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RESEARCH ARTICLE

Antibacterial activity of male and female sperm-storage organs in ants

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ABSTRACT

Bacteria can damage sperm and thus reduce the reproductive success of both males and females; selection should therefore favour the evolution of antimicrobial protection. Eusocial hymenopterans might be particularly affected by such bacterial infections because of their mating ecology. In both sexes, mating is restricted to a short window early in the adult stage; there are no further chances to mate later in life. Males die shortly after mating, but queens use the acquired sperm to fertilise their eggs for years, sometimes decades. The reproductive success of both sexes is, thus, ultimately sperm-limited, which maintains strong selection for high sperm viability before and after storage. We tested the antibacterial activity of the contents of the male and female sperm-storage organs – the accessory testes and the spermatheca, respectively. As our study species, we used the bacterium *Escherichia coli* and the garden ant *Lasius niger*, whose queens can live for several decades. Our results provide the first empirical evidence that male and female sperm-storage organs display different antibacterial activity. While the contents of the accessory testes actually enhanced bacterial growth, the contents of the spermatheca strongly inhibited it. Furthermore, mating appears to activate the general immune system in queens. However, antimicrobial activity in both the spermatheca and the control tissue (head–thorax homogenate) declined rapidly post-mating, consistent with a trade-off between immunity and reproduction. Overall, this study suggests that ejaculates undergo an immune 'flush' at the time of mating, allowing storage of sperm cells free of bacteria.

KEY WORDS: Mating, Immunity, Sperm, Spermatheca, Accessory testes, Social insects, *Lasius niger*

INTRODUCTION

Microbes are environmentally ubiquitous and thus impose great selective pressure on organisms to protect themselves from infections. In sexually reproducing animals, sperm can be exposed to a variety of pathogens, including bacteria, fungi and viruses, via several pathways (reviewed in Knell and Webberley, 2004; Otti, 2015). First, male reproductive tissues may become infected by pathogens prior to copulation. Second, during copulation, pathogens may enter the reproductive tract through genital openings. Third, sperm may be exposed to pathogens in the female reproductive tract, which harbours diverse microbes (Otti, 2015). Infection can negatively affect sperm function (e.g. motility, viability; Otti et al., 2013; Otti, 2015) either directly by the action of pathogens on sperm cells or indirectly via the costs associated with activation of the immune system on reproduction (e.g. Losdat et al., 2011; Simmons, 2011; Radhakrishnan and Fedorka, 2012), thereby potentially jeopardizing reproductive success in both sexes (Lung et al., 2001; Poiani, 2006). However, ejaculates display antibacterial activity against Gram-positive and Gram-negative bacteria, a phenomenon observed in several taxa, including mammals (Hankiewicz and Swierczek, 1974; Bourgeon et al., 2004), fish (Lahnsteiner and Radner, 2010), birds (Sotirov et al., 2002; Rowe et al., 2011, 2013) and insects (Samakovlis et al., 1991; Jothy et al., 2005; Avila et al., 2011; Otti et al., 2013).

In this context, the reproductive system of eusocial Hymenoptera (ants, bees and wasps) imposes unique selective pressures (reviewed in Hölldobler and Bartz, 1985; Boomsma and Ratnieks, 1996; Baer, 2003, 2005; Boomsma et al., 2005; Boomsma et al., 2009; Baer, 2011). Mating occurs during a brief period early in adult life for both sexes. Females (queens) mate with one or a few males and store a lifetime's supply of semen in a specialised organ, the spermatheca. They never remate, even though they may live for more than a decade. Males have usually completed spermatogenesis by the time they reach sexual maturity. Their testes degenerate shortly after they emerge as adults. Sperm cells are stored in their accessory testes, the content of which is mixed with seminal fluid from the accessory glands during ejaculation. Males die shortly after copulation but persist posthumously as sperm stored in queens' spermathecae. In such a system, it may be critical to keep sperm free of pathogens as sperm quality could directly affect the lifetime reproductive success of both sexes.

