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

Francisco Dávila[a*], Anne Botteaux[b], David Bauman[c], Sarah Chérasse[a], Serge Aron[a]

[a]Evolutionary Biology and Ecology, Université Libre de Bruxelles, 1050 Brussels, Belgium

[b]Laboratory of Molecular Bacteriology, Université Libre de Bruxelles, 1070 Brussels, Belgium

[c]Laboratory of Plant Ecology and Biogeochemistry, Université Libre de Bruxelles, 1050 Brussels, Belgium

Corresponding author
Serge Aron
Evolutionary Biology and Ecology, CP 160/12
Université Libre de Bruxelles
B-1050 Brussels, Belgium
e-mail: saron@ulb.ac.be
Tel: +32 (0)2 650 30 37
Fax: +32 (0)2 650 24 45

Summary statement

Male and female sperm-storage organs display different antibacterial activity in ants. This activity varies over time in females after mating, allowing long-term storage of sperm cells free of bacteria.

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

Antibacterial activity, sperm, spermatheca, accessory testes, social insects, ant

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, like 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 since 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 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 organs [SSO]). 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 SSO was 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 reduce 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., 2015). Although *E. coli* was not reported to infect 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 hemolymph 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 SSO of males sampled prior to the mating flight and of fluid taken from the SSO of both virgin and mated queens. Since 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 SSO one day, one week, two weeks, and four 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 (one day after collection; *n*= 18) and one-day- (*n*= 28), one-week- (*n*= 30), two-week- (*n*= 30), and four-week- (*n*= 30) mated queens. Ants were dissected under 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 that 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 hemolymph would have been a better control, the amount of hemolymph 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) 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. Fifty µl of LB was added; the homogenate was vortexed for 30 sec and centrifuged for 10 min at 13,000 rpm. Five 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 Gram-negative bacterium *Escherichia coli*. We used the *E. coli* K-12 strain D31 (CGSC 5165, Coli Genetic Stock Centre, Yale University), 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). 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 $µl^{-1}$ of LB. To test ABA, the 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 were added to 5 µl of the *E. coli* suspension, which allowed free bacterial growth. All these mixtures were incubated for 2 hours 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). It is based on the ability of bacteria to reduce water-soluble tetrazolium dye (MTT), a substrate that replaces succinate in the respiration reaction. This assay allows to quantify only living bacteria, thereby avoiding measure bias due to dead cells (Botsford, 1998).

Colorimetric assay

Bacteria were centrifuged for 10 min at 4,000 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 the ABA was measured once. Twenty µl of MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide; 5mg ml^{-1} 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 one-day-, one-week-, two-week-, and four-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 by plate).

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

Statistical analysis

Statistical analyses were conducted using R v. 3.4 (R Development Core Team, 2013). Males and queens were analysed separately. Virgin queens and mated queens at different time points (one day, one week, two weeks and four 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 head-thorax homogenates, and the negative controls.

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, data of ABA 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 (9,999 permutations; function *anova.2way.unbalanced*; Legendre, 2005) that calculates type III sums of squares, as suggested for unbalanced designs (Legendre, 2015). Because the results of this global model were significant (see Results), we carried out multiple comparisons of means. Since *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 (9,999 permutations; function *t.perm*; Legendre, 2005), and the ensuing *p*-values were corrected using the Šidák method (Šidák, 1967). Overall, 26 tests were performed: we compared the ABA of (i) each type of tissue versus the negative controls for males, on the one hand, and versus the negative controls for females at each time point, on the other hand (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 electronic supporting information (Appendix S1).

Results

The ABA index of negative controls (n = 34) did not differ significantly from zero (linear model: t = 0, P = 1), meaning 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 head-thorax homogenates, and the negative controls are shown Fig. 1. The statistical results are given in Table 1.

Antibacterial activity of sperm-storage organ contents

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 one-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 one-week-mated queens favoured bacterial growth (P = 0.023). Two weeks after mating, SSO contents of queens showed no significant activity (ABA index statistically indistinguishable from zero; P = 1); this pattern held for four-week-mated queens.

Antibacterial activity of head-thorax homogenates

The head-thorax homogenates displayed no significant 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 one-week-mated queens (P = 0.021) and, finally, increased significantly P = 0.003) such that in two-week- and four-week-mated queens, head-thorax

homogenates showed no significant activity (ABA index not different from zero; $P = 1$).

Comparison of antibacterial activity between sperm-storage organ contents and head-thorax homogenates

The ABA index differed significantly between SSO contents and head-thorax homogenates for virgin males, virgin queens, and one-day-mated queens (all $P \leq 0.05$). In males, this difference stemmed from the fact that antibacterial activity 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 one-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 homogenates for one-week-, two-week-, and four-week-mated queens.

Discussion

Our results show, to our knowledge for the first time in eusocial Hymenoptera, substantial differences in antibacterial activity 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 one week after mating, no ABA was detected in the spermatheca anymore. 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, since 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 byproduct 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, since 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 one-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 one-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 hours 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 one-day-mated queens of the wood ant *Formica paralugubris*. Likewise, Baer et al. (2006) reported that the encapsulation response (a measure of an immune defence that results from the phenoloxidase cascade) did not differ between virgin queens and one-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 one 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 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 electronic supporting information, Fig. S1). This enhanced investment in reproduction between one and 20 days after mating mirrors the strong decrease in queen immune defences one 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 one 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 two-week- and four-week-mated *L. niger* queens. This result is consistent with the high levels of prophenoloxidase previously found in head-thorax homogenates of two-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 likely 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 since mating is restricted to a single event early in life, after which males die and queens store a lifetime supply of sperm. Our study shows that, in the ant *L. niger*, the content of the

male accessory testes favours 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 likely 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 queen spermatheca. 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 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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Conflicts of interest

We have no conflict of interest to declare.

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Data accessibility.

The datasets supporting this article have been uploaded as part of the Suppementary material.

