Title

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Journal

Journal of Experimental Biology, 221(6)

ISSN

0022-0949

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Publication Date

2018-03-15

DOI

10.1242/jeb.173435

Peer reviewed

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RESEARCH ARTICLE

Differential immune gene expression in sperm storage organs of leaf-cutting ants

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ABSTRACT

Leaf-cutting ant queens mate with multiple males during a single nuptial flight and store sperm for up to two decades. During mating, males transfer sperm from their accessory testes to the queen bursa copulatrix from where it enters the spermatheca, an insect sperm storage organ that has become highly specialized in long-lived ant queens who never re-mate later in life. Long-term storage without the possibility to obtain new sperm creates an immune defence dilemma, because recognition of non-self cells eliminates infections but may also target irreplaceable sperm and reduce lifetime reproductive success. We therefore hypothesized that non-specific immune responses, like pathogen melanization, should be silenced in the spermatheca, because they rely on general non-self recognition, and that specific responses such as antimicrobial peptides are activated instead as they specifically target pathogenic bacteria and/or fungi. The maintenance of uninfected sperm cells by males before mating is not constrained by non-self recognition, meaning immune regulation might be more liberal in male reproductive organs. To test this hypothesis, we measured gene expression of two antimicrobial peptides, abaecin and defensin, and prophenoloxidase, an important enzyme of the melanization pathway, in male accessory glands and testes and in queen bursae copulatrix and spermathecae of *Acromyrmex echinatior* and *Atta colombica* leaf-cutting ants. As expected, prophenoloxidase expression was low in reproductive organs that sustain prolonged contact with sperm, whereas antimicrobial peptides showed average to high expression, indicating that leaf-cutting ants invest in specific rather than generalist immune defences for pathogen protection in organs that store sperm.

KEY WORDS: Antimicrobial peptides, Abaecin, Defensin, Melanization, Prophenoloxidase

INTRODUCTION

The journey that sperm cells undertake to fertilize eggs within the female sexual tract is full of pitfalls. After copulation, females may be exposed to sexually transmitted pathogens, resulting in the expression of immune responses even though these may also potentially harm non-self sperm. Realizing this general dilemma, Birkhead et al. (1993) suggested that female immune responses could have become co-opted for cryptic female choice of the most preferred sperm, consistent with later empirical findings that immune responses of promiscuous female insects indeed reduce sperm viability (Eady, 1994; Bernasconi et al., 2002; Demary, 2005).

The perennial social Hymenoptera are exceptional in that breeding females (queens) mate only during a single mating episode early in adult life and store the acquired sperm in a specialized organ, the spermatheca, without ever re-mating once they have started laying eggs (Boomsma et al., 2005a; Boomsma, 2016). This implies that selection pressure for maximizing sperm survival and prudent sperm use can be very strong (Cole, 1983; den Boer et al., 2009a; Baer et al., 2016), particularly in ants whose queens can live for up to two decades (Keller, 1998; Kramer et al., 2016). Newly fertilized queens of many ants found their colonies alone, and their families will not reach an adequate size for producing males and virgin queens until several years later (Oster and Wilson, 1978). The continued availability of viable stored sperm beyond the founding and ergonomic stages of colony growth, before a colony can make any attempt to reproduce, is therefore crucial for both male and female mating partners (Baer and Boomsma, 2004; Boomsma et al., 2005a). Long-term sperm storage thus aligns the reproductive interests of stored ejaculates in not compromising their mate's survival and future reproductive efficiency, which gives queens considerable power to control sperm competition and maximize survival of all stored sperm (den Boer et al., 2010; Boomsma, 2013, 2017). In line with this, both male seminal fluid and female spermathecal fluid have been shown to efficiently keep sperm viable in honeybees (den Boer et al., 2009b) and *Atta* leaf-cutting ants (den Boer et al., 2008, 2010).

The expression of queen immune defences during colony founding represents a significant cost emanating from the maintenance of massive sperm stores without the assistance of workers (Baer et al., 2006; Castella et al., 2009). However, these studies investigated general subcuticular encapsulation immune reactions, but did not consider immune defences present in the organs that store sperm. Although specialized sexually transmitted diseases are unlikely to be able to maintain themselves in social insect populations because of strict sexual partner commitment and complete lack of re-mating promiscuity (Knell and Webberley, 2004; Boomsma et al., 2005b), honeybee male reproductive organs have been found to harbour fungal spores and viruses that can be sexually transmitted to queens (Fievet et al., 2006; Yue et al., 2006; de Miranda and Fries, 2008; Peng et al., 2015; Roberts et al., 2015). Unless they are effectively controlled, such infections may imply health risks for queens or damage to transferred sperm, so targeted immune defences should be in place. Such specific anti-microbial defences are present in honeybee male seminal fluid and are activated after infection to effectively kill fungal spores (Baer et al., 2009b; Peng et al., 2016; Grassl et al., 2017), suggesting that males (drones) can reduce the risk of transferring pathogens to queens, but it is unknown whether this also applies in ants.