Although the advantages of antimicrobial defences against sperm-associated pathogens have been repeatedly mentioned in the literature, the mechanisms for protecting sperm from infections have received surprisingly little attention in ants, bees and wasps. Analyses of proteomes (Collins et al., 2006; Baer et al., 2009a,b; Poland et al., 2011; Malta et al., 2014) and gene expression (via RNA sequencing; Gotoh et al., 2017) in the honeybee and in ants have revealed that male ejaculates and female spermathecal fluid contain a number of antifungal and antibacterial peptides. However, thus far a single study has explored their actual efficiency in protecting sperm against pathogens; Peng et al. (2016) showed that the seminal fluid of honeybee drones kills spores of the fungus *Nosema apis* in two ways: the protein fraction disrupts the fungus' life cycle by inducing extracellular spore germination, while the non-protein fraction reduces the viability of intact spores. Given the strong sperm-damaging effect of bacteria (Otti et al., 2013) and the irreplaceable nature of stored sperm in eusocial Hymenoptera, we predict that sperm storage organs should also exhibit antibacterial activity.

Here, we investigated the antibacterial activity (ABA) of the contents of male accessory testes and female spermathecae (hereafter referred to as the male and female sperm-storage

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organs, SSOs). Our study species was the garden ant *Lasius niger* (Linnaeus). Queens of this ant species can live up to 29 years (Kutter and Stumper, 1969) and are capable of laying fertilised eggs their entire lives. ABA of SSOs were tested against the bacterium *Escherichia coli*. This Gram-negative bacterium has been shown to damage sperm cells by negatively affecting the mitochondrial membrane potential, and reducing sperm motility, velocity and viability in various mammals (mice: Bhardwaj et al., 2015; rams: Yániz et al., 2010; boars: Maroto et al., 2010; humans: Fraczek et al., 2007; Tremellen, 2008; Diemer et al., 2000; Prabha et al., 2010; Barbonetti et al., 2013). Although *E. coli* was not reported to infect the reproductive organs of arthropods (Otti, 2015), it was shown to activate the insect immune system (e.g. Radhakrishnan and Fedorka, 2012), and to be susceptible to anti-bacterial activity of peptides from the haemolymph of termites and moths (Lockey and Ourth, 1996; Coutinho et al., 2009) and the male genital tract in *Drosophila* (Samakovlis et al., 1991; Lung et al., 2001).

We measured the ABA of sperm taken from the SSOs of males sampled prior to the mating flight and of fluid taken from the SSOs of both virgin and mated queens. As female immunity levels are known to increase within a few days of mating (Baer et al., 2006; Dávila et al., 2015), we also characterised antibacterial activity in female SSOs 1 day, 1 week, 2 weeks and 4 weeks post-mating.

MATERIALS AND METHODS

Sampling

Lasius niger virgin males and virgin queens were collected from field colonies in Brussels (Belgium) before they carried out their mating flights. Freshly mated queens were caught on the day of the flight by hand, when they started landing after mating. They were placed in laboratory nests with *ad libitum* water, and fed sugar water and mealworms.

We took samples from six types of individuals: virgin males (n=39) and virgin queens (1 day after collection; n=18) and 1 day-mated (n=28), 1 week-mated (n=30), 2 week-mated (n=30) and 4 week-mated (n=30) queens. Ants were dissected in a laminar flow cabinet using sterilised forceps (i.e. rinsed with 70% ethanol and flame sterilised between dissections). For each individual, we tested the ABA of two tissue types: (i) the contents of the SSO [i.e. sperm stored in the accessory testes of males (this product does not contain the seminal fluid from the accessory glands, which is added after the sperm leaves the accessory testes at ejaculation) or fluid in the spermathecae of virgin and mated queens] and (ii) a homogenate of head and thorax tissues, which served as a control. Although haemolymph would have been a better control, the amount of haemolymph that can be extracted from *L. niger* is insufficient for accurately testing ABA (Dávila et al., 2015).

First, the SSO were carefully dissected in lysogeny broth (LB; 10 g tryptone, 10 g NaCl and 5 g yeast extract; Sigma L3022, St Louis, MO, USA) to avoid hampering bacterial growth, and placed in a clean 5 µl drop of the same broth. The SSO envelopes were ruptured and removed; the contents released were transferred to a vial and stored at 4°C for subsequent ABA analyses. Second, the head and thorax of each individual were separated from the abdomen, placed together in a 1.5 ml vial, and homogenised in liquid nitrogen. Then, 50 µl of LB was added and the homogenate was vortexed for 30 s and centrifuged for 10 min at 13,000 rpm; 5 µl of the supernatant was sampled and stored at 4°C.