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Figures

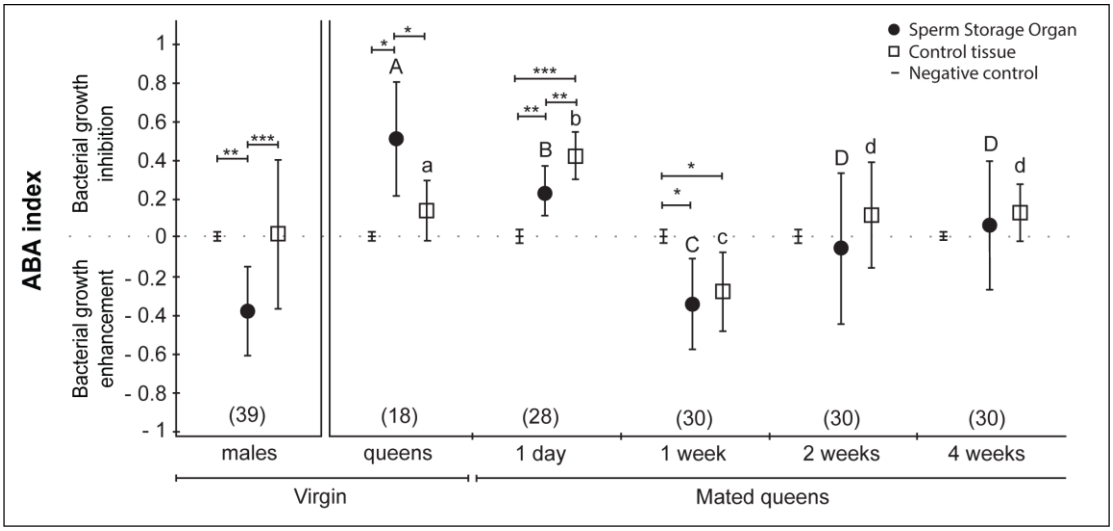

Figure 1. Antibacterial activity (ABA) index of the contents of sperm-storage organs (i.e., the accessory testes of virgin males and the spermathecae of virgin and mated queens) and of control tissues (i.e., head-thorax homogenates) in the ant *Lasius niger*.

The mean antibacterial activity (ABA) index values (± s.d.) are given; they reflect ABA relative to that of the negative control (i.e., free growth; see Methods). Values > 0 mean ABA index was present; values = 0 mean there was no difference in activity between the sample type and the negative control; and values < 0 indicate that bacterial growth was enhanced. Significant differences in ABA indexes between the contents of sperm-storage organs and control tissues are shown for virgin males (single time point) and for queens (multiple time points). Differences in ABA indexes between consecutive time points were tested. Statistical differences in spermathecal contents and control tissues are indicated by capital and lower-case letters, respectively. Sample size is indicated in brackets. The level of significance is as follows *: $p \leq 0.05$; **: $p \leq 0.01$; and ***: $p \leq 0.003$.

Table 1. Results of the permutational *t*-tests comparing antibacterial activity. Comparison of 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 *p*-values are corrected by the Šidák correction for multiple tests.

Tissue		Time Point	*t* value	*p*-value (corrected)
i)	Negative control vs. Sperm-storage organ	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 weeks mated queens	-0.422	1
		4 weeks mated queens	0.388	1
	Negative control *vs*. Head-thorax homogenate	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 weeks mated queens	1.151	1
		4 weeks mated queens	1.854	0.84
ii)	Sperm-storage organ *vs.* Head-thorax homogenate	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 weeks mated queens	2.159	0.602
		4 weeks mated queens	1.142	1
iii)	Sperm-storage organ	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 weeks mated queens	-3.39	**0.033**
		2 week mated queens *vs.* 4 weeks mated queens	-1.251	0.998
	Head-thorax homogenate	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 weeks mated queens	-6.527	**0.003**
		2 week mated queens *vs.* 4 weeks mated queens	-0.253	1

Supporting Information

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

Figure S1. Antibacterial activity in spermathecae and control tissues of mated *Lasius niger* queens with corresponding egg laying rates.

The mean antibacterial activity (ABA) values (± SD) in the mated queen spermathecae (black dots) and control tissues (i.e. head-thorax homogenates; white squares) during the first 30 days after mating are given in the lower part of the figure. They reflect ABA relative to that of the negative control (i.e., free growth; see Methods). Values > 0 mean ABA was present; values = 0 mean there was no difference in activity between the sample type and the negative control; and values < 0 indicate that bacterial growth was enhanced. Statistically significant differences in ABA between consecutive time points are indicated in spermathecal contents and control tissues by capital and lower-case letters, respectively.

Mated queen egg laying rate over the first 30 days after mating is shown in the upper part of the figure. Horizontal bars represent the mean number of eggs laid per day (± SD) over the following periods of time: 0-1 day, 5-10 days, 10-20 and 20-30 days after mating. Given that mated queen egg laying rate was calculated as part of another study, these queens are not the same as the ones used for ABA measures. Sample sizes are indicated above (egg laying) or beneath (ABA) error bars.

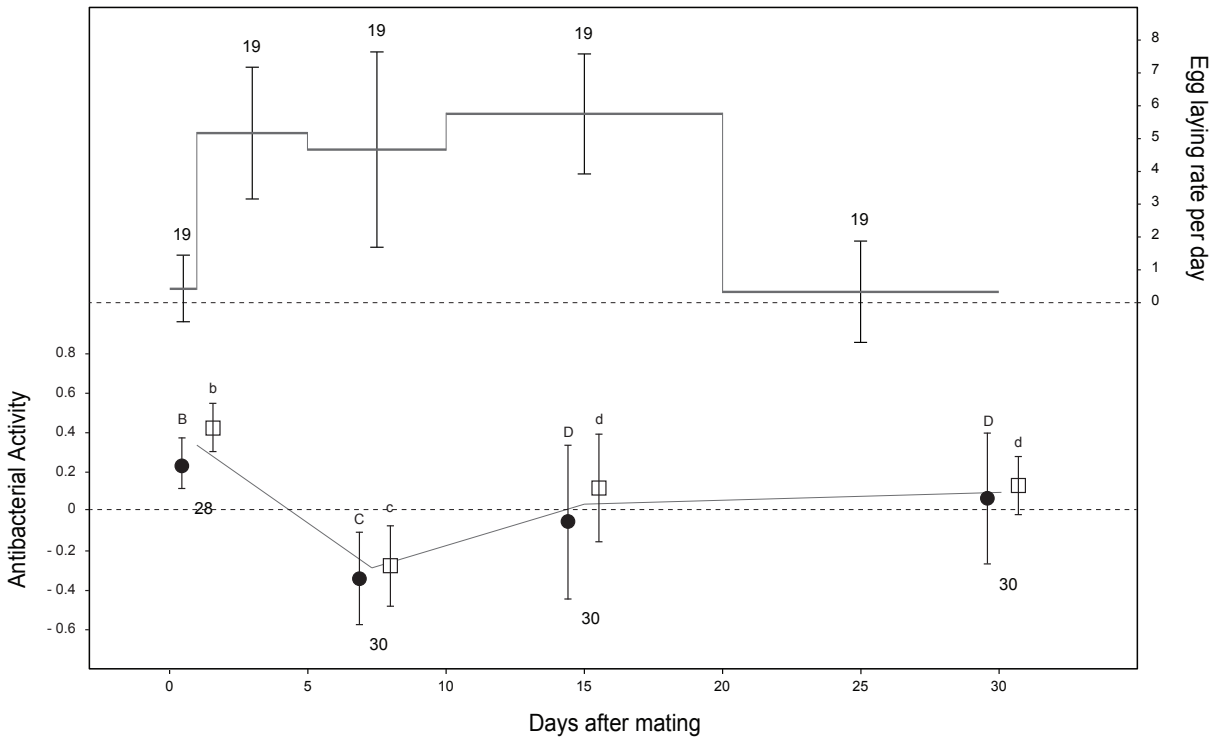

Table S1. Antibacterial activity (ABA) index of sperm-storage organ contents from males (accessory testes) and females (spermatheca of virgin queens (i.e., gynes), and of 1-day, 1-week, 2-week and 4-week mated queens), and of head-thorax homogenates (homo), in the ant *Lasius niger*.