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Received 1 November 2017; Accepted 1 February 2018

Journal of Experimental Biology (2018) 221, jeb173435. doi:10.1242/jeb.173435

List of symbols and abbreviations

AMPs	antimicrobial peptides
cDNA	complementary DNA
C_t	threshold cycle
ef1β	elongation factor 1β
mRNA	messenger RNA
NTC	no template control
proPO	prophenoloxidase
rpL18	ribosomal protein L18

Insect immune systems do not rely on the same highly adaptive and specific antibody-based memory as found in vertebrates. Insect immune activation is triggered by self versus non-self recognition, and is mediated by the identification of pathogen-associated molecular patterns (Siva-Jothy et al., 2005). Following initial recognition, insects can then mount an immune reaction that can be either constitutive or induced and therefore vary in specificity and response time (Schmid-Hempel, 2005). Melanin is often involved in such immune reactions and can be deposited either directly around small invading non-self bodies such as bacteria and fungal spores, or around nodules or capsules of immune effector cells that have captured clusters of micro-parasites or single larger macro-parasites (Sugumaran, 2002; Cerenius and Söderhäll, 2004). Melanin synthesis is activated through a complex enzymatic cascade that involves serine proteinases and phenoloxidase enzymatic activity, and releases cytotoxic by-products such as reactive intermediates of oxygen and nitrogen that can participate in eliminating pathogens (Nappi and Christensen, 2005; Cerenius et al., 2008). Through pathogen sequestration and cytotoxic activity, melanization thus serves as an unspecific defence. Melanization in female reproductive organs can be triggered by mating in both non-social and social insects. In *Drosophila*, copulation sometimes induces the formation of a dark reaction mass in the bursa copulatrix (also called the uterus) (Patterson, 1946) that seems to be related to melanogenesis (Asada and Kitagawa, 1988a,b; Asada and Fukumitsu, 1990), and the spermathecae of bumblebee queens have been observed to become melanized after mating (Greeff and Schmid-Hempel, 2008).

In addition to defences based on self/non-self recognition, invertebrates are also capable of pathogen-specific immune defences, which mainly encompass antimicrobial peptides (AMPs) that are synthesized in the fat body, hemocytes or epithelial cells (Zasloff, 2002). They are classified according to their structure and to the prevalence of specific amino acids. In insects, the main families identified are α-helical peptides (e.g. cecropins), cysteine-rich peptides (e.g. defensins), proline-rich peptides (e.g. drosocin, pyrrhocoricin, apidaecin, formaecin, abaecin, lebocin) and glycine-rich peptides (e.g. coleoptericin, attacin, hemiptericin, hymenoptaecin, gloverin) (Bulet et al., 1999; Otvos, 2000; Bulet and Stocklin, 2005; Yi et al., 2014; Mylonakis et al., 2016). Exploiting the availability of seven published ant genomes, including the leaf-cutting ants *Acomyrmex echinatior* and *Atta cephalotes*, Zhang and Zhu (2012) identified five ant AMP families: abaecin, hymenoptaecins, crustins, defensins and ICK-type. However, compared with the solitary wasp *Nasonia vitripennis*, the number of AMPs in ants seems to be relatively low (Gupta et al., 2015). AMPs are effective against a variety of bacteria and fungi (Bulet et al., 1999; Otvos, 2000; Bulet and Stocklin, 2005; Uvell and Engström, 2007) and their production is mediated by gene expression activation (Gillespie et al., 1997; Lemaitre and Hoffmann, 2007). Although AMP synthesis is usually induced after pathogen recognition, they have also been found to be constitutively expressed in the oviduct and sperm storage organs of *Drosophila* females (Charlet et al., 1996; Ferrandon et al., 1998; Tzou et al., 2000) and in the female accessory glands of the medfly *Ceratitis capitata* (Marchini et al., 1991, 1993, 1995; Rosetto et al., 1996), suggesting the need for rapid availability in the female reproductive tract.

