establishing a colony they may live for several decades, whereas males die on the day of mating but 'survive' as stored sperm. Queens never re-mate later in life, which implies that they are not promiscuous in the usual sense even when mating with multiple males. As the lifetime reproductive success of ant queens is ultimately constrained by the availability of stored sperm, there has been strong selection for keeping sperm viable and using only a few sperm to fertilize each egg[11]. However, it has remained unclear what factors have constrained the evolution of larger sperm stores. Although mating with more males might incur prohibitive costs of longer exposure to predators and diseases, it seems hard to understand why ant males have not evolved larger ejaculates that would allow queens to store more sperm without having to increase the number of matings[12].

We propose that the long-term storage and maintenance of large quantities of sperm incurs a significant metabolic cost and that this cost is a major factor explaining sperm limitation in ant queens. Suggestive indications of such costs have been reported for the honeybee (*Apis mellifera*)[3] and the ant *Crematogaster opuntiae*[3], but this idea has never been tested explicitly. A complication is that the cost of sperm storage may not significantly affect the fitness of queens in established colonies. During colony founding, however, solitary queens are severely resource-limited until their first workers emerge, so that metabolic costs of maintaining large quantities of stored sperm may interfere with other vital functions. This implies that evolutionary trade-offs may become apparent that are not expressed later in life when workers provide ample resources and care.

In contrast to established queens, founding queens are exposed to a variety of soil-borne pathogens that would be unlikely to reach them in the protected nest environment of mature colonies[13]. Mortality rates are typically more than 95% during colony founding[14,15], with pathogens accounting for 74% of failures during colony foundation in the leaf-cutting ant *Atta cephalotes*[15]. The importance of a well-functioning immune system early in colony life has been documented for bumblebees (*Bombus terrestris*)[16,17], in which the encapsulation response of the first worker brood is a significant predictor for eventual colony size and fitness. There is also evidence that maintaining immune responses in insects is costly[18]. We therefore expect that founding ant queens should increase their investment into immune defence shortly after mating to avoid premature death from fungal or bacterial infections, and that this adaptive response might be compromised by the metabolic costs of sperm storage in this crucial solitary period of a queen's life.

We tested this hypothesis on founding queens of the Panamanian leaf-cutting ant *A. colombica*, which are among the most long-lived and fertile insects known. Queens store up to several hundred million sperm, usually from two or three males[19]. After dispersal and mating they dig a burrow and seal it. Soon afterwards they lay their first eggs and start building a fungus garden from a fragment of mycelium that they take along from their maternal colony[14] (Fig. 1a). In the months to follow, they nurse their brood and incipient fungus garden with trophic eggs and faecal droplets, while recycling their flight-muscle proteins and abdominal fat reserves until the first workers eclose and start foraging[20]. Once founding colonies have reached this stage, they may grow to contain several million workers and will produce yearly clutches of thousands of winged new queens and males until they die one or several decades later[14].

As expected, immune defence measured as encapsulation response (see Methods and Supplementary Information) was significantly higher ($t = -3.299$, d.f. $= 44$, $P = 0.002$) in queens that were excavated nine days after the mating flight (48.61 ± 4.48) than in queens excavated after one day (30.69 ± 3.08; Fig. 1b). After nine days, the mortality of queens in their burrows had increased to 21%. This corresponds to an average of 2.6% per day, very close to the 3% mortality observed in the queen sample taken after one day (Fig. 1b) and consistent with 95% mortality being reached after about 3.5 months, in agreement with previous estimates[14,15]. Additional data on founding queens from the same site in the following year showed that the average encapsulation response and number of haemocytes (another measure of the potential of immune defence) one day after mating were not significantly different from values obtained for winged virgin queens before mating (encapsulation response: $F_{1,67} = 1.771$, $P = 0.188$; haemocyte number: $F_{1,67} = 1.536$, $P = 0.220$), whereas both responses were significantly elevated nine days after mating (multivariate analysis of covariance $F_{2,62} = 6.618$,

