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Impact of immune activation on stored sperm viability in ant queens

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Ant queens mate on a single occasion early in life and store millions of sperm cells in their spermatheca. By carefully using stored sperm to fertilize eggs, they can produce large colonies of thousands of individuals. Queens can live for decades and their lifetime reproductive success is dependent on their ability to keep stored sperm alive. Maintaining high sperm viability requires metabolic energy which could trade-off with other costly processes such as immunity. We tested the impact of immune activation on the survival of stored sperm by prompting *Lasius niger* ant queens to mount a melanization response and subsequently measuring sperm viability in their spermatheca. Since queens face different challenges that influence energy allocation depending on the life stage of their colony, we measured sperm viability after immune activation in both newly mated queens (incipient) and in queens 1 year after mating (established). We found that immune activation reduced sperm viability in established queens but not in incipient queens, showing that the cost of immunity on sperm preservation depends on the life stage. Unexpectedly, established queens had significantly higher sperm viability in their spermatheca compared to incipient queens suggesting that ant queens are able to remove dead sperm from their spermatheca.

1. Introduction

Female sperm storage is widespread in internally fertilizing animals. It has been documented in numerous taxa, including mammals, fishes, amphibians, reptiles, birds and insects (reviewed in [1–3]). Sperm storage provides many advantages for females by lowering the costs of additional matings and increasing fertilization success. Birds and reptiles maintain sperm in tubules whereas insects and amphibians use a specialized organ, the spermatheca. Storage time is also species dependent and ranges from several days in mammals to years in some reptiles. Ants take sperm storage to exceptional levels, as mated females (i.e. queens) are capable of storing sperm for up to several decades making them the ultimate record holders [4,5].

In ants, mating occurs on a single occasion early in queen life. Males die soon after copulation, but survive posthumously as spermatozoa stored in the queen spermatheca. Once mated, queens found new colonies using the sperm cells they acquired to fertilize eggs that develop into worker ants and, once the colony is mature, also future queens as in ants and other Hymenoptera, males arise from unfertilized eggs. Ant queens can be extremely long-lived—decades in some species [6]. Workers, on the other hand, have a much shorter lifespan [7,8] and a queen will need to renew her workforce on a continuing basis by laying newly fertilized eggs. This ongoing process gradually depletes the sperm stock and because ant queens never mate again later in life they must use their stored sperm parsimoniously [9,10]. Besides sperm economy, queens also need to keep spermatozoa alive in order to ensure successful fertilization. The mating strategy of ants entails that the reproductive success of both sexes, and of the colony as a whole, depends entirely on the ability of the queen to maintain sperm cells alive throughout her decades-long life.

The mechanisms underlying the maintenance of sperm viability during storage are still unclear; however, it is becoming increasingly evident that this process is associated with substantial energy costs [11–18].

Depending on their life stage, ant queens experience different levels of pathogenic pressure. During, and right after mating, when they scurry on the ground to find a nest burrow, queens have a high risk of becoming infected and/or wounded [19–22]. Once inside their new nest, it takes a few months for the first workers to hatch and queens survive on their own body reserves. Young queens therefore experience strenuous conditions and queens of several species have been shown to have an elevated immune capacity shortly after mating [19,21,23,24]. A couple of months later, workers hatch and start to feed, groom and protect the queen. Thus, during the pre-worker hatching phase, queens with higher immune capacity have a selective advantage, whereas after worker hatching, queens can rely on the combined effort of workers for protection against pathogens as part of social immunity [25] and are expected to invest less in their individual immune system.

Several studies have demonstrated the existence of a trade-off between sperm viability and immunity in insects. In fruit flies, immune activation negatively affects sperm viability in both males and females [26], whereas in crickets, this effect was only found in males [27–29]. In contrast to these species that mate on several occasions throughout their life, Hymenoptera males and queens mate only during a single event. The selective pressure for maintaining high sperm viability in the spermatheca should thus be strong as queens can never replenish their sperm stock. Queens of the leaf-cutting ant *Atta colombica* have been shown to mount a lower immune response when they store more sperm suggesting a metabolic cost of sperm storage [19]. Male leaf-cutting ants as well as honeybee drones experiencing an immune challenge have lower sperm viability compared with unchallenged controls [30,31]. Surprisingly enough, despite the central role of ant queens in determining colony reproductive success, it remains completely unknown whether sperm viability in the spermatheca suffers from the activation of the queen immune system.