We hypothesized that long-term queen sperm storage organs should have been under selection to (1) minimize the expression of non-specific immune responses as they might damage sperm cells either directly after non-self recognition and/or indirectly via cytotoxic by-products, and (2) preferentially express immune responses that specifically target sexually transmitted bacterial and fungal cells that may be introduced with ejaculates. In male reproductive organs, sperm cells are recognized as self and (1) selection for specific immune responses should not operate, but (2) sperm should still be shielded from the cytotoxic by-products produced by certain immune responses as these could compromise sperm quality. We tested these hypotheses in the polyandrous leaf-cutting ants *Acromyrmex echinatior* Forel 1899 and *Atta colombica* (Guérin-Méneville 1844) (Villesen et al., 2002) by measuring gene expression of two antimicrobial peptides, abaecin and defensin, and of prophenoloxidase (proPO), the inactive precursor of phenoloxidase, in reproductive organs involved in sperm storage in virgin queens, males and artificially inseminated queens. We selected abaecin and defensin as candidate AMPs because *A. echinatior* workers upregulate abaecin and downregulate defensin expression after a fungal immune challenge (Yek et al., 2013), implying that these two AMPs differ in their modes and/or ranges of action. Abaecin and defensin also belong to two different AMP families (Zhang and Zhu, 2012), which might somehow influence gene expression. A third peptide of interest was hymenoptaecin, which was also identified as being upregulated after the same fungal immune challenge (Yek et al., 2013). Unfortunately, we were unable to develop adequate primers for this gene so we had to exclude this AMP from the present study (see 'Primer design' section in Materials and methods). Abaecin was first characterized in the honeybee *Apis mellifera* and is active against gram-positive and gram-negative bacteria (Casteels et al., 1990) and probably also against fungi (Yek et al., 2013). Defensin has been identified in many insects and seems to be active only against bacteria (Reddy et al., 2004; Viljakainen and Pamilo, 2005; Yek et al., 2013). Prophenoloxidase has been studied in a variety of social insects, usually indirectly by measuring encapsulation responses of experimental nylon inserts (Armitage et al., 2003; Vainio et al., 2004; Zuk et al., 2004; Baer et al., 2005, 2006; Baer and Schmid-Hempel, 2006).

The virgin queen organs that we tested were the bursa copulatrix, a receptacle through which ejaculates transit before reaching final storage, and the spermatheca, where sperm is stored for up to several decades after mating. Male organs were the accessory testes, where sperm is stored before mating (i.e. a few weeks), and the accessory glands, through which the sperm passes and mixes with seminal fluid upon ejaculation. For queens of *Atta*, we also investigated immune gene expression in the spermatheca after artificial insemination. To test whether *Atta* queens can adjust the intensity or composition of their immune response depending on the male product that enters the spermatheca, we artificially inseminated queens with entire ejaculates (sperm mixed with seminal fluid) or ejaculate components (sperm or seminal fluid separately). The process of queen sperm storage during and shortly after mating differs between the two leaf-cutting ant species because sperm is

directly mass-transferred to the spermatheca in *Atta* (Baer and Boomsma, 2006), whereas sperm spends several hours in the bursa copulatrix before individually migrating to the spermatheca for final storage in *Acromyrmex* (Reichardt and Wheeler, 1996; Liberti et al., 2016). The bursa of *Acromyrmex* queens might thus play a major role in sperm protection against sexually transmitted pathogens while this is unlikely to be the case in *Atta*. We used quantitative polymerase chain reaction (PCR) to measure gene expression of the three focal immune genes and compared levels of immune gene expression in reproductive organs with levels in flight muscles where gene expression in response to pathogen exposure should not be constrained by possible immune damage to sperm.

MATERIALS AND METHODS

Sample collection, dissection and artificial insemination

Virgin queens and males of *Acromyrmex echinatior* originated from five laboratory colonies (Ae168, Ae342, Ae406, Ae420A and Ae451) that were collected in Gamboa, Panama, in May 2002, 2007, 2009 and 2010 and kept in climate rooms at approximately 25°C and 70% relative humidity in Copenhagen. *Atta colombica* virgin queens and males were collected from five colonies at the same field site in May 2013 and 2014. *Acromyrmex echinatior* and *A. colombica* virgin queens were dissected to obtain spermathecae and bursae copulatrix, and males were dissected to obtain accessory testes and accessory glands. Flight muscle samples were dissected for both sexes and species. *Acromyrmex echinatior* tissues were stored at −80°C prior to further use, but *A. colombica* had to be dissected under field conditions, so tissues were stored in RNAlater (Qiagen, Venlo, The Netherlands) at −20°C.

For the artificial inseminations, we collected additional *A. colombica* virgin queens and males from the same source colonies and during the same sampling campaigns described above. Virgin queens were anaesthetized with CO_2 and artificially inseminated with either Hayes saline (9.0 g l^{-1} NaCl, 0.2 g l^{-1} $CaCl_2$, 0.2 g l^{-1} KCl, 0.1 g l^{-1} $NaHCO_3$), seminal fluid, sperm, or a complete male ejaculate (seminal fluid mixed with sperm) following established techniques (den Boer et al., 2013). Sperm was collected directly from dissected male accessory testes and seminal fluid was collected as pre-ejaculatory fluid by gently squeezing male gasters (terminal part of the abdomen) between two fingers. Complete ejaculates were collected using the same procedure but here we allowed males to fully ejaculate. Artificially inseminated queens were allowed to recover and were kept in Petri dishes with moist tissue paper for 12 h after which their spermathecae and flight muscles were dissected and stored in RNAlater (Qiagen) at −20°C. All dissections were carried out under a Leica stereomicroscope. Flight muscle samples were used as control tissue that we expected to show normal immune gene expression, not constrained by possible trade-offs with sperm preservation. In queens, these muscles are histolysed after insemination so their ephemeral existence suggests that selection for expressing high levels of immune defence in this tissue is unlikely. Using these muscles as reference organs therefore implicitly (and we feel reasonably) assumes that they are exposed to neither extremely high nor very low pathogen pressure. However, we have no formal proof for this contention, so it is important to bear in mind that the gene expression results we present are relative measures.