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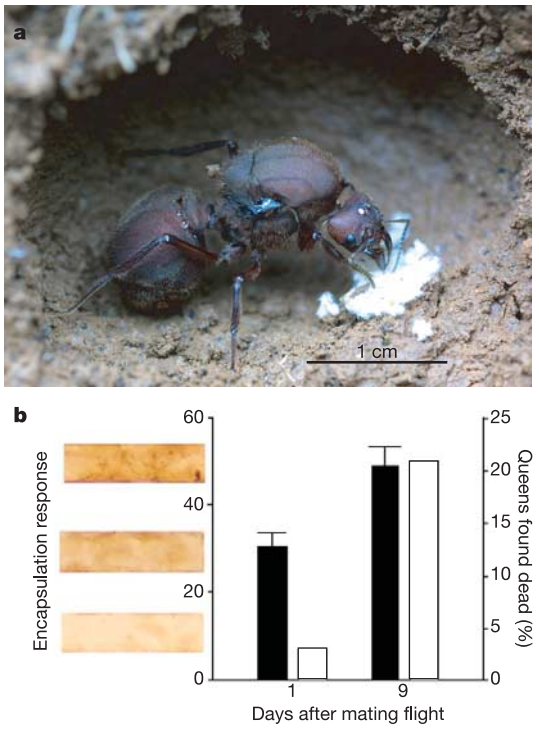

Figure 1 | Increase in immune response during colony founding. a, A large (about 2.2 cm) founding queen of *A. colombica* in her initial brood chamber with the incipient fungus garden. **b**, The encapsulation response (filled bars; means ± s.e.m. for 23 queens each) and mortality rates (open bars) of colony-founding queens one and nine days after their mating flight. Horizontal bars at the left are representative examples of melanization of the nylon inserts that were used to quantify encapsulation (see Methods and Supplementary Information). Encapsulation differed significantly between queens collected one and nine days after their mating flight (see the text), and this difference remained significant when including queen weight (not significant) as a covariate in the analysis ($F_{1,43} = 11.712$, $P = 0.001$).

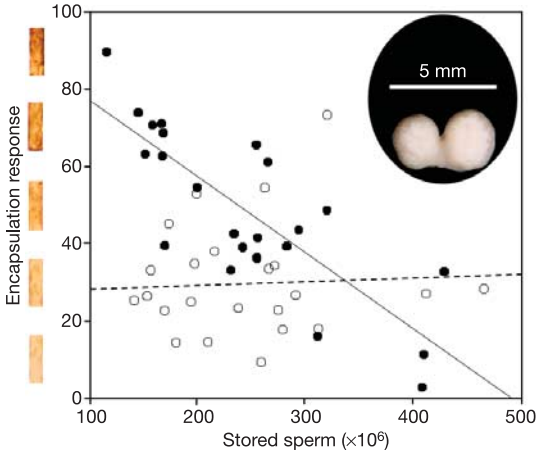

Figure 2 | Effect of stored sperm on immune response. The encapsulation response (indicated with bars along the *y* axis as in Fig. 1) as a function of the amount of sperm stored in a queen's spermatheca. Open circles refer to queens collected one day after mating, at which time there is no relationship between the two variables (dashed linear regression line: $F_{1,21} = 0.063$, $P = 0.804$, $r^2 = 0.003$). Filled circles refer to the encapsulation responses of queens collected nine days after mating. At this time mean encapsulation responses were elevated by 63% (from 30.7 to 48.6 on average; Fig. 1) and showed a significant negative correlation with the number of sperm stored in the spermatheca (solid regression line: $F_{1,21} = 39.282$, $P < 0.0001$, $r^2 = 0.652$). The inset illustrates the large size of the sperm storage organ (white) relative to the approximate outline of a queen gaster (black).

$P = 0.002$; see Supplementary Information). This indicates that the encapsulation response is indeed upregulated after colony founding and not downregulated during the mating flight. These results are consistent with a recent gene expression study in *Solenopsis* fire ants, in which two precursors of antibacterial peptides were shown to be upregulated in queens one day after their mating flight[21].

It was not possible to count stored sperm in dead queens, but the 46 queens sampled alive had stored up to 465×10^6 sperm. The overall mean was $(244 \pm 12) \times 10^6$ sperm (mean ± s.e.m.) per queen, with no significant difference for queens sampled one and nine days after mating ($t = 0.025$, d.f. = 44, $P = 0.981$). For the queens collected after nine days, 65% of the variation in encapsulation response could be explained by the amount of stored sperm, whereas no such relationship was present in the sample of founding queens taken one day after mating (Fig. 2). Queens with higher than average sperm stores ($(313 \pm 14) \times 10^6$) were apparently unable to increase their immune response (difference between day 1 and day 9 after founding for 22 queens with more than 244×10^6 sperm: $t = -0.637$, $P = 0.532$), whereas the remaining 24 queens with sperm stores lower than average ($(181 \pm 7) \times 10^6$) increased their encapsulation response by 100% (from 29.86 to 59.49 on average) between day 1 and day 9 ($t = -4.922$, $P < 0.0001$).