The ABA index is calculated as ABA = 1 - (sample OD/negative control OD) (see Methods).

ABA	Tissue	Individual
-0.484793585	sperm	male
-0.071197236	sperm	male
-0.588625304	sperm	male
-0.142148911	sperm	male
-0.175028955	sperm	male
-0.342890235	sperm	male
-0.325584948	sperm	male
-0.555745259	sperm	male
-0.211370057	sperm	male
-0.124843625	sperm	male
-0.493446228	sperm	male
-0.453644069	sperm	male
-0.439799839	sperm	male
-0.114460453	sperm	male
-0.931269977	sperm	male
-0.150801554	sperm	male
-0.102346752	sperm	male
0.02398184	sperm	male
-0.277130146	sperm	male
-0.265016445	sperm	male
-0.348075188	sperm	male
-0.437137213	sperm	male
-0.639550905	sperm	male
-0.58557392	sperm	male
-0.542392333	sperm	male
-0.644948603	sperm	male
-0.107877607	sperm	male
-0.558585428	sperm	male
-0.653045151	sperm	male
-0.331882093	sperm	male
-0.561284277	sperm	male
-0.730997014	sperm	male
-0.334987893	sperm	male
-0.280986649	sperm	male
-0.242414332	sperm	male
-0.766997843	sperm	male
-0.378703185	sperm	male
-0.05726721	sperm	male
-0.622994526	sperm	male
-0.1957953	homo	male
-0.310010191	homo	male
-0.306549133	homo	male
0.151211918	homo	male
-0.79608416	homo	male
-0.751568916	homo	male
-0.345273844	homo	male

0.15268962	homo	male
0.498478626	homo	male
0.222458535	homo	male
0.236717876	homo	male
0.262180984	homo	male
0.05847612	homo	male
0.242829022	homo	male
0.539219598	homo	male
0.083939228	homo	male
-0.094302528	homo	male
0.562645658	homo	male
0.059494644	homo	male
-0.024449566	control-	male
-0.023194831	control-	male
0.011175651	control-	male
0.006032675	control-	male
0.030436071	control-	male
0.295103764	sperm	gyne
0.446152957	sperm	gyne
0.784869331	sperm	gyne
0.217290543	sperm	gyne
0.052509605	sperm	gyne
0.556006916	sperm	gyne
0.298808645	sperm	gyne
0.162363564	sperm	gyne
0.103116837	sperm	gyne
0.281372019	sperm	gyne
0.299730943	sperm	gyne
0.766560337	sperm	gyne
0.853528055	sperm	gyne
0.873210222	sperm	gyne
0.793108377	sperm	gyne
0.815079169	sperm	gyne
0.772968485	sperm	gyne
0.804551498	sperm	gyne
0.034200611	homo	gyne
0.272217522	homo	gyne
0.372916985	homo	gyne
-0.043612609	homo	gyne
-0.01614912	homo	gyne
0.272217522	homo	gyne
0.01131437	homo	gyne
0.061664101	homo	gyne
0.276794771	homo	gyne
-0.024449566	control-	gyne
-0.023194831	control-	gyne
0.011175651	control-	gyne
0.006032675	control-	gyne
0.030436071	control-	gyne
0.030049563	sperm	q1d
0.11728664	sperm	q1d
0.395008864	sperm	q1d
0.157706307	sperm	q1d
0.316777252	sperm	q1d
0.082203971	sperm	q1d
0.106855759	sperm	q1d

0.099154154	sperm	q1d
0.065132232	sperm	q1d
0.320688833	sperm	q1d
0.391097284	sperm	q1d
0.159010167	sperm	q1d
0.17987193	sperm	q1d
0.252888102	sperm	q1d
0.232026339	sperm	q1d
0.091209436	sperm	q1d
0.301130929	sperm	q1d
0.009066243	sperm	q1d
0.202037554	sperm	q1d
0.269838285	sperm	q1d
0.342854456	sperm	q1d
0.469328896	sperm	q1d
0.106855759	sperm	q1d
0.424997649	sperm	q1d
0.321992693	sperm	q1d
0.318081112	sperm	q1d
0.344158316	sperm	q1d
0.336335155	sperm	q1d
0.288092327	sperm	q1d
0.411959047	sperm	q1d
0.594499476	homo	q1d
0.271142145	homo	q1d
0.415870628	homo	q1d
0.55929525	homo	q1d
0.478455918	homo	q1d
0.554079809	homo	q1d
0.265926704	homo	q1d
0.483671358	homo	q1d
0.487582939	homo	q1d
0.580157014	homo	q1d
0.581460874	homo	q1d
0.541041207	homo	q1d
0.413262907	homo	q1d
0.530610326	homo	q1d
0.314169532	homo	q1d
0.316777252	homo	q1d
0.372843241	homo	q1d
0.554079809	homo	q1d
0.454986434	homo	q1d
0.477152057	homo	q1d
0.461505735	homo	q1d
0.385881843	homo	q1d
0.314169532	homo	q1d
0.298523209	homo	q1d
0.067739953	homo	q1d
0.418478348	homo	q1d
0.452378713	homo	q1d
0.310257951	homo	q1d
0.301130929	homo	q1d
0.358500779	homo	q1d
-0.052391843	control-	q1d
0.007803625	control-	q1d
0.017899075	control-	q1d

0.026689143	control-	q1d
-0.122775046	sperm	q1w
-0.475067893	sperm	q1w
-0.736119427	sperm	q1w
-0.231757725	sperm	q1w
-0.307792153	sperm	q1w
-0.705705656	sperm	q1w
-0.206412916	sperm	q1w
-0.18867155	sperm	q1w
-0.315395595	sperm	q1w
-0.401567946	sperm	q1w
-0.538429916	sperm	q1w
-0.649947076	sperm	q1w
-0.469998931	sperm	q1w
-0.122775046	sperm	q1w
0.014086924	sperm	q1w
-0.63220571	sperm	q1w
-0.072085427	sperm	q1w
-0.376223137	sperm	q1w
-0.335671443	sperm	q1w
0.03182829	sperm	q1w
-0.120240565	sperm	q1w
-0.414240351	sperm	q1w
-0.307792153	sperm	q1w
-0.16332674	sperm	q1w
-0.231757725	sperm	q1w
-0.693033252	sperm	q1w
-0.214016359	sperm	q1w
-0.82736074	sperm	q1w
-0.302723191	homo	q1w
-0.533360954	homo	q1w
-0.419309313	homo	q1w
-0.196274992	homo	q1w
-0.297654229	homo	q1w
-0.462395488	homo	q1w
-0.02393029	homo	q1w
-0.269774939	homo	q1w
-0.173464664	homo	q1w
-0.368619695	homo	q1w
-0.312861114	homo	q1w
-0.049275099	homo	q1w
-0.791878008	homo	q1w
-0.310326634	homo	q1w
-0.267240458	homo	q1w
-0.363550733	homo	q1w
-0.672757404	homo	q1w
-0.267240458	homo	q1w
-0.018861328	homo	q1w
-0.406636908	homo	q1w
-0.198809473	homo	q1w
-0.330602481	homo	q1w
-0.016326847	homo	q1w
0.039431733	homo	q1w
-0.026464771	homo	q1w
-0.092361275	homo	q1w
-0.525757511	homo	q1w