We measured the viability of sperm stored in queens' spermatheca after an immune challenge. Our study species was the black garden ant *Lasius niger*. Queens of this species store approximately 2.6 million sperm cells (this study; [32]), they are extremely long lived (29 years; [33]) and can produce colonies of up to 55 000 workers [34]. We artificially induced queens to mount an immune response and measured sperm viability in the spermatheca using flow cytometry. To determine if queen life stage influences sperm preservation, we tested immune activation in both newly mated queens (incipient) and in queens 1 year after mating (established).

2. Methods

(a) Queen collection and rearing

Newly mated queens were collected from the ground during mating flights in July 2016 and 2017 around an area of *ca* 0.2 km^2 on the Solbosch campus of the Université Libre de Bruxelles, Brussels, Belgium. After collection, mated queens were placed in individual nest tubes. These tubes were half filled with water held back by a cotton plug thus providing a moderately humid nest space for queens. Queens were kept at room temperature in the dark and reared for either two weeks, hereafter referred to as *incipient queens*, or 1 year, hereafter referred to as *established queens* before experiments were carried out. To maintain queens in conditions mimicking as closely as possible their natural feeding habits, queens were not fed until their first workers hatched, about two months after mating. Then, queens were fed ad libitum sugar water and meal worms. To avoid biases introduced by immune fluctuations in the days following mating [19,21,24,35], we tested the effect of immune activation on stored sperm viability two weeks after mating for incipient queens.

First, because queens were collected in two different years, we tested whether collection year impacted stored sperm viability. We compared sperm viability between established queens collected in July 2016 and 2017. These groups are referred to as *established_2016* ($n = 16$) and *established_2017* ($n = 9$), hereafter.

Second, we tested if the effect of queen age on stored sperm viability was dependent on collection year, by comparing sperm viability between incipient queens collected in July 2017, hereafter referred to as *incipient_2017* ($n = 18$), and the two previous groups of established queens, *established_2016* ($n = 16$) and *established_2017* ($n = 9$).

Finally, we tested the impact of immune activation on stored sperm viability in both incipient and established queens. We used two additional groups of queens: incipient queens collected in 2017 ($n = 32$) and established queens collected in 2016 ($n = 32$). Incipient queens were not fed because workers had not hatched yet. To limit variation caused by nutritional status between incipient and established queens, we stopped feeding established queens two weeks before the experiment. However, we were unable to fully control this aspect as the workers in the established queen colonies might have fed their queens by trophallaxis using food still remaining in their crop.

(b) Immune challenge

To test the effect of immune activation on stored sperm viability, the two additional groups of incipient queens collected in 2017 ($n = 32$) and established queens collected in 2016 ($n = 32$) were each divided into two treatment groups: *immune challenge* or *control*. We, thus, ended up with four distinct age and treatment groups: *incipient-control*, *incipient-immune challenge*, *established-control* and *established-immune challenge*. Each group consisted of $n = 16$ queens. In the immune-challenged groups, queens were removed from their nests and pricked with a sterile needle tip (Neolus Hyperdermic Needles, 0.5 × 16 mm, Terumo) through the intersegmental membrane of the abdomen between 2nd and 3rd tergite. The prick induced queens to mount a melanization response, which is a common immune reaction in arthropods where melanin is deposited around wounds or pathogens [36]. To control for stress during the treatment, control queens were handled in the same way as immune-challenged queens except they were not subjected to the needle prick. Control and immune challenge treatments were carried out two weeks after mating for incipient queens and 1 year after mating for established queens. After each treatment, queens were placed back into their respective nest tubes. All queens were weighed before the treatments, *weight at treatment* hereafter, and before starting the sperm viability measures, *weight at measure* hereafter. The abdomens of control and immune-challenged queens were inspected under a Leica EZ4 stereomicroscope before sperm viability measure, that is right before dissecting the spermatheca (see Sperm viability measure section) to confirm that immune-challenged queens showed melanized scarring around the area were the prick was inflected, whereas control queens did not (figure 1).