Antibacterial activity

The ABA of the SSO contents and of the head–thorax homogenates was tested using the *E. coli* K-12 strain D31 (CGSC 5165, Coli Genetic Stock Center, Yale University, USA), which is lipopolysaccharide defective and thus sensitive to antimicrobial peptides (Monner et al., 1971; Papo and Shai, 2005). It also expresses resistance to ampicillin and streptomycin. The strain was cultured overnight in LB at 37°C in a shaker (180 rpm) in the presence of ampicillin (100 µg ml^{-1}) and streptomycin (100 µg ml^{-1}). The amount of bacteria present was then estimated by measuring absorbance at 600 nm using a spectrophotometer (TriStar LB941, Berthold Technologies, Bad Wildbad, Germany). The bacterial culture was diluted in LB (OD_{600}=0.1), and the bacteria were grown until they reached the exponential growth phase (OD_{600}=0.4–0.6). The bacteria were then pelleted and resuspended to obtain a concentration of ~10^6 cells 100 l^{-1} of LB. To test ABA, 5 µl samples of the SSO contents or the head–thorax homogenates were added to 5 µl of the *E. coli* suspension. To establish negative controls, 5 µl of LB was added to 5 µl of the *E. coli* suspension, which allowed free bacterial growth. All these mixtures were incubated for 2 h at 37°C, and ABA was quantified by determining the end quantity of bacteria. A colorimetric method was used to quantify the amount of bacteria in the samples and controls after incubation (see below). This method is based on the ability of bacteria to reduce water-soluble tetrazolium dye (MTT), a substrate that replaces succinate in the respiration reaction, and thus allows quantification of only living bacteria, thereby avoiding measure bias due to dead cells (Botsford, 1998).

Colorimetric assay

Bacteria were centrifuged for 10 min at 4000 rpm, rinsed twice with 100 µl of PBS (pH 7.4) and resuspended in 100 µl of PBS. The bacterial suspensions were loaded onto 96-well plates. Each well contained one sample or one control, and ABA was measured once. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 20 µl of 5 mg ml^{-1} MTT in PBS] was added to each well (including negative controls) and the plates were incubated for 20 min at 37°C. MTT is transformed into formazan (purple in colour; absorbance measured at 600 nm) by succinate dehydrogenase during cell respiration (Mosmann, 1983). Preliminary studies indicated that formazan absorbance was highly correlated with the amount of bacteria (linear regression: r^2>0.96). One 96-well plate was used for each sample type (i.e. virgin males, virgin queens and 1 day-mated, 1 week-mated, 2 week-mated and 4 week-mated queens). Each plate had its own negative control wells. A total of 342 samples and 34 controls were tested. To account for possible variation among plates, the absorbance values of the samples were divided by the mean absorbance of the negative controls from the same plate (n=5–8 negative controls per plate).

The ABA index was calculated as per Rowe et al. (2011): ABA=1−(sample OD/negative control OD). ABA index values can vary from 1 to ∞ (see Table S1): values that are greater than zero indicate antibacterial activity is present; zero indicates there is no difference in activity between the samples and the negative controls; and values that are less than zero indicate that bacterial growth has been enhanced.

Statistical analysis

Statistical analyses were conducted using R v.3.4 (http://www.R-project.org/). Males and queens were analysed separately. Virgin queens and mated queens at different time points (1 day, 1 week, 2 weeks and 4 weeks after collection) were treated as different levels of the 'time point' variable. A second variable – 'tissue' – had three levels, corresponding to the SSO contents, the control tissue (head–thorax homogenates) and the negative control.