Microsatellite data for the number of father haplotypes represented in the stored sperm showed that queens had mated with two to five males (see Supplementary Information for details), confirming earlier data obtained from the same population[19]. The expected positive correlation between the inferred number of fathers and the number of stored sperm was weak ($r = 0.211$; one-tailed $P = 0.085$; $n = 44$), which is consistent with a previous study[19]. However, the correlation between immune response and the inferred number of fathers was negative and significant ($r = -0.314$; $P = 0.038$; $n = 44$).

In a multiple regression analysis with backwards elimination of non-significant terms, all but one of the interaction terms and queen weight ($F_{1,38} = 0.083$, $P = 0.775$) were excluded. This produced an overall model for encapsulation response with a significant positive effect of day of sampling (day 1 versus day 9; $F_{1,39} = 31.334$, $P < 0.001$, $r^2 = 0.45$), significant negative effects of both the number of stored sperm ($F_{1,39} = 10.410$, $P = 0.003$, $r^2 = 0.21$) and the number of fathers ($F_{1,39} = 5.854$, $P = 0.020$, $r^2 = 0.13$), and a significant sampling-day × sperm-number interaction term ($F_{1,39} = 18.886$, $P < 0.001$, $r^2 = 0.33$), showing that all singular relationships (Figs 1 and 2) were robust in partial analysis and that being inseminated by more males induced a significant additional immunity cost (Fig. 3).

Our results indicate that a founding queen's risk of dying from disease might be proportional to the amount of sperm stored during her mating flight because maintaining stored sperm carries considerable metabolic costs, which trade off against the needs of immune defences during solitary colony founding. A corollary would be that the average number of sperm stored by a queen that has survived the colony-founding stage is lower than the amount observed in an average newly mated queen, because of differential mortality related to the cost of sperm storage. This seems to be true, because a sample from the same site of young *A. colombica* queens that had already produced their first hundreds of workers[22] had significantly smaller numbers of stored sperm ($(124 \pm 11) \times 10^6$; range $(57–255) \times 10^6$) than the average of 244×10^6 observed in the present study. Storing more sperm might thus increase the risk of failure early in life but be beneficial later, when the prolonged ability to fertilize eggs might add several years of colony life and reproduction. However, our results are not consistent with the assumption that the sperm affects the immune system directly, because this should have implied an increase in immune response with the number of stored sperm.

Atta leaf-cutting ants are among the few clades of social insects that

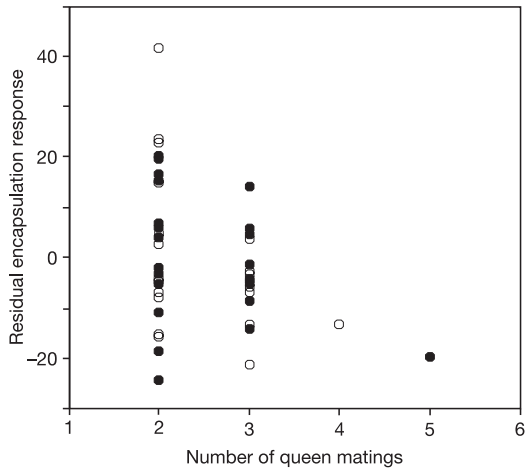

Figure 3 | Effect of number of mates on immune response. Residual encapsulation responses based on the regressions from day 1 (open circles) and day 9 (filled circles) in Fig. 2, plotted as a function of the number of fathers that contributed to the stored sperm. Differences between day 1 and day 9 for queens that mated with two males ($t = 0.662$, $P > 0.5$) or with three to five males ($t = 1.047$, $P > 0.2$) were not significant (tested separately because of unequal variances). The overall partial regression ($F_{1,38} = 5.396$, $P = 0.026$, $r^2 = 0.12$) across the pooled data set was significant (see the text). When distinguishing between only two fathers and more than two fathers, the average residual encapsulation response for queens that mated with two males (+3.403; $n = 25$) and queens that mated with three to five males (−4.680; $n = 19$) remained significantly different (after adjustment for unequal variances: $t = 2.144$, $P = 0.040$).