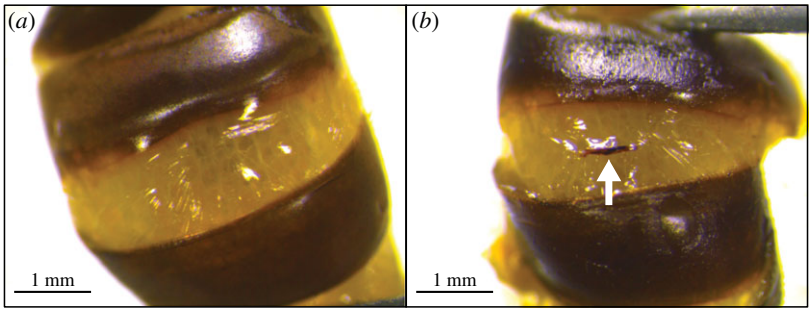

Figure 1. Melanized scarring caused by the immune challenge. (*a*) Control queens did not mount a melanization reaction. (*b*) The needle prick through the intersegmental membrane of the 2nd and 3rd tergite of the abdomen (immune challenge) caused queens to mount a melanization reaction, indicated by the white arrow. (Online version in colour.)

(c) Sperm viability measure

The time between the immune challenge treatment and sperm viability measurement is likely to be an important factor as the immune response elicited by the needle prick is expected to change over time. To make sure that the immune response had taken place, we waited 24 h after the immune challenge or control treatments to start sperm viability measurements. However, it took 32 h to carry out the sperm viability measurements for each individual age and treatment group. Sperm viability was, therefore, measured between 24 and 56 h after immune challenge or control treatments. To account for the variability that this measurement timespan might induce, we quantified sperm viability at the same points in time for each age and treatment groups during the 32 h timespan. These points in time were included as a random variable in the statistical analysis (see *time point* variable in Statistical analysis section).

For the queen groups used to test the effect of collection year and queen age on sperm viability (*incipient_2017*, *established_2016* and *established_2017*), there was no actual treatment inflicted on queens and there was therefore no need to include a variable to control for time after treatment. Thus, we simply started sperm viability measurements two weeks after mating for incipient queens and 1 year after mating for established queens. The timespan needed to carry out the sperm viability measures was between 8 and 33 h.

Sperm viability was measured by flow cytometry by counting the number of live and dead sperm cells present in each spermatheca sample (range: (15 549–279 268) sperm cells analyzed per queen). To distinguish live from dead cells, sperm was stained using the Live/Dead Sperm Viability Kit (ThermoFisher Scientific). The kit contains two separate dyes, SYBR 14 (1 mM), that is membrane permeable and therefore stains all cells, and propidium iodide (PI) (2.4 mM) that only penetrates membrane-compromised cells and therefore stains only dead cells. Queens were killed by decapitation and their spermatheca was dissected in semen diluent (188.3 mM sodium chloride, 5.6 mM glucose, 574.1 nM arginine, 684.0 nM lysine and 50 mM tris(hydroxymethyl)aminomethane, pH 8.7) [37] under a Leica EZ4 stereomicroscope. The spermatheca was pierced in 550 µl semen diluent to allow the sperm to flow out and the spermathecal membrane was removed. This volume was then transferred to an empty 1.5 ml tube and the sperm was mixed with the semen diluent by gently inverting the tube until homogenization. Then 150 µl of the sperm solution was further diluted into 850 µl semen diluent. This step was repeated with another 150 µl of the sperm solution in order to have two technical replicates. Five microlitres of SYBR 14 (50-fold dilution of stock solution, final concentration: 100 nM) was added to each technical replicate and the tubes were incubated at room temperature in the dark for 10 min. At the end of the 10 min SYBR 14 incubation, 5 µl of PI (final concentration: 12 µM) was added and the mix was incubated at room temperature in the dark for another 10 min after which sperm fluorescence was immediately measured on a CyFlow Space flow cytometer (Sysmex Partec). Incubation time for SYBR and PI was therefore identical for all samples, that is 10 min for each dye. For each sample, cells were counted at a flow rate of 1 µl sec^{-1} and the stream was allowed to stabilize for 25 s before counting initiation. SYBR 14 and PI were excited by a 488 nm blue solid state 50 mW laser. SYBR 14 fluorescent emission was acquired between 516 and 556 nm (536/40 bandpass filter) and PI above 630 nm (630 long-pass filter). PI fluorescent emission was compensated for 2% spectral overlap. Paynter *et al*. [37] used height of the voltage pulse, instead of area, in their methodological study on viability quantification of bee sperm and because ant sperm morphology is very similar to that of bees, we did the same. Sperm cell populations were identified by characteristic forward and side scatter, and a gating strategy for live and dead sperm cell populations was determined by running reference samples of known proportions of live and dead sperm, as described in [37]. We obtained two distinct populations: one for dead sperm with high PI and low SYBR 14, and another for live sperm with low PI and high SYBR 14. Gating and sperm cell population counts were carried out using FlowMax Software version 2.9 (Sysmex Partec). We used the mean of the two technical replicates as the final count of live and dead sperm cells per queen sample. The mean difference in sperm viability between technical replicates was of 4.97 ± 4.1% (mean ± standard deviation). We also estimated the total number of sperm cells stored in each spermatheca by multiplying the sperm cell concentration in each sample (number of sperm cells/ml given by the flow cytometer) by 550/150 (spermatheca opened in 550 µl semen diluent/150 µl aliquot taken and diluted in 850 µl semen diluent).