-0.089826794	homo	q1w
-0.46746445	homo	q1w
0.041341495	control-	q1w
-0.038241206	control-	q1w
-0.025568802	control-	q1w
-0.045303257	control-	q1w
0.013201583	control-	q1w
0.023013496	control-	q1w
0.03155669	control-	q1w
0.36060628	sperm	q2w
0.560562134	sperm	q2w
-0.136958288	sperm	q2w
0.869796188	sperm	q2w
0.035096749	sperm	q2w
0.049047158	sperm	q2w
0.093223451	sperm	q2w
-0.01372968	sperm	q2w
-0.506644112	sperm	q2w
-0.857729391	sperm	q2w
-0.615922312	sperm	q2w
0.26295342	sperm	q2w
0.009521	sperm	q2w
0.342005735	sperm	q2w
0.221102195	sperm	q2w
0.002545796	sperm	q2w
0.23970274	sperm	q2w
-0.399690981	sperm	q2w
-0.036980361	sperm	q2w
-0.43689207	sperm	q2w
-0.906555821	sperm	q2w
-0.44851741	sperm	q2w
-0.306688258	sperm	q2w
-0.127658016	sperm	q2w
0.13042454	sperm	q2w
0.062997566	sperm	q2w
0.142049881	sperm	q2w
0.121124268	sperm	q2w
-0.120682812	sperm	q2w
-0.36481496	sperm	q2w
-0.216010603	homo	q2w
0.207151787	homo	q2w
0.19087631	homo	q2w
0.258303284	homo	q2w
0.165300561	homo	q2w
-0.408991253	homo	q2w
0.090898383	homo	q2w
0.018821273	homo	q2w
-0.102082267	homo	q2w
-0.197410058	homo	q2w
-0.164859105	homo	q2w
0.056022362	homo	q2w
0.016496205	homo	q2w
0.346655871	homo	q2w
0.195526446	homo	q2w
0.093223451	homo	q2w
0.132749608	homo	q2w

0.755867852	homo	q2w
0.074622906	homo	q2w
0.167625629	homo	q2w
0.067647702	homo	q2w
-0.067206246	homo	q2w
-0.248561556	homo	q2w
0.137399744	homo	q2w
0.36060628	homo	q2w
-0.360164824	homo	q2w
-0.129983084	homo	q2w
-0.181134582	homo	q2w
0.541961589	homo	q2w
0.639614448	homo	q2w
0.655889925	homo	q2w
0.435008458	homo	q2w
0.342005735	homo	q2w
-0.132308152	homo	q2w
0.165300561	homo	q2w
0.332705463	homo	q2w
-0.171834309	homo	q2w
0.439658594	homo	q2w
0.093223451	homo	q2w
-0.060231042	homo	q2w
0.024140604	control-	q2w
-0.018556399	control-	q2w
-0.006931059	control-	q2w
-0.025034944	control-	q2w
0.002596357	control-	q2w
0.0376371	control-	q2w
-0.038231519	control-	q2w
0.024379858	control-	q2w
0.602836879	sperm	q4w
-0.169795578	sperm	q4w
0.14226116	sperm	q4w
0.309136421	sperm	q4w
0.41093033	sperm	q4w
0.337505215	sperm	q4w
0.280767626	sperm	q4w
0.627868169	sperm	q4w
0.087192324	sperm	q4w
-0.393408427	sperm	q4w
0.352523988	sperm	q4w
0.157279933	sperm	q4w
-0.341677096	sperm	q4w
-0.113057989	sperm	q4w
-0.189820609	sperm	q4w
0.028785982	sperm	q4w
0.195661243	sperm	q4w
0.182311222	sperm	q4w
-0.513558615	sperm	q4w
0.043804756	sperm	q4w
0.217355027	sperm	q4w
0.212348769	sperm	q4w
0.048811014	sperm	q4w
-0.745515227	sperm	q4w
0.48769295	sperm	q4w

0.073842303	sperm	q4w
-0.386733417	sperm	q4w
-0.221526909	sperm	q4w
-0.251564456	sperm	q4w
0.280767626	sperm	q4w
0.170629954	homo	q4w
0.289111389	homo	q4w
0.229036295	homo	q4w
0.113892365	homo	q4w
-0.034626617	homo	q4w
-0.021276596	homo	q4w
0.130579892	homo	q4w
0.017104714	homo	q4w
-0.073007927	homo	q4w
-0.009595327	homo	q4w
0.200667501	homo	q4w
0.167292449	homo	q4w
0.135586149	homo	q4w
-0.021276596	homo	q4w
0.053817272	homo	q4w
-0.071339174	homo	q4w
0.192323738	homo	q4w
-0.0880267	homo	q4w
0.022110972	homo	q4w
0.138923655	homo	q4w
0.18731748	homo	q4w
-0.189820609	homo	q4w
0.242386316	homo	q4w
-0.188151856	homo	q4w
-0.12640801	homo	q4w
0.11055486	homo	q4w
0.098873592	homo	q4w
0.118898623	homo	q4w
0.183979975	homo	q4w
0.112223613	homo	q4w
0.265748853	homo	q4w
0.377555277	homo	q4w
0.247392574	homo	q4w
0.21902378	homo	q4w
0.287442637	homo	q4w
0.304130163	homo	q4w
0.350855236	homo	q4w
0.280767626	homo	q4w
0.335836462	homo	q4w
0.284105131	homo	q4w
-0.019607843	control-	q4w
-0.018940342	control-	q4w
0.031122236	control-	q4w
0.007425949	control-	q4w
0	control-	q4w