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First, we verified that the ABA of the controls was not different from 0; a linear model was used to assess that none of the intercepts of the negative controls differed significantly from 0. Second, ABA data were analysed using a two-way crossed-factor analysis of variance (ANOVA) adapted to unbalanced designs to test for an interaction between the factors time point and tissue. A permutation approach was used (9999 permutations; function *anova.2way.unbalanced*; http://adn.biol.umontreal.ca/~numericalecology/Rcode/) that calculates type III sums of squares, as suggested for unbalanced designs (http://adn.biol.umontreal.ca/~numericalecology/Rcode/). Because the results of this global model were significant (see Results), we carried out multiple comparisons of means. As *P*-value corrections for multiple tests must be applied in such contexts to control for type I error rate (Bretz et al., 2011), we only performed the comparisons relevant to our hypotheses of interest to maintain our statistical power as high as possible. These comparisons were carried out using permutation *t*-tests (9999 permutations; function *t.perm*; http://adn.biol.umontreal.ca/~numericalecology/Rcode/), and the resulting *P*-values were corrected using the Šidák method (Šidák, 1967). Overall, 26 tests were performed: we compared the ABA (i) of each type of tissue versus the negative controls for males and versus the negative controls for females at each time point (12 tests); (ii) between the different types of tissue within each time point (6 tests); and (iii) between consecutive time points for each tissue type for females (8 tests) (Table 1). For these 26 tests, the Šidák-corrected significance threshold was 0.002. The R codes used in this study are provided in the supplementary information (Script 1).

Table 1. Results of the permutational *t*-tests comparing antibacterial activity

ABA comparison	Time point	*t*	*P* (corrected)
Tissue type vs negative control			
SSO vs negative control	Virgin males	−3.738	**0.008**
	Virgin queens	3.776	**0.028**
	1 day-mated queens	3.601	**0.005**
	1 week-mated queens	−3.87	**0.023**
	2 week-mated queens	−0.422	1
	4 week-mated queens	0.388	1
Control tissue vs negative control	Virgin males	0.081	1
	Virgin queens	1.906	0.941
	1 day-mated queens	6.747	**0.003**
	1 week-mated queens	−3.593	**0.021**
	2 week-mated queens	1.151	1
	4 week-mated queens	1.854	0.84
Between tissue types			
SSO versus control tissue	Virgin males	4.947	**0.003**
	Virgin queens	−3.498	**0.048**
	1 day-mated queens	5.594	**0.003**
	1 week-mated queens	1.146	1
	2 week-mated queens	2.159	0.602
	4 week-mated queens	1.142	1
Tissue type at different time points			
SSO	Virgin queens vs 1 day-mated queens	4.386	**0.005**
	1 day-mated queens vs 1 week-mated queens	11.859	**0.003**
	1 week-mated queens vs 2 week-mated queens	−3.39	**0.033**
	2 week-mated queens vs 4 week-mated queens	−1.251	0.998
Tissue control	Virgin queens vs 1 day-mated queens	−5.676	**0.003**
	1 day-mated queens vs 1 week-mated queens	16.036	**0.003**
	1 week-mated queens vs 2 week-mated queens	−6.527	**0.003**
	2 week-mated queens vs 4 week-mated queens	−0.253	1

Comparison of antibacterial activity (ABA) index values (i) between each tissue type and the corresponding negative control at each time point; (ii) between tissue types at each time point; and (iii) between consecutive time points for each tissue type. The tissue control is the head–thorax homogenate. SSO, sperm-storage organ. *P*-values were corrected by the Šidák correction for multiple tests.

RESULTS

The ABA index of negative controls (n=34) did not differ significantly from zero (linear model: t=0, P=1), indicating neither inhibition nor enhancement of bacterial growth. Consequently, the results below will be discussed in terms of significant differences from an ABA value of zero. For the queens, there was a significant time point by tissue interaction: the ABA index differed between tissues depending on the time point after mating (two-way ANOVA: time point F=19.28, P=0.0001; tissue F=2.04, P=0.13; interaction F=7.03, P=0.0001).

The ABA of the SSO contents, the control tissue (head–thorax homogenates) and the negative control are shown in Fig. 1. The statistical results are given in Table 1.