(d) Statistical analysis

All statistical analyses were carried out in R, v. 3.4.3 [38]. First, we tested the effect of queen collection year and queen age on weighted sperm viability (by using the *cbind* function as follows: cbind(number alive, number dead)) with a generalized linear mixed model (*glmer* function, lme4 package in R) with binomial errors and a logit link function [39]. We defined one explanatory variable with three levels corresponding to the queen groups used to test the effect of queen collection year and age: *incipient_2017*, *established_2016* and *established_2017*. The *cbind* function creates a two-column matrix with the number of live sperm cells in the first column and dead sperm cells in the second column. The rows correspond to each queen sample. The *glmer* function uses this matrix to build the model by coding the number of live sperm cells as successes and the number of dead sperm cells as failures. The function also extracts

Table 1. The effect of queen collection year and age (*incipient_2017*, *established_2016* and *established_2017*) on sperm viability determined by a generalized linear mixed model with binomial errors and a logit link function. (Queen identity (ID) was used as an observation-level random effect to account for overdispersion.)

random effects	variance	s.d.		
queen ID	1.218	1.104		
fixed effects	**estimate**	**s.e.**	***Z*-value**	***p*-value**
intercept	4.8661	0.2762	17.619	<0.001
established_2017	0.3023	0.4606	0.656	0.512
incipient_2017	−3.4068	0.3794	−8.979	<0.001

the weight of each sample, which is the sum of live and dead sperm cells, from the matrix.

Second, we evaluated the impact of immune activation on stored sperm viability in incipient and established queens. We defined two explanatory variables: treatment (*control* or *immune challenge*) and age (*incipient* or *established*), and tested their effect and that of their interaction on stored sperm viability also using a generalized linear mixed model with binomial errors and a logit link function. Here again, weighted sperm viability was the response variable. To control for unwanted variability, we included the *time point* during the 32 h timespan needed to carry out the flow cytometry measures as a random effect in the model. *Time point* was defined as an ordinal variable ordered from the first to the last measure during the 32 h timespan (see the electronic supplementary material, Supporting data: Time of measure: a < b< ⋯ <o < p).

To correct for overdispersion [40], we included the identity of each queen sample as an observation-level random effect in both models. Significance of the effect of queen collection year, age and treatment was assessed with Wald *Z*-tests [41].

To determine whether queen weight was affected by the treatment in the immune challenge experiment, we used the ln of the ratio of weight right before the measure and the treatment (ln(*weight at measure*/*weight at treatment*)) and tested if it differed between age and treatment groups with a one-way within-subject ANOVA with four levels: *incipient-control*, *incipient-immune challenge*, *established-control* and *established-immune challenge*; and *time point* as within-subject factor. Post-hoc pairwise differences between age and treatment groups were determined with multiple paired *t*-tests using the Benjamini–Hochberg procedure to correct for multiple comparisons.