Appendix S1: R code for the analyses of the data.

```
###############
#### R code ####
###############

# Useful functions:
# *****************
sidak <- function (p, n) {
  P <- 1-(1-p)^n
  return(P)
}
source("anova.2way.unbalanced.R")
source("t.perm.R")

# Data:
#******
data  <- read.table("data.txt",  h = TRUE, sep = " ")
dataf <- read.table("dataf.txt", h = TRUE, sep = " ")

# Do the negative control differ from 0?
# ************************************
control <- subset(data, tissue == "c-")
mod_cont <- lm(ABA ~ 1, data = control)
summary(mod_cont)

# I. General significance test of the model: comparisons between females
# ************************************
test <- anova.2way.unbalanced(dataf$ABA, dataf$tissue, dataf$ind, model="direct",
nperm = 9999,                                    strata=FALSE, silent=FALSE)
test

# II. Multiple comparisons by permutations:
# ************************************
# We create a list for the dataframe subsets. The subsets are combinations of levels of
our two
# factors.
listdata <- vector("list", 18)
names(listdata) <- c("homo_male", "homo_gyne", "homo_q1d", "homo_q1w",
"homo_q2w", "homo_q4w",  "sperm_male", "sperm_gyne", "sperm_q1d", "sperm_q1w",
"sperm_q2w", "sperm_q4w","control_male", "control_gyne", "control_q1d",
"control_q1w", "control_q2w", "control_q4w")

homo <- subset(data, tissue == "homo")
listdata[[1]] <- subset(homo, ind == "male")
listdata[[2]] <- subset(homo, ind == "gyne")
listdata[[3]] <- subset(homo, ind == "q1d")
listdata[[4]] <- subset(homo, ind == "q1w")
listdata[[5]] <- subset(homo, ind == "q2w")
listdata[[6]] <- subset(homo, ind == "q4w")
```

```
sperm <- subset(data, tissue == "sperm")
listdata[[7]] <- subset(sperm, ind == "male")
listdata[[8]] <- subset(sperm, ind == "gyne")
listdata[[9]] <- subset(sperm, ind == "q1d")
listdata[[10]] <- subset(sperm, ind == "q1w")
listdata[[11]] <- subset(sperm, ind == "q2w")
listdata[[12]] <- subset(sperm, ind == "q4w")
control <- subset(data, tissue == "c-")
listdata[[13]] <- subset(control, ind == "male")
listdata[[14]] <- subset(control, ind == "gyne")
listdata[[15]] <- subset(control, ind == "q1d")
listdata[[16]] <- subset(control, ind == "q1w")
listdata[[17]] <- subset(control, ind == "q2w")
listdata[[18]] <- subset(control, ind == "q4w")
# Matrix of comparisons defining the elements of listdata to be compared:
matcomp <- matrix(c(1, 13,
                    2, 14,
                    3, 15,
                    4, 16,
                    5, 17,
                    6, 18,
                    7, 13,
                    8, 14,
                    9, 15,
                    10, 16,
                    11, 17,
                    12, 18,
                    1, 7,
                    2, 8,
                    3, 9,
                    4, 10,
                    5, 11,
                    6, 12,
                    2, 3,
                    3, 4,
                    4, 5,
                    5, 6,
                    8, 9,
                    9, 10,
                    10, 11,
                    11, 12), ncol = 2, byrow = TRUE)
row.names(matcomp) <- c("homo_male-control", "homo_gyne-control", "homo_q1d-
control",
                        "homo_q1w-control", "homo-q2w_control", "homo q4w_control",
                        "sperm male_control", "sperm gyne_control", "sperm q1d_control",
                        "sperm q1w_control", "sperm q2w_control", "sperm q4w_control",
                        "homo_male - sperm_male", "homo_gyne - sperm_gyne",
                        "homo_q1d - sperm_q1d", "homo_q1w - sperm_q1w",
                        "homo_q2w - sperm_q2w", "homo_q4w - sperm_q4w",
                        "homo_gyne - homo_q1d", "homo_q1d - homo_q1w",
```

```
                "homo_q1w - homo_q2w", "homo_q2w - homoq4w",
                "sperm_gyne - sperm_q1d", "sperm_q1d - sperm_q1w",
                "sperm_q1w - sperm_q2w", "sperm_q2w - spermq4w")

# We create a result matrix containing three columns: 'tref' for the observed t.Student

# statistic values, 'p_uncorr' for the uncorrected p-value as obtained with the
permutation
# test, and 'p_corr' for the p-value after performing a Sidak correction for multiple
# comparisons (here, nb of tests = 30). The latter prevents an inflation of the type I error

# rate and is therefore necessary.
matresults <- matrix(ncol = 3, nrow = nrow(matcomp))
colnames(matresults) <- c("t.ref","p_uncorr", "p_corr")
row.names(matresults) <- row.names(matcomp)
for (i in 1:nrow(matcomp)) {
   t <- t.perm(listdata[[matcomp[i, 1]]]$ABA, listdata[[matcomp[i, 2]]]$ABA, nperm =
9999,
            silent = TRUE)
 matresults[i, 1] <- t$t.ref
 matresults[i, 2] <- t$p.perm
 matresults[i, 3] <- sidak(t$p.perm, nrow(matcomp))
}
write.table(matresults, file = "Results - Mult. comparisons.txt", sep = "\t")
```

Fig. S1. Antibacterial activity in spermathecae and control tissues of mated *Lasius niger* queens with corresponding egg laying rates.

The mean antibacterial activity (ABA) values (± SD) in the mated queen spermathecae (black dots) and control tissues (i.e. head-thorax homogenates; white squares) during the first 30 days after mating are given in the lower part of the figure. They reflect ABA relative to that of the negative control (i.e., free growth; see Methods). Values > 0 mean ABA was present; values = 0 mean there was no difference in activity between the sample type and the negative control; and values < 0 indicate that bacterial growth was enhanced. Statistically significant differences in ABA between consecutive time points are indicated in spermathecal contents and control tissues by capital and lower-case letters, respectively.

Mated queen egg laying rate over the first 30 days after mating is shown in the upper part of the figure. Horizontal bars represent the mean number of eggs laid per day (± SD) over the following periods of time: 0-1 day, 5-10 days, 10-20 and 20-30 days after mating. Given that mated queen egg laying rate was calculated as part of another study, these queens are not the same as the ones used for ABA measures. Sample sizes are indicated above (egg laying) or beneath (ABA) error bars.