Antibacterial activity of SSO contents

The SSO contents of virgin males significantly enhanced bacterial growth (permutational *t*-tests: t=−3.738, P=0.008). In contrast, the SSO contents of virgin queens showed marked antibacterial activity (P=0.028). The ABA index for 1 day-mated queens was significantly lower than that for virgin queens (P=0.005) but remained significantly different from zero (P=0.005). It continued to decrease over time and the SSO contents of 1 week-mated queens favoured bacterial growth (P=0.023). Two weeks after mating, the SSO contents of queens showed no significant activity (ABA index statistically indistinguishable from zero; P=1); this pattern held for 4 week-mated queens.

Antibacterial activity of control tissue

The head–thorax homogenates displayed no significant antibacterial activity on bacterial growth in either virgin males or virgin queens. However, antibacterial activity increased significantly after mating (P=0.003). The ABA index then decreased and became negative in 1 week-mated queens (P=0.021) and, finally, increased significantly (P=0.003) such that in 2 week- and 4 week-mated queens, head–thorax homogenates showed no significant activity (ABA index not different from zero; P=1).

Comparison of antibacterial activity between SSO contents and control tissue

The ABA index differed significantly between SSO contents and head–thorax homogenates for virgin males, virgin queens and 1 day-mated queens (all $P \leq 0.05$). In males, this difference stemmed from the fact that ABA in the accessory testes was negative, suggesting enhanced bacterial growth in the SSO (see above), but head–thorax homogenates displayed no activity. In contrast, in virgin queens, while head–thorax homogenates also displayed no activity, SSO contents had strong antibacterial effects. In 1 day-mated queens, head–thorax homogenates displayed much greater antibacterial activity than did SSO contents, suggesting that mating triggers an increase in general immune function. Finally, the ABA index did not differ between SSO contents and head–thorax

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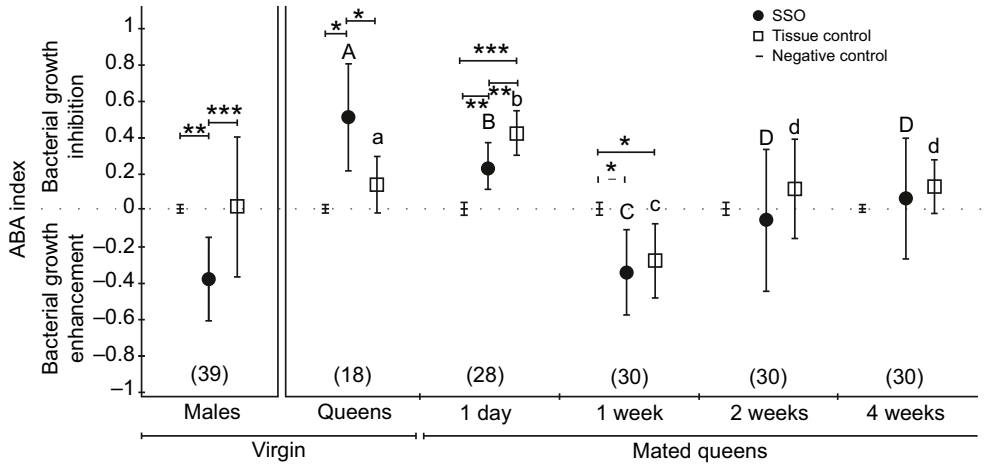

Fig. 1. Antibacterial activity index of the contents of sperm-storage organs. Data are given for the sperm-storage organs (SSOs; accessory testes of virgin males and the spermathecae of virgin and mated queens) and control tissues (i.e. head–thorax homogenates) in the ant *Lasius niger*. Values are mean (±s.d.) antibacterial activity (ABA) index values; they reflect ABA relative to that of the negative control (i.e. free growth; see Materials and methods). Values >0 indicate that ABA was present; values equal to 0 indicate there was no difference in ABA between the SSO sample and the negative control; and values <0 indicate that bacterial growth was enhanced in the SSOs. Significant differences in ABA index between the contents of SSOs and control tissues are shown for virgin males (single time point) and for queens (multiple time points). Differences in ABA index between consecutive time points were tested. Statistical differences in spermathecal content and control tissues are indicated by capital and lowercase letters, respectively. Sample size is indicated in parentheses. The level of significance is as follows: *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.003$.

homogenates for 1 week-mated, 2 week-mated and 4 week-mated queens.