We tested if the percentage of live sperm was influenced by the total number of sperm stored in the spermatheca using Spearman's correlation tests for each individual queen group (*incipient_2017*, *established_2016*, *established_2017*, *incipient-control*, *incipient-immune challenge*, *established-control* and *established-immune challenge*). Finally, we used a Welsh *t*-test to determine if there was a difference in the total number of sperm cells present in the spermatheca of all incipient and established queens tested together.

3. Results

There was no effect of queen collection year, but a significant effect of queen age on stored sperm viability (table 1). First, the percentage of live sperm cells (mean ± standard deviation) measured in the spermathecae of established queens were as follows: *established_2016* = 98.6 ± 1.5%; *established_2017* = 98.4 ± 2.8% (electronic supplementary material, figure S1). Post-hoc pairwise comparisons between parameter estimates showed that the year of collection did not significantly affect sperm viability in established queens ($z = 0.66$, $p = 0.79$). Unfortunately, we were unable to test the effect of collection year on sperm viability in incipient queens. Nevertheless, the lack of an effect of collection year on queen sperm viability in established queens suggests that the year in which queens were collected and mated does not influence sperm viability.

Second, the percentage of live sperm cells measured in *incipient_2017* queens was 78.8 ± 8% (electronic supplementary material, figure S1). Post-hoc pairwise comparisons showed that incipient queens had significantly lower stored sperm viability than established queens (*established_2016* > *incipient_2017*: $z = -8.98$, $p < 0.001$; *established_2017* > *incipient_2017*: $z = -8.22$, $p < 0.001$), indicating that incipient queens have lower stored sperm viability than established ones independently of collection year.

Finally, our test of the impact of immune activation on stored sperm viability in incipient and established queens showed that sperm viability was significantly affected by treatment, age and their interaction (table 2). The percentage of live sperm cells (mean ± standard deviation) measured in queen spermathecae were as follows: *incipient-control* = 75.1 ± 7.5%; *incipient-immune challenge* = 76.4 ± 6.3%; *established-control* = 88.6 ± 4.6%; *established-immune challenged* = 83.3 ± 6.3% (figure 2). All queens in the immune-challenged groups had melanized scarring where the needle prick was inflicted (figure 1). Post-hoc pairwise comparisons between parameter estimates showed that immune activation significantly lowered sperm viability in established queens ($z = -0.52$, $p < 0.001$) but had no influence in incipient queens ($z = 0.06$, $p = 0.96$). Established queens always had higher sperm viability than incipient ones both in the control ($z = -0.99$, $p < 0.001$) and immune-challenged groups ($z = -0.42$, $p = 0.004$). Moreover, queen weight was significantly affected by treatment ($F_{3,36} = 17.12$, $p < 0.001$). Incipient queens lost more weight after the immune challenge (ln(*weight at measure*/*weight at treatment*) = −0.117 ± 0.052, mean ± standard deviation) than after the control treatment (−0.073 ± 0.054) ($p = 0.046$); this difference was marginally significant in established queens (immune challenged = −0.031 ± 0.042; control = −0.004 ± 0.014; $p = 0.05$). Three out of the 16 queens in the established-immune challenge group died within 24 h of the treatment against none of the incipient queens, however this result was not statistically significant (Fisher's exact test: $p = 0.22$).

There was no relationship between the percentage of live sperm and the total number of sperm stored in the spermatheca in any of the individual queen groups (Spearman's correlation tests: $p > 0.15$ for all groups). The average number of sperm cells stored in the spermatheca of all incipient and established queens was 2 856 980 ± 1 022 054 and 2 232 548 ± 806 923 (mean ± standard deviation), respectively. Incipient queens had significantly more sperm cells stored in their spermatheca than established queens (Welsh *t*-test: $t_{93.18} = -3.44$, $p < 0.001$).