RNA isolation and reverse transcription

There was considerable size variation between the different organs investigated depending on the species and the organ in question. To start out with similar amounts of tissue for RNA isolation, we pooled organs of a varying number of individuals so that gene expression comparisons between organs would be as comparable as possible (Pfaffl, 2006). Each biological replicate thus consisted of the focal organs of several individuals (detailed below) from the same colony, which we assumed to be comparable (i.e. to have limited variation) because same colony individuals are always same-cohort siblings raised under identical environmental conditions. We always collected individuals from five different colonies, ending up with five replicate samples per organ of interest. Following this procedure, every *A. echinatior* replicate consisted of either 30 individuals (spermatheca), 10 individuals (bursae copulatrix, queen flight muscles, accessory glands and male flight muscles) or three individuals (accessory testes). For the larger *A. colombica* individuals, it was sufficient to pool three individuals for spermatheca, queen flight muscles, accessory glands and male flight muscles, four for bursa copulatrix, and two for accessory testes measurements. For the spermatheca and flight muscle samples in the artificial insemination treatment of *A. colombica* queens, we pooled three individuals (Hayes insemination and seminal fluid insemination) and used a single individual per replicate for the sperm-only and full ejaculate inseminations. We were unable to obtain bursa copulatrix samples of artificially inseminated *Atta* queens, but these are merely bypassed by ejaculates on their way to the spermatheca (Baer and Boomsma, 2006) so that gene expression patterns in the bursa are not expected to be affected by insemination.

For each replicate sample we isolated total RNA. Tissues were first homogenized in RLT lysis buffer (included in the RNeasy Plant Mini Kit; Qiagen) with 1% β-mercaptoethanol and 0.5 mm glass beads using a FastPrep instrument (Thermo Electron, Waltham, MA, USA). Samples were then further homogenized using Qiashredder Mini Spin columns (included in the RNeasy Plant Mini Kit; Qiagen). After homogenization, a phenol:chloroform: isoamyl alcohol (25:24:1), pH 8, extraction step with phase lock gel tubes (5PRIME, Hilden, Germany) was carried out to remove contaminating proteins with maximal possible efficiency. Samples were then purified with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol, starting from the step where the extract is loaded on an RNeasy Mini Spin column. To remove contaminating genomic DNA, extracted total RNA samples were treated with Dnase I from the RNeasy Plant Mini Kit. The amount and quality of total RNA in each sample was measured with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and RNA integrity was verified with electrophoresis on a 2% agarose gel.

Reverse transcription of mRNA to cDNA was carried out on 86 ng of total RNA for each organ replicate with Superscript III Reverse Transcriptase (Thermo Fisher Scientific), RNaseOUT Recombinant Ribonuclease Inhibitor (Thermo Fisher Scientific), and 3′ RACE anchored oligo(dT) primer (see Table S1 for primer sequence). In a final step, cDNA products were diluted ten times in AE buffer (Qiagen) and stored at −20°C until further use.

Primer design

For each species, primers were designed for two stably expressed (confirmed on our samples using geNorm; Vandesompele et al., 2002) housekeeping genes, *ribosomal protein L18* (rpL18) and *elongation factor 1β* (ef1β) (Cheng et al., 2013; Moreira et al., 2017), and for the three genes of interest, *abaecin*, *defensin* and *proPO* (see Table S1 for primer sequences). Primers were designed by BLAST (Altschul et al., 1990; tBLASTn) searching protein sequences known from other Hymenoptera against published genomes of *A. colombica* (Nygaard et al., 2016) and *A. echinatior* (Nygaard et al., 2011). All primers were positioned at the 3′ end of

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the genes and they amplified DNA fragments of approximately 150 bp with annealing temperatures of 56.5°C. The forward or reverse primer, or both, were designed to span an intron in order to avoid amplification of contaminating genomic DNA. Primer specificity was verified by PCR followed by electrophoresis on a 2% agarose gel that produced single bands of the desired length for each gene and was further checked by melt curve analysis after quantitative PCR.