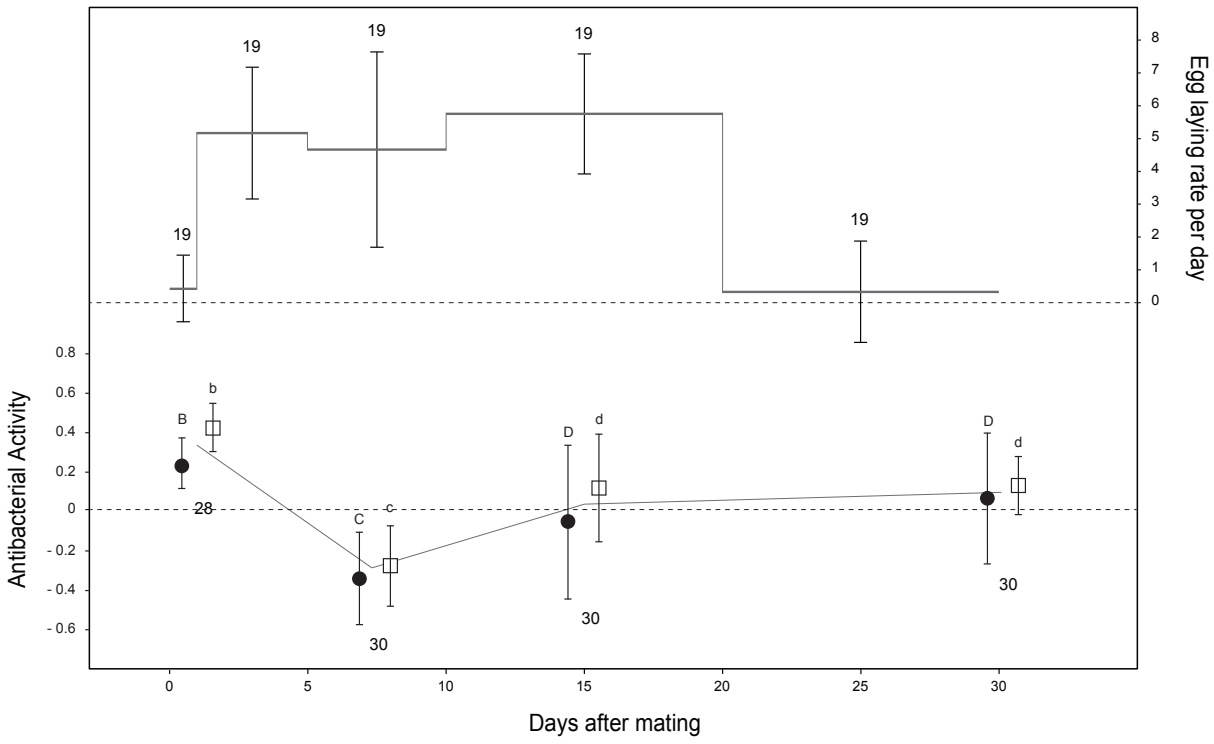

Table S1. Antibacterial activity (ABA) index of sperm-storage organ contents from males (accessory testes) and females (spermatheca of virgin queens (i.e., gynes), and of 1-day, 1-week, 2-week and 4-week mated queens), and of head-thorax homogenates (homo), in the ant *Lasius niger*.

The ABA index is calculated as ABA = 1 - (sample OD/negative control OD) (see Methods).

ABA	Tissue	Individual
-0.484793585	sperm	male
-0.071197236	sperm	male
-0.588625304	sperm	male
-0.142148911	sperm	male
-0.175028955	sperm	male
-0.342890235	sperm	male
-0.325584948	sperm	male
-0.555745259	sperm	male
-0.211370057	sperm	male
-0.124843625	sperm	male
-0.493446228	sperm	male
-0.453644069	sperm	male
-0.439799839	sperm	male
-0.114460453	sperm	male
-0.931269977	sperm	male
-0.150801554	sperm	male
-0.102346752	sperm	male
0.02398184	sperm	male
-0.277130146	sperm	male
-0.265016445	sperm	male
-0.348075188	sperm	male
-0.437137213	sperm	male
-0.639550905	sperm	male
-0.58557392	sperm	male
-0.542392333	sperm	male
-0.644948603	sperm	male
-0.107877607	sperm	male
-0.558585428	sperm	male
-0.653045151	sperm	male
-0.331882093	sperm	male
-0.561284277	sperm	male
-0.730997014	sperm	male
-0.334987893	sperm	male
-0.280986649	sperm	male
-0.242414332	sperm	male
-0.766997843	sperm	male
-0.378703185	sperm	male
-0.05726721	sperm	male
-0.622994526	sperm	male
-0.1957953	homo	male
-0.310010191	homo	male
-0.306549133	homo	male
0.151211918	homo	male
-0.79608416	homo	male
-0.751568916	homo	male
-0.345273844	homo	male

0.15268962	homo	male
0.498478626	homo	male
0.222458535	homo	male
0.236717876	homo	male
0.262180984	homo	male
0.05847612	homo	male
0.242829022	homo	male
0.539219598	homo	male
0.083939228	homo	male
-0.094302528	homo	male
0.562645658	homo	male
0.059494644	homo	male
-0.024449566	control-	male
-0.023194831	control-	male
0.011175651	control-	male
0.006032675	control-	male
0.030436071	control-	male
0.295103764	sperm	gyne
0.446152957	sperm	gyne
0.784869331	sperm	gyne
0.217290543	sperm	gyne
0.052509605	sperm	gyne
0.556006916	sperm	gyne
0.298808645	sperm	gyne
0.162363564	sperm	gyne
0.103116837	sperm	gyne
0.281372019	sperm	gyne
0.299730943	sperm	gyne
0.766560337	sperm	gyne
0.853528055	sperm	gyne
0.873210222	sperm	gyne
0.793108377	sperm	gyne
0.815079169	sperm	gyne
0.772968485	sperm	gyne
0.804551498	sperm	gyne
0.034200611	homo	gyne
0.272217522	homo	gyne
0.372916985	homo	gyne
-0.043612609	homo	gyne
-0.01614912	homo	gyne
0.272217522	homo	gyne
0.01131437	homo	gyne
0.061664101	homo	gyne
0.276794771	homo	gyne
-0.024449566	control-	gyne
-0.023194831	control-	gyne
0.011175651	control-	gyne
0.006032675	control-	gyne
0.030436071	control-	gyne
0.030049563	sperm	q1d
0.11728664	sperm	q1d
0.395008864	sperm	q1d
0.157706307	sperm	q1d
0.316777252	sperm	q1d
0.082203971	sperm	q1d
0.106855759	sperm	q1d

0.099154154	sperm	q1d
0.065132232	sperm	q1d
0.320688833	sperm	q1d
0.391097284	sperm	q1d
0.159010167	sperm	q1d
0.17987193	sperm	q1d
0.252888102	sperm	q1d
0.232026339	sperm	q1d
0.091209436	sperm	q1d
0.301130929	sperm	q1d
0.009066243	sperm	q1d
0.202037554	sperm	q1d
0.269838285	sperm	q1d
0.342854456	sperm	q1d
0.469328896	sperm	q1d
0.106855759	sperm	q1d
0.424997649	sperm	q1d
0.321992693	sperm	q1d
0.318081112	sperm	q1d
0.344158316	sperm	q1d
0.336335155	sperm	q1d
0.288092327	sperm	q1d
0.411959047	sperm	q1d
0.594499476	homo	q1d
0.271142145	homo	q1d
0.415870628	homo	q1d
0.55929525	homo	q1d
0.478455918	homo	q1d
0.554079809	homo	q1d
0.265926704	homo	q1d
0.483671358	homo	q1d
0.487582939	homo	q1d
0.580157014	homo	q1d
0.581460874	homo	q1d
0.541041207	homo	q1d
0.413262907	homo	q1d
0.530610326	homo	q1d
0.314169532	homo	q1d
0.316777252	homo	q1d
0.372843241	homo	q1d
0.554079809	homo	q1d
0.454986434	homo	q1d
0.477152057	homo	q1d
0.461505735	homo	q1d
0.385881843	homo	q1d
0.314169532	homo	q1d
0.298523209	homo	q1d
0.067739953	homo	q1d
0.418478348	homo	q1d
0.452378713	homo	q1d
0.310257951	homo	q1d
0.301130929	homo	q1d
0.358500779	homo	q1d
-0.052391843	control-	q1d
0.007803625	control-	q1d
0.017899075	control-	q1d