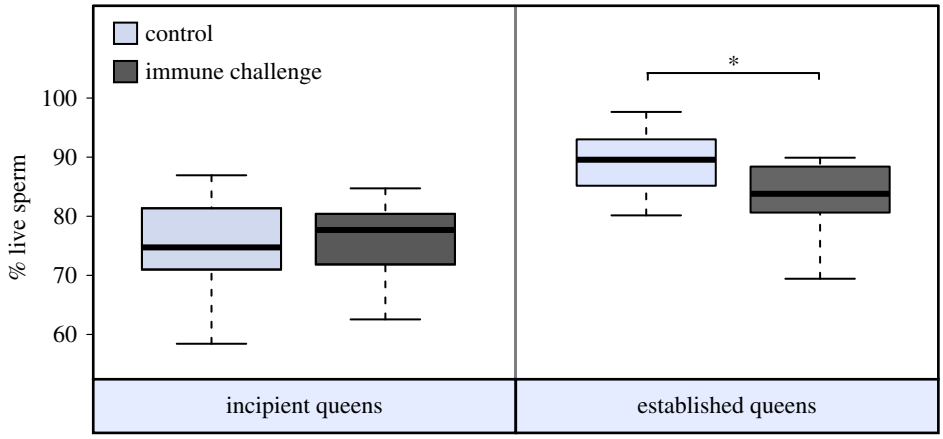

Figure 2. Sperm viability of control and immune-challenged incipient and established queens. The percentage of live sperm cells (box and whiskers plot) in *Lasius niger* ant queen spermatheca is represented for age, *incipient* and *established*, and treatment, *control* and *immune challenge*, groups. The midline of the box and whiskers plot is the median of each data group; the lower and upper edges of the boxes are the first and third quartiles, respectively. As there are no outliers, the whiskers extend to the lowest and highest data points in each group. Statistically significant differences between control and immune-challenged queens are shown with an asterisk. (Online version in colour.)

Table 2. The effect of queen age (*incipient* or *established*), treatment (*control* or *immune challenge*) and their interaction on sperm viability determined by a generalized linear mixed model with binomial errors and a logit link function. (Time point (ordinal variable with 16 levels: a < b< ··· <o < p) was used as a random effect to account for variation introduced by the time at which sperm viability was measured. Queen identity (ID) was used as an observation-level random effect to account for overdispersion.)

random effects	variance	s.d.		
queen ID	0.1115	0.3339		
time point	0.0445	0.2110		
fixed effects	**estimate**	**s.e.**	**Z-value**	***p*-value**
intercept	2.1429	0.1016	21.101	<0.001
age	−0.9974	0.1204	−8.282	<0.001
treatment	−0.5173	0.1290	−4.011	<0.001
age*treatment	0.5736	0.1749	3.280	0.001

4. Discussion

Ant queens mate on a single occasion and can therefore never renew their sperm stock. This implies that queens are under strong selection to maximize the survival of stored sperm. We show that immune activation has a negative impact on sperm viability in established queens: immune-challenged queens had lower sperm viability than controls. By contrast, incipient queen sperm viability was not affected by the immune challenge. Thus, depending on their life stage, queens do not have the same ability to prevent sperm maintenance from trading-off with the activation of an immune response.

Young ant queens are exposed to numerous pathogens during mating and early colony foundation. It has been shown that ant queens upregulate their immune system early in life [19,21,23,24], indicating that selection favours a strong immune capacity through the maintenance of high levels of immune effectors readily available upon infection. On the other hand, established queens that never leave the colony and benefit from social immunity are not subject to high pathogen pressure and we can expect their immune system to be downregulated. Our data show that three of the 16 initial established queens, but not a single incipient queen, died after the immune challenge. Although not statistically different, this could suggest that queens invest less in immunity after worker hatching. The fact that the immune challenge caused incipient queens to lose proportionally more weight than established queens demonstrates that the stronger immune activation in incipient queens takes place at the expense of body weight. An alternative explanation could be that established queens were fed by workers with food still remaining in their crop, thereby limiting the effect of the immune challenge on their weight.

The strong immune capacity of incipient queens would allow them to prevent stored sperm viability from trading-off with immune activation. In established queens, an immune challenge probably requires the de novo activation of the immune system based on energy originally allocated to other processes. Long-term sperm conservation could be one of the latter, thus explaining the reduced sperm viability in established immune-challenged queens. Accordingly, social Hymenoptera males that spend the majority of their lives in the protected nest environment have reduced sperm viability after an immune challenge [30,31]. The possibility that established queens have a lower ability to maintain sperm viability stable because they are older seems unlikely

as *L. niger* queens can live up to 29 years [33] so that a 1 year old queen can hardly be considered as old. It has also been suggested that stored sperm might suffer from an autoimmune effect [26] caused by immune effectors themselves or by side-products such as the reactive oxygen species produced by melanization [42]. Under this scenario, we would expect incipient and established queens to both show reduced sperm viability after an immune challenge. This was not the case in our experiments, ruling out that reduced sperm viability was caused by autoimmunity.