DISCUSSION

Our results show, to our knowledge for the first time in eusocial Hymenoptera, substantial differences in ABA among the SSO of *Lasius niger* virgin males, virgin queens and mated queens. Whereas the content of male accessory testes did not display ABA against the Gram-negative bacterium *E. coli*, queen spermathecal fluid showed high levels both before and shortly after copulation. From 1 week after mating, no ABA was detected in the spermatheca. Below, we discuss these variations of ABA in the SSO and in control tissues (head–thorax homogenates) in relation to the putatively high selective pressure for sperm protection that exists in ants.

Unexpectedly, we found that the contents of the males' accessory testes enhanced bacterial growth. The possibility that the negative value of ABA comes from the addition of bacteria naturally present in the SSO seems unlikely, as the amount of bacteria potentially present in the testes – if any – should be considerably lower than that added in our experiments. Enhanced bacterial growth suggests that bacteria utilise natural sperm-associated compounds (e.g. proteins, carbohydrates, lipids), and possibly even sperm themselves, as energy sources. However, we did not characterise the exact substances found in the accessory testes. Proteome analysis of ejaculates in *Apis mellifera* (Baer et al., 2009b) revealed the presence of 31 types of peptides that are involved in cell nutrition and metabolism. Some could potentially be produced in the accessory testes and help sustain the sperm, which are stored for days or weeks before copulation. These nutrients could allow the enhanced bacterial growth we observed. Antimicrobial peptides (AMPs) and other immune cells found in male ejaculate are typically contained in the seminal fluid produced by the accessory glands (Lung et al., 2001; Poiani, 2006; Baer et al., 2009a,b; Perry et al., 2013). Unfortunately, we were unable to induce ejaculation of *L. niger* males and could not therefore compare ABA between the contents of the accessory testes, the seminal fluid and whole ejaculates. However, it has been shown that AMPs in the seminal fluid can have cytotoxic effects on sperm cells (Boman, 2003). Consequently, sperm may be stored separately from such peptides in the accessory testes to prevent any such degradation.

We also found that the SSO contents of virgin queens displayed strong ABA, which contrasts with the lack of activity in the head–thorax homogenates. This result indicates that such ABA is localised within the spermatheca and is not a by-product of the higher general immune function that arises post-mating. It could have been selected to preserve sperm from pathogens introduced during copulation. This 'hygienic' mechanism may be critical in eusocial Hymenoptera, as mating is restricted to a brief period early in life and queens do not later replenish their sperm stores. In the honeybee, the AMPs in male ejaculate are transferred to females during mating (Baer et al., 2009a,b), and it was recently shown that they have antifungal effects on *Nosema* spores (Peng et al., 2016). Collectively, these results support the argument that males and females of social hymenopterans both attempt to protect sperm from microorganisms.

In 1 day-mated queens, the SSO contents still showed marked ABA. At this point in time, the SSO may contain a mixture of AMPs from the female's spermathecal fluid and AMPs from the male's seminal fluid, transferred during copulation. In addition, ABA in 1 day-mated queens could also be heightened because the general immune system has been activated as a result of copulation and/or in response to pathogen exposure during mating. Up-regulation of immune function after mating could be an adaptive response to reduce the risk of sexually transmitted diseases and other microbial infections (McGraw et al., 2004; Valtonen et al., 2010; Fedorka et al., 2004, 2007; Shoemaker et al., 2006). Our results show that *L. niger* queens displayed a significant increase in ABA in head–thorax homogenates within 24 h of copulation, lending support to the idea that the general immune system is activated post-mating (Fig. 1). Thus far, two studies have investigated this idea in ants using zone of inhibition assays and phenoloxidase measurements. Castella et al. (2009) found no ABA in 1 day-mated queens of the wood ant *Formica paralugubris*. Likewise, Baer et al. (2006)