0.026689143	control-	q1d
-0.122775046	sperm	q1w
-0.475067893	sperm	q1w
-0.736119427	sperm	q1w
-0.231757725	sperm	q1w
-0.307792153	sperm	q1w
-0.705705656	sperm	q1w
-0.206412916	sperm	q1w
-0.18867155	sperm	q1w
-0.315395595	sperm	q1w
-0.401567946	sperm	q1w
-0.538429916	sperm	q1w
-0.649947076	sperm	q1w
-0.469998931	sperm	q1w
-0.122775046	sperm	q1w
0.014086924	sperm	q1w
-0.63220571	sperm	q1w
-0.072085427	sperm	q1w
-0.376223137	sperm	q1w
-0.335671443	sperm	q1w
0.03182829	sperm	q1w
-0.120240565	sperm	q1w
-0.414240351	sperm	q1w
-0.307792153	sperm	q1w
-0.16332674	sperm	q1w
-0.231757725	sperm	q1w
-0.693033252	sperm	q1w
-0.214016359	sperm	q1w
-0.82736074	sperm	q1w
-0.302723191	homo	q1w
-0.533360954	homo	q1w
-0.419309313	homo	q1w
-0.196274992	homo	q1w
-0.297654229	homo	q1w
-0.462395488	homo	q1w
-0.02393029	homo	q1w
-0.269774939	homo	q1w
-0.173464664	homo	q1w
-0.368619695	homo	q1w
-0.312861114	homo	q1w
-0.049275099	homo	q1w
-0.791878008	homo	q1w
-0.310326634	homo	q1w
-0.267240458	homo	q1w
-0.363550733	homo	q1w
-0.672757404	homo	q1w
-0.267240458	homo	q1w
-0.018861328	homo	q1w
-0.406636908	homo	q1w
-0.198809473	homo	q1w
-0.330602481	homo	q1w
-0.016326847	homo	q1w
0.039431733	homo	q1w
-0.026464771	homo	q1w
-0.092361275	homo	q1w
-0.525757511	homo	q1w

-0.089826794	homo	q1w
-0.46746445	homo	q1w
0.041341495	control-	q1w
-0.038241206	control-	q1w
-0.025568802	control-	q1w
-0.045303257	control-	q1w
0.013201583	control-	q1w
0.023013496	control-	q1w
0.03155669	control-	q1w
0.36060628	sperm	q2w
0.560562134	sperm	q2w
-0.136958288	sperm	q2w
0.869796188	sperm	q2w
0.035096749	sperm	q2w
0.049047158	sperm	q2w
0.093223451	sperm	q2w
-0.01372968	sperm	q2w
-0.506644112	sperm	q2w
-0.857729391	sperm	q2w
-0.615922312	sperm	q2w
0.26295342	sperm	q2w
0.009521	sperm	q2w
0.342005735	sperm	q2w
0.221102195	sperm	q2w
0.002545796	sperm	q2w
0.23970274	sperm	q2w
-0.399690981	sperm	q2w
-0.036980361	sperm	q2w
-0.43689207	sperm	q2w
-0.906555821	sperm	q2w
-0.44851741	sperm	q2w
-0.306688258	sperm	q2w
-0.127658016	sperm	q2w
0.13042454	sperm	q2w
0.062997566	sperm	q2w
0.142049881	sperm	q2w
0.121124268	sperm	q2w
-0.120682812	sperm	q2w
-0.36481496	sperm	q2w
-0.216010603	homo	q2w
0.207151787	homo	q2w
0.19087631	homo	q2w
0.258303284	homo	q2w
0.165300561	homo	q2w
-0.408991253	homo	q2w
0.090898383	homo	q2w
0.018821273	homo	q2w
-0.102082267	homo	q2w
-0.197410058	homo	q2w
-0.164859105	homo	q2w
0.056022362	homo	q2w
0.016496205	homo	q2w
0.346655871	homo	q2w
0.195526446	homo	q2w
0.093223451	homo	q2w
0.132749608	homo	q2w

0.755867852	homo	q2w
0.074622906	homo	q2w
0.167625629	homo	q2w
0.067647702	homo	q2w
-0.067206246	homo	q2w
-0.248561556	homo	q2w
0.137399744	homo	q2w
0.36060628	homo	q2w
-0.360164824	homo	q2w
-0.129983084	homo	q2w
-0.181134582	homo	q2w
0.541961589	homo	q2w
0.639614448	homo	q2w
0.655889925	homo	q2w
0.435008458	homo	q2w
0.342005735	homo	q2w
-0.132308152	homo	q2w
0.165300561	homo	q2w
0.332705463	homo	q2w
-0.171834309	homo	q2w
0.439658594	homo	q2w
0.093223451	homo	q2w
-0.060231042	homo	q2w
0.024140604	control-	q2w
-0.018556399	control-	q2w
-0.006931059	control-	q2w
-0.025034944	control-	q2w
0.002596357	control-	q2w
0.0376371	control-	q2w
-0.038231519	control-	q2w
0.024379858	control-	q2w
0.602836879	sperm	q4w
-0.169795578	sperm	q4w
0.14226116	sperm	q4w
0.309136421	sperm	q4w
0.41093033	sperm	q4w
0.337505215	sperm	q4w
0.280767626	sperm	q4w
0.627868169	sperm	q4w
0.087192324	sperm	q4w
-0.393408427	sperm	q4w
0.352523988	sperm	q4w
0.157279933	sperm	q4w
-0.341677096	sperm	q4w
-0.113057989	sperm	q4w
-0.189820609	sperm	q4w
0.028785982	sperm	q4w
0.195661243	sperm	q4w
0.182311222	sperm	q4w
-0.513558615	sperm	q4w
0.043804756	sperm	q4w
0.217355027	sperm	q4w
0.212348769	sperm	q4w
0.048811014	sperm	q4w
-0.745515227	sperm	q4w
0.48769295	sperm	q4w