An unexpected result of our study was that established queens had significantly higher sperm viability in their spermatheca than incipient queens. This suggests that ant queens are capable of eliminating dead spermatozoa from their spermatheca. In our study, queens laid on average 88 eggs during the first year of their life (S. Chérasse 2017, personal observation). Ant queens use only two or three sperm cells to fertilize each egg [9,10] meaning that established 1 year old queens should have used between 176 and 264 sperm cells. However, established queens had around 600 000 less sperm cells in their spermatheca as compared to incipient queens (see Results). This large difference indicates that ant queens might in fact be able to remove dead sperm cells from storage. This process might also be cumulated with high levels of egg mortality (dead eggs may be eliminated by workers, hence cannot be censused) and/or less parsimonious sperm use in *L. niger*. The underlying mechanism for dead sperm removal from the spermatheca has yet to be identified. It may involve phagocytosis of dead cells and ensuing debris [43]; during cell necrosis, intracellular material spills into the extracellular space through the damaged plasma membrane [44]. This process triggers an inflammatory response that might have deleterious consequences for the surrounding cells especially when these are 'non-self' like sperm cells. By eliminating dead or dying cells in the spermatheca, queens would thus ensure that optimal conditions are maintained for the live sperm that remains in storage. Consistently, stingless bee queens seem capable of digesting sperm cells in their spermathecal membrane [45]. If these sperm cells are dead, as suggested by our results, this indicates that Hymenoptera queens are able to 'clean' their spermatheca from useless sperm, a hypothesis that certainly deserves further investigation. Ant queens use only a few spermatozoa to fertilize each egg [9,10]. Maintaining high sperm viability in the spermatheca by discarding dead cells from storage allows queens to keep using sperm parsimoniously as it minimizes the proportion of dead sperm cells released to fertilize each egg. Another explanation for the higher sperm viability measured in established queens is that sperm cells stored by incipient queens are more susceptible to damage during the experimental protocol. In honeybees, stored sperm differs from ejaculated sperm [14] and it is likely that a similar difference exists between newly stored sperm and sperm cells that have been stored for a year. If sperm cells undergo a maturation process to prepare them for long-term storage, this might also make them more resistant to experimental damage, thus explaining the higher sperm viability in established queens.

In their study on bumblebees, Greeff & Schmid-Hempel [46] found no difference in stored sperm viability between newly mated founding queens and 1 year old queens at the end of their life. However, this does not exclude the fact that bumblebee queens might also be able to eliminate dead sperm cells from storage. These diverging results might stem from the different methods used; while Greeff & Schmid-Hempel [46] used fluorescence microscopy, we used flow cytometry—which analyses consistently greater numbers of cells per sample resulting in higher statistical power. In any case, the discrepancy between our results and theirs indicates that the difference in sperm viability between Hymenoptera queens of different ages deserves to be tested in species with varying life-history traits.

The percentage of viable sperm we measured in *L. niger* queen spermathecae was comparable to other studies in Hymenoptera [16,30,31,47–50]. An important aspect to consider when measuring sperm viability is that the methods, such as dilution or pipetting, cause sperm mortality, meaning one should be careful when interpreting such data [39]. Sperm viability in Hymenoptera queen storage organs might therefore be much higher than what we are able to measure so far, especially considering the importance of maintaining high sperm viability in these species.

Data accessibility. The raw data can be found in the electronic supplementary material file. The flow cytometry results files have been deposited in Flow Repository (see https://flowrepository.org/id/FR-FCM-ZYNJ).

Authors' contributions. S.C. and S.A. designed the study and wrote the manuscript. S.C. carried out the fieldwork, the experimental work, the data analysis and the statistical analysis. Both authors gave final approval for the publication.

Competing interests. We have no competing interests to declare.

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