As mentioned in the Introduction, we had initially planned to also study the expression of hymenoptaecin, but the gene coding for this AMP being composed of multiple repetitions of the same nucleotide sequence (Ratzka et al., 2012) precluded inclusion because the primer sets that we designed always produced multiple amplicons of varying sizes. When carrying out quantitative PCR using SYBR Green (which binds to all double-stranded DNA), this is problematic as it is impossible to distinguish between variation in fluorescence caused by differences in amplicon lengths or by actual differences in gene expression.

Immune gene expression

Quantitative PCRs were run on an Mx3000P QPCR system (Agilent, Santa Clara, CA, USA). We used 1 µl cDNA in a 20 µl reaction with 10 µl of 2× SYBR Premix Ex Taq (TaKaRa, Kusatsu, Shiga, Japan), 0.4 µl of each primer solution (10 µmol l^{-1}), 0.4 µl of 50× ROX Reference Dye II (TaKaRa) and 7.8 µl H_2O. The reaction consisted of an initial step of 95°C for 2 min, followed by 40 cycles with three steps each: 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. Each sample was run in triplicate and melt curves were run after each reaction and always produced single products. Threshold cycle (C_t) values were determined with MxPro QPCR software version 4.1 (Agilent) and the mean C_t values of the triplicate samples were used to estimate expression ratios as explained below. For each primer and plate, a 'no template control' (NTC), containing all the quantitative PCR products but water instead of cDNA, was added to ensure that amplification did not take place in the absence of cDNA template. Gene-specific standard curves were run using template dilutions of 1×, 2×, 50×, 100× and 500×, after which primer efficiencies were determined (Pfaffl, 2001). These efficiencies and mean C_t values for each sample were used to calculate expression ratios between genes of interest and housekeeping genes using the equation:

$$\text{Expression ratio} = \frac{\sqrt{[\,(E_{\text{rpL18}})^{C_t\ \text{sample}}(E_{\text{ef1}\beta})^{C_t\ \text{sample}}\,]}}{(E_{\text{target}})^{C_t\ \text{sample}}}, \quad (1)$$

where E_{rpL18} and $E_{\text{ef1}\beta}$ are the primer efficiencies of the housekeeping genes: *ribosomal protein L18* (rpL18) and *elongation factor 1β* (ef1β) and E_{target} is the primer efficiency of the target genes (*abaecin*, *defensin* or *proPO*). These primer efficiencies are all elevated to the power of the sample C_t, which is the mean C_t value of the sample triplicate for the gene in question (housekeeping genes or target genes). The numerator represents the geometric mean between $(E_{\text{rpL18}})^{C_t\ \text{sample}}$ and $(E_{\text{ef1}\beta})^{C_t\ \text{sample}}$ (Vandesompele et al., 2002). Eqn 1 was slightly modified from Pfaffl (2001) to exclude the flight muscle control samples, because we intended to show the relative differences between flight muscles and reproductive organs on our figures rather than hide them in ratios. The expression ratios in the different organs remained comparable relative to each other because flight muscles and reproductive organs were always run on the same quantitative PCR plate, which excluded noise due to variation between runs.

Statistical analyses

In order to meet assumptions of normality, sphericity and homoscedasticity, we used $\log_{10}$-transformed expression ratios for the statistical analyses. Expression ratios for *abaecin*, *defensin* and *proPO* were considered as dependent variables in separate tests. We compared gene expression between the different organs of virgin queens and males of both species by using one-way within-subjects ANOVA with organ as the between-subject factor (three levels: flight muscles, bursa copulatrix and spermatheca for virgin queens and flight muscles, accessory testes and accessory glands for males) and the different colonies as the within-subject factor.

For the analysis of artificially inseminated *A. colombica* queens, we included the spermatheca and flight muscles of the previously used virgin *A. colombica* queens as controls for immune defence expression before artificial insemination and used a two-way within-subjects ANOVA with treatment (five levels: virgin queen, Hayes insemination, seminal fluid insemination, sperm insemination and full ejaculate insemination) and organs (two levels: flight muscles and spermatheca) as between-subject factors, and colony as a within-subject factor. Because field colonies offered virgin queens in variable numbers beyond our control, we were unable to use queens from the same colonies in all treatments. This implied that replicates were always paired between spermatheca and flight muscles within the same treatment but not between treatments.

Post hoc tests consisted of multiple paired *t*-tests for virgin queens and males. For artificially inseminated queens, we used independent sample *t*-tests for comparisons between insemination treatments and paired *t*-tests for comparisons between flight muscles and spermathecae within each treatment. *Post hoc P*-values were adjusted using the Benjamini–Hochberg false discovery rate procedure to account for multiple comparisons. Statistical tests were carried out using RStudio (https://www.rstudio.com/).