0.073842303	sperm	q4w
-0.386733417	sperm	q4w
-0.221526909	sperm	q4w
-0.251564456	sperm	q4w
0.280767626	sperm	q4w
0.170629954	homo	q4w
0.289111389	homo	q4w
0.229036295	homo	q4w
0.113892365	homo	q4w
-0.034626617	homo	q4w
-0.021276596	homo	q4w
0.130579892	homo	q4w
0.017104714	homo	q4w
-0.073007927	homo	q4w
-0.009595327	homo	q4w
0.200667501	homo	q4w
0.167292449	homo	q4w
0.135586149	homo	q4w
-0.021276596	homo	q4w
0.053817272	homo	q4w
-0.071339174	homo	q4w
0.192323738	homo	q4w
-0.0880267	homo	q4w
0.022110972	homo	q4w
0.138923655	homo	q4w
0.18731748	homo	q4w
-0.189820609	homo	q4w
0.242386316	homo	q4w
-0.188151856	homo	q4w
-0.12640801	homo	q4w
0.11055486	homo	q4w
0.098873592	homo	q4w
0.118898623	homo	q4w
0.183979975	homo	q4w
0.112223613	homo	q4w
0.265748853	homo	q4w
0.377555277	homo	q4w
0.247392574	homo	q4w
0.21902378	homo	q4w
0.287442637	homo	q4w
0.304130163	homo	q4w
0.350855236	homo	q4w
0.280767626	homo	q4w
0.335836462	homo	q4w
0.284105131	homo	q4w
-0.019607843	control-	q4w
-0.018940342	control-	q4w
0.031122236	control-	q4w
0.007425949	control-	q4w
0	control-	q4w

Script 1. R code for the analyses of the data.

```
###############
#### R code ####
###############

# Useful functions:
# *****************
sidak <- function (p, n) {
  P <- 1-(1-p)^n
  return(P)
}
source("anova.2way.unbalanced.R")
source("t.perm.R")

# Data:
#******
data  <- read.table("data.txt",  h = TRUE, sep = " ")
dataf <- read.table("dataf.txt", h = TRUE, sep = " ")

# Do the negative control differ from 0?
# ************************************
control <- subset(data, tissue == "c-")
mod_cont <- lm(ABA ~ 1, data = control)
summary(mod_cont)

# I. General significance test of the model: comparisons between females
# ************************************
test <- anova.2way.unbalanced(dataf$ABA, dataf$tissue, dataf$ind, model="direct",
nperm = 9999,                                    strata=FALSE, silent=FALSE)
test

# II. Multiple comparisons by permutations:
# ************************************
# We create a list for the dataframe subsets. The subsets are combinations of levels of
our two
# factors.
listdata <- vector("list", 18)
names(listdata) <- c("homo_male", "homo_gyne", "homo_q1d", "homo_q1w",
"homo_q2w", "homo_q4w",  "sperm_male", "sperm_gyne", "sperm_q1d", "sperm_q1w",
"sperm_q2w", "sperm_q4w","control_male", "control_gyne", "control_q1d",
"control_q1w", "control_q2w", "control_q4w")

homo <- subset(data, tissue == "homo")
listdata[[1]] <- subset(homo, ind == "male")
listdata[[2]] <- subset(homo, ind == "gyne")
listdata[[3]] <- subset(homo, ind == "q1d")
listdata[[4]] <- subset(homo, ind == "q1w")
listdata[[5]] <- subset(homo, ind == "q2w")
listdata[[6]] <- subset(homo, ind == "q4w")
```

```
sperm <- subset(data, tissue == "sperm")
listdata[[7]] <- subset(sperm, ind == "male")
listdata[[8]] <- subset(sperm, ind == "gyne")
listdata[[9]] <- subset(sperm, ind == "q1d")
listdata[[10]] <- subset(sperm, ind == "q1w")
listdata[[11]] <- subset(sperm, ind == "q2w")
listdata[[12]] <- subset(sperm, ind == "q4w")
control <- subset(data, tissue == "c-")
listdata[[13]] <- subset(control, ind == "male")
listdata[[14]] <- subset(control, ind == "gyne")
listdata[[15]] <- subset(control, ind == "q1d")
listdata[[16]] <- subset(control, ind == "q1w")
listdata[[17]] <- subset(control, ind == "q2w")
listdata[[18]] <- subset(control, ind == "q4w")
# Matrix of comparisons defining the elements of listdata to be compared:
matcomp <- matrix(c(1, 13,
                    2, 14,
                    3, 15,
                    4, 16,
                    5, 17,
                    6, 18,
                    7, 13,
                    8, 14,
                    9, 15,
                    10, 16,
                    11, 17,
                    12, 18,
                    1, 7,
                    2, 8,
                    3, 9,
                    4, 10,
                    5, 11,
                    6, 12,
                    2, 3,
                    3, 4,
                    4, 5,
                    5, 6,
                    8, 9,
                    9, 10,
                    10, 11,
                    11, 12), ncol = 2, byrow = TRUE)
row.names(matcomp) <- c("homo_male-control", "homo_gyne-control", "homo_q1d-
control",
                        "homo_q1w-control", "homo-q2w_control", "homo q4w_control",
                        "sperm male_control", "sperm gyne_control", "sperm q1d_control",
                        "sperm q1w_control", "sperm q2w_control", "sperm q4w_control",
                        "homo_male - sperm_male", "homo_gyne - sperm_gyne",
                        "homo_q1d - sperm_q1d", "homo_q1w - sperm_q1w",
                        "homo_q2w - sperm_q2w", "homo_q4w - sperm_q4w",
                        "homo_gyne - homo_q1d", "homo_q1d - homo_q1w",
```

```
                "homo_q1w - homo_q2w", "homo_q2w - homoq4w",
                "sperm_gyne - sperm_q1d", "sperm_q1d - sperm_q1w",
                "sperm_q1w - sperm_q2w", "sperm_q2w - spermq4w")

# We create a result matrix containing three columns: 'tref' for the observed t.Student

# statistic values, 'p_uncorr' for the uncorrected p-value as obtained with the
permutation
# test, and 'p_corr' for the p-value after performing a Sidak correction for multiple
# comparisons (here, nb of tests = 30). The latter prevents an inflation of the type I error

# rate and is therefore necessary.
matresults <- matrix(ncol = 3, nrow = nrow(matcomp))
colnames(matresults) <- c("t.ref","p_uncorr", "p_corr")
row.names(matresults) <- row.names(matcomp)
for (i in 1:nrow(matcomp)) {
   t <- t.perm(listdata[[matcomp[i, 1]]]$ABA, listdata[[matcomp[i, 2]]]$ABA, nperm =
9999,
            silent = TRUE)
 matresults[i, 1] <- t$t.ref
 matresults[i, 2] <- t$p.perm
 matresults[i, 3] <- sidak(t$p.perm, nrow(matcomp))
}
write.table(matresults, file = "Results - Mult. comparisons.txt", sep = "\t")
```