reported that the encapsulation response (a measure of immune defence that results from the phenoloxidase cascade) did not differ between virgin queens and 1 day-mated queens of the leaf-cutter ant *Atta colombica*. However, our data are not entirely comparable with these results for at least three reasons. First, different methods were used. We directly measured ABA by examining the inhibition of bacterial growth. Castella et al. (2009) used a different direct approach – inhibition zone assays – as well as an indirect approach – the quantification of phenoloxidase levels. Baer et al. (2006) used only an indirect approach – the measurement of the encapsulation response. Second, Castella et al. (2009) used queens mated under laboratory conditions, whereas we used queens that had mated in the field during the nuptial flight. In ants, mating under natural conditions may increase the risk of pathogen exposure, as pairs in copula can land, and their genitalia come into contact with the soil. Microbes may then enter the reproductive tract via genital openings, triggering the immune system. Finally, mating has been shown to differentially affect immunity in invertebrates, by inducing or suppressing different components of the immune response depending on the species (Lawniczak et al., 2007). Species may also suffer from post-mating immunosuppression in a pathogen-dependent manner (i.e. bacteria; Short and Lazzaro, 2010).

We found a dramatic decrease in general ABA in queens 1 week after mating: ABA in the SSO contents and the head–thorax homogenates was significantly lower than zero. This decrease might result from a trade-off between queen immunity and other physiological traits, particularly reproductive effort (Stearns, 1992; Schwenke et al., 2016). Indeed, the allocation of limited resources to reproduction has been shown to be coupled with a decrease in immune function in a variety of taxonomic groups (Sheldon and Verhulst, 1996; Rolff and Siva-Jothy, 2002; Fedorka et al., 2004; Gwynn et al., 2005), including ants (Pull et al., 2013; von Wyschetzki et al., 2016). Reproductive costs play a critical role in social hymenopteran species in which queens found their colonies alone (i.e. without the help of workers), as is the case in *L. niger*. Queens must rapidly produce a worker force to ensure nest construction, colony defence and food collection. After their nuptial flights, queens remain sealed within a chamber and rear their brood in isolation; they do not eat, but rather histolyse their wing muscles to feed their larvae until the first workers emerge, which may occur several weeks later (Hölldobler and Wilson, 1990). Under laboratory conditions, *L. niger* queens start to lay eggs 1–3 days after mating (Aron and Passera, 1999). The egg-laying rate then rapidly peaks and remains high for the next few weeks before drastically declining 20 days after mating (see Fig. S1). This enhanced investment in reproduction between 1 and 20 days after mating mirrors the strong decrease in queen immune defences 1 week after mating. Here again, our results diverge from those of Baer et al. (2006) and Castella et al. (2009), who found that immune function was up-regulated 1 week after mating in *A. colombica* and *F. paralugubris* queens, respectively. The reason for this discrepancy is unknown; it may arise from methodological differences (see above).

Finally, no ABA was detected in the SSO contents and head–thorax homogenates of 2 week-mated and 4 week-mated *L. niger* queens. This result is consistent with the high levels of prophenoloxidase previously found in head–thorax homogenates of 2 week-mated queens (Dávila et al., 2015), which indicated that reserves of the inactive precursor to phenoloxidase were full and that the immune system was inactive. In our study, mated queens were kept in clean, artificial laboratory nests and thus probably faced few to no pathogens.

In eusocial Hymenoptera, the protection of sperm cells against pathogens may be an essential component for the reproductive success of both sexes as mating is restricted to a single event early in life, after which males die and queens store a lifetime's supply of sperm. Our study shows that, in the ant *L. niger*, the contents of the male accessory testes favour bacterial growth. In contrast, there is high ABA in the spermatheca around the time of mating, showing that females play a key role in sperm preservation. ABA in the spermathecal fluid probably acts in concert with immune defences present in the seminal fluid of males that is transferred to females during copulation. In the weeks following mating, ABA disappears from the queens' spermathecae. Altogether, this suggests that ejaculates undergo an immune 'flush' at the time of mating, allowing long-term storage of sperm cells free of bacteria. Future research should focus on testing the activity of sperm storage organs against more prevalent pathogens of social Hymenoptera, as well as identifying the antibacterial compound(s) involved and their functional activity.

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Author contributions
Conceptualization: F.D., A.B., S.A.; Methodology: F.D., A.B.; Formal analysis: F.D., D.B.; Data curation: D.B., S.C.; Writing - original draft: F.D., S.A.; Writing - review & editing: F.D., S.A.; Supervision: S.A.; Project administration: S.A.; Funding acquisition: S.A.

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