RESULTS

AMP expression in reproductive organs of *A. echinatior* and *A. colombica* virgin queens and males

As shown in Fig. 1, we found a significant effect of organ type on *abaecin* ($F_{2,8}$=7.6, P=0.014) and *defensin* ($F_{2,8}$=8.67, P=0.010) expression in virgin queens of *A. echinatior*. The bursa copulatrix expressed *abaecin* more abundantly than the flight muscles, but there was no difference between flight muscles and the spermatheca, nor between the bursa copulatrix and spermatheca. However, *defensin* was downregulated in both the spermatheca and the bursa copulatrix relative to flight muscles but expression of *abaecin* did not vary between the bursa and the spermatheca. AMPs had similar expression levels across the three investigated organs in *A. echinatior* males (*abaecin*: $F_{2,8}$=1.63, P=0.25; *defensin*: $F_{2,8}$=3.66, P=0.07).

In *A. colombica* virgin queens (Fig. 1), *abaecin* and *defensin* expression differed significantly between organs (*abaecin*: $F_{2,8}$=8.5, P=0.01; *defensin*: $F_{2,8}$=170, P<0.001). *Defensin* was clearly downregulated in the bursa and even more so in the spermatheca compared with flight muscles. *Abaecin* levels showed a similar pattern as *defensin* levels in all three tissues but here significance was lost after the Benjamini–Hochberg adjustments. *Atta colombica* males also had significantly different *abaecin* and *defensin* levels between organs (*abaecin*: $F_{2,8}$=19.7, P<0.001; *defensin*: $F_{2,8}$=99.1, P<0.001). *Defensin* expression was equivalent in accessory glands and testes, but always lower than in flight muscles. *Abaecin* expression was higher in the accessory testes than in the accessory glands and flight muscles, but did not differ between the latter two.

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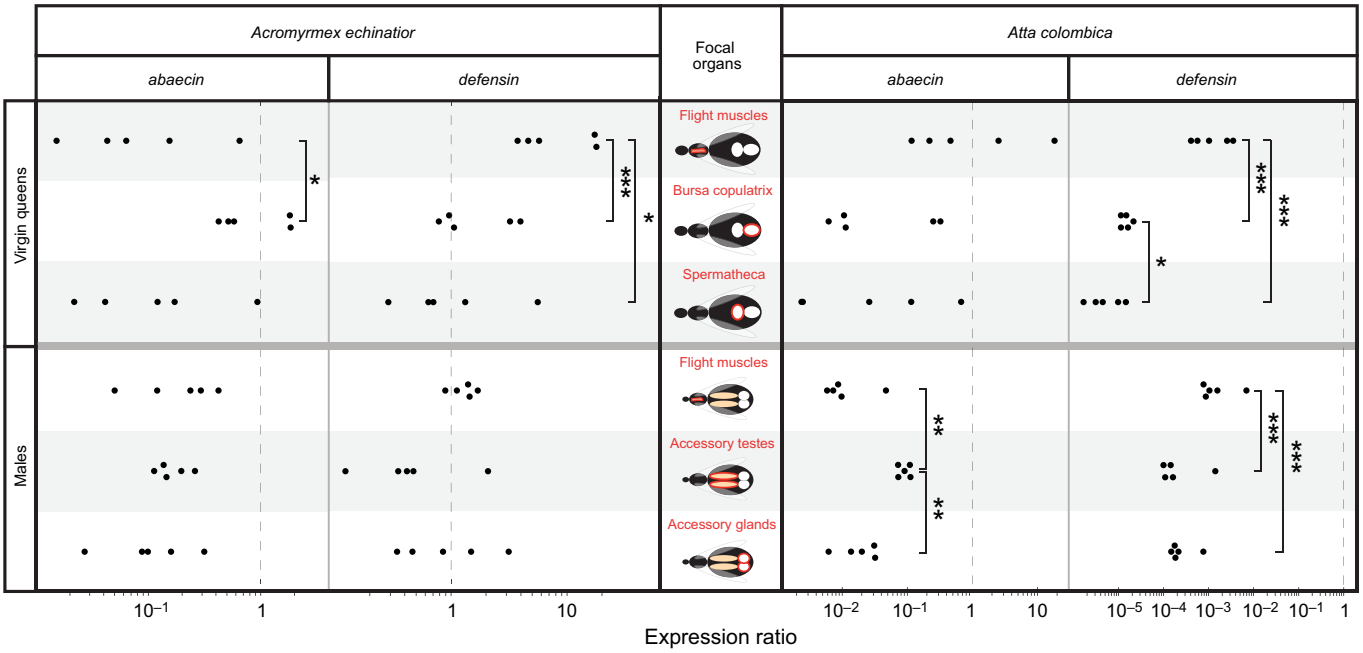