have obligatory multiple mating of queens[10,23]. Because colonies need several years of ergonomic growth to have enough workers to start reproducing, it has been proposed that sexually transmitted diseases should be absent or non-virulent because they would otherwise eliminate their own transmission[13]. Similarly, male accessory gland compounds that would reduce the survival of competing ejaculates or have directly negative effects on female fitness later in life are expected to be of limited significance in social insects[10,23,24]. Our finding that storing a specific amount of sperm of n males is more costly than storing the same amount of sperm from $n - 1$ males (Fig. 3) is therefore interesting in two ways. It may indicate that male–male conflicts over sperm storage have not been completely eliminated, in spite of queen mates being lifetime committed and having a joint interest in building a strong colony of sterile workers before reproduction can start some years later. These conflicts may therefore be similar to those social insects for which single mating of the queen is the rule, as recent work on bumblebees has shown that mixed artificially inseminated sperm from more than one male significantly reduced queen fitness relative to the same number of sperm from a single male[25]. Alternatively, the negative effect of the number of inseminations on immune response in founding queens may reflect a delayed cost of mating, because mating with more males is likely to imply more hours of flight activity so that fewer resources may be left to mount immune defences during subsequent colony founding. This possibility is intriguing because this type of mating cost has never been empirically quantified in any social insect.

METHODS

Sampling. On 18 May 2004 we marked 120 entrance holes of *A. colombica* burrows on a single lawn in Gamboa, Panama, about 30 h after a nocturnal mating flight. A random subset of 38 of these queens was excavated immediately afterwards and taken to the laboratory, where each queen was maintained at ambient temperature in a Petri dish with moist cotton wool. For a random sample of 23 of these queens, the encapsulation response over a 24-h period was measured, and sperm counts and microsatellite paternity data of the stored sperm were obtained (see below). Nine days after the mating flight a second sample of 53 live queens was excavated, of which we used a random subset of 23 for the same measurements. Mortality after one and nine days was assessed at excavation (see also Supplementary Information).

Measuring the immune response. We estimated the efficiency of immune defence by experimentally eliciting an encapsulation response (see Supplementary Information for a more extensive overview of this technique). This was done by inserting a small piece of nylon (0.13 mm × 0.50 mm) through the queen's intersegmental membrane between the 6th and 7th sternites. For the implantation we adapted machinery normally used for artificially inseminating bumblebees[25]. After 24 h the queens were freeze-killed and the encapsulated nylon filaments were dissected out and mounted on microscope slides, then photographed with a Canon EOS D30 digital camera connected to a Leica stereomicroscope at ×62.5 magnification. Shades of grey measure the deposition of dead melanized haemocytes. Melanin is synthesized from the amino acid tyrosine, a reaction that is in part catalysed by the enzyme phenoloxidase, producing reactive intermediates of oxygen and nitrogen that have been implicated in parasite death (see Supplementary Information for further details). The extent of grey coloration on the implants was analysed with the Image 1.62 software developed by the US National Institutes of Health (http://rsb.info.nih.gov/nih-image/). To verify the generality of our 2004 estimations, encapsulation responses after one and nine days were also obtained from founding queens in 2005, and compared with encapsulation responses of virgin queens just before mating, and also with haemocyte counts for the same individuals to examine whether alternative measures of immune response gave similar results (see Supplementary Information).

Sperm counts. Spermathecae were dissected out of frozen queens and transferred to 2 ml of culture medium for boar semen (Merk III diluant, purchased from Minitüb). They were ruptured with watchmaker forceps after which solvent and sperm were mixed by vortex-mixing the solution for 20–30 s. Subsamples (50 μl) of this stock solution were transferred to 1.95 ml of distilled water and vortex-mixed again for 20–30 s. Single drops of 1 μl of this solution were placed on a microscope slide and air-dried. Sperm counts were performed after staining with 4′,6-diamidino-2-phenylindole (Merck) by counting all fluorescence-marked sperm heads within a single 1-μl drop of diluted sperm, using an Olympus fluorescence microscope at ×400 magnification.

Number of matings and statistics. The number of fathers that contributed to the stored sperm was estimated after amplifying two highly variable microsatellite loci Etta5-6TF and Etta7-8TF (ref. 26) from both a sample of the stock solution of sperm and a leg of each corresponding queen that the sperm came from. Reliable estimates of the number of inseminations were obtained for 44 of the 46 queens (see Supplementary Information for details), so mate number could be used as an additional predictor for encapsulation response in multiple regression analysis with stored sperm, queen weight and date of excavation as other predictor variables. The listed r^2 values for the multiple regression analysis refer to partial proportions of explained variance. All means are given with s.e.m.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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