Fig. 1. Antimicrobial peptide gene expression in focal organs of virgin queens and males of *Acromyrmex echinatior and Atta colombica*. Focal organs are outlined in red in the central column. Antimicrobial gene expression is shown in the top rows for virgin queens and in the bottom rows for males. Data points are organ-specific expression ratios for genes of interest (*abaecin* and *defensin*) relative to housekeeping genes (N=5 biological replicates for each organ). The vertical dashed lines at value '1' represent no difference between expression of *abaecin* and *defensin* and housekeeping genes. Significant differences in gene expression between organs are marked with asterisks (*P<0.05; **P<0.01; ***P<0.001).

proPO expression in reproductive organs of *A. echinatior* and *A. colombica* virgin queens and males

For some spermatheca, bursa copulatrix and accessory testes replicates, we were unable to detect any *proPO* PCR product despite repeated quantitative PCR runs of the samples in question. Analyses of the melt curves showed that these samples often contained only primer dimers, similar to what the NTC controls can produce because they contain water instead of cDNA, suggesting that the focal samples contained no *proPO* cDNA. The fact that the samples in question worked fine for the other genes investigated makes erroneous reverse transcription unlikely as a general explanation for this phenomenon. This inference is reinforced by the fact that *proPO* always amplified in flight muscle samples so that the primers and PCR conditions must have been adequate throughout. These results thus suggest that the lack of *proPO* amplification reflects an actual absence of transcription of the gene in some of our replicates, consistent with an earlier study (Dávila et al., 2015), which showed that prophenoloxidase enzymatic activity was absent in the reproductive organs of the same ants collected at the same field site. As the quantitative PCRs always consisted of 40 cycles and because C_t values of 30 or above usually represent very low levels of gene expression, we decided to attribute a C_t value of 40 to the samples that failed to amplify (open circles in the figures). This allowed us to include them in the analyses by calculating their expression ratios (following Eqn 1) and thus to account for the extremely low, if not absent, *proPO* expression in these samples.

In virgin queens of both species (Fig. 2), *proPO* expression differed significantly between organs (*A. echinatior*: $F_{2,8}$=12.25, P=0.004; *A. colombica*: $F_{2,8}$=127.5, P<0.001), because *proPO* was always downregulated to the same extent in the spermatheca and the bursa copulatrix compared with flight muscles. In addition to this consistent downregulation, *proPO* amplification was only detected in two out of the five spermatheca samples for both species and in two out of the five bursa copulatrix samples in *A. colombica*. However, males of both species (Fig. 2) showed a different pattern of *proPO* regulation. Similar to virgin queens, *proPO* expression was significantly affected by the type of organ considered (*A. echinatior*: $F_{2,8}$=16.6, P=0.001; *A. colombica*: $F_{2,8}$=27.2, P<0.001), but although *proPO* remained downregulated in the accessory testes relative to flight muscles, the accessory glands expressed *proPO* at levels similar to those of flight muscles and higher than those of the accessory testes. Also here, amplification failed in three out of five accessory testes samples for *A. echinatior*.

AMP expression in spermathecae of artificially inseminated *A. colombica* queens

For artificially inseminated queens of *A. colombica* (Fig. 3), the interaction term between organ and treatment was marginally significant for both AMPs (*abaecin*: $F_{4,20}$=2.95, P=0.046; *defensin*: $F_{4,20}$=4.4, P=0.01). However, for *abaecin*, we could not detect any significant pairwise differences in the *post hoc* tests after Benjamini–Hochberg correction, so we concluded that *abaecin* expression levels were similar between the spermathecae and the flight muscles within and across virgin and artificially inseminated queens. For *defensin*, the *post hoc* tests confirmed that expression was higher in the flight muscles than in the spermatheca of virgin queens but there was no difference between organs within or across insemination treatments.

proPO expression in spermathecae of artificially inseminated *A. colombica* queens

proPO had an overall lower expression in the spermathecae than in the flight muscles regardless of the treatment ($F_{1,20}$=145.98, P<0.001; Fig. 3). However, neither the treatment ($F_{4,20}$=1.25, P=0.32) nor its interaction with the organ type ($F_{4,20}$=2.14,

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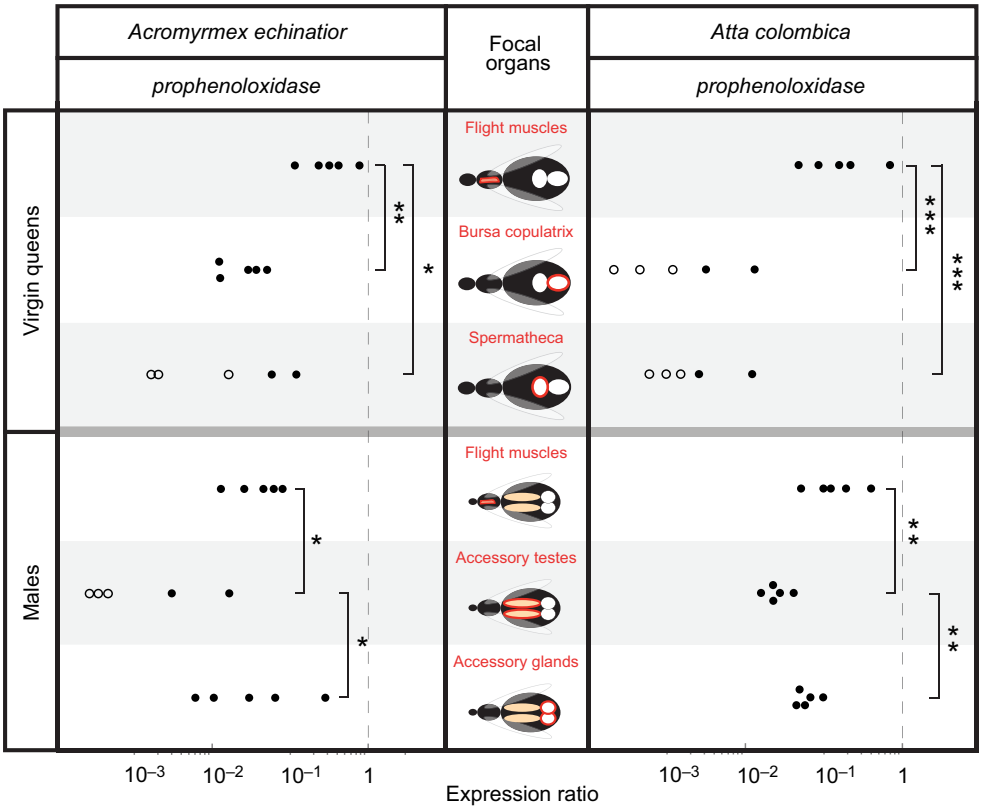

Fig. 2. *Prophenoloxidase* gene expression in focal organs of virgin queens and males of *A. echinatior* and *A. colombica*. Focal organs are outlined in red in the central column. Antimicrobial gene expression is shown in the top rows for virgin queens and in the bottom rows for males. Data points are organ-specific expression ratios for the gene of interest (*prophenoloxidase*) relative to housekeeping genes (N=5 biological replicates for each organ). The vertical dashed lines at value '1' represent no difference between expression of *prophenoloxidase* and housekeeping genes. Significant differences in *prophenoloxidase* expression between organs are marked with asterisks (*P<0.05; **P<0.01; ***P<0.001). Open circles represent samples that did not give a PCR product.

P=0.113) had a significant effect on *proPO* expression. Most artificial insemination samples gave a PCR product with the exception of one out of the five spermatheca samples inseminated with sperm and a full ejaculate.

DISCUSSION

We provide consistent evidence for our hypothesis that the expression of non-specific insect immune defences, such as the prophenoloxidase-driven melanization response, is minimized in male and queen sperm storage organs, and for AMPs, particularly abaecin, being expressed in sperm storage organs consistent with pathogen-specific immune responses being important for eliminating infections from stored sperm. We discuss these findings and their implications in the three sections below, focusing on the specific challenges of sperm maintenance and disease protection before copulation, during copulation, and after insemination. However, before proceeding we would like to highlight that a previous study in *A. echinatior* and *A. colombica* (Dávila et al., 2015) found that prophenoloxidase enzymatic activity was absent in the accessory testes of males and in the bursae copulatrix and spermathecae of queens compared with hemolymph controls. Against this background we felt it was reasonable to accept that our failures to amplify *proPO* in some reproductive organ replicates indeed represent zero scores, and also because none of our validations (repeated quantitative PCR runs of problematic samples; exclusion of faulty reverse transcription as the same samples could readily be used for amplification of the other genes; successful amplification of *proPO* in control tissues) produced any indications of this interpretation being liable. This explanation is further supported by the fact that the expression ratios of replicates that failed to amplify (open circles in figures) followed the same trend as successful replicates (filled circles in figures), and that significant differences between organs were identical in both species.

Preparation for mating

Virgin queens of *Atta* and *Acromyrmex* leaf-cutting ants spend the first weeks of their adult life inside the protected environment of their natal colony. They therefore experience low pathogen pressure (Stürup et al., 2014) during sexual maturation that ends when they leave for their nuptial flight some weeks later. Once inseminated, they shed their wings and dig a nest burrow. At this stage, queens will never re-mate, so the amount of viable sperm stored will ultimately determine their reproductive success once their colonies have survived the founding stage and several years of ergonomic colony development (Oster and Wilson, 1978; Cole, 1983; Baer et al., 2006). We found that *abaecin* was generally expressed similarly or in excess compared with flight muscles in virgin queen spermathecae and bursae copulatrix (Fig. 1), implying that this AMP is rapidly available upon insemination to eliminate sexually transmitted pathogens, which will probably increase the survival and quality of sperm. This seems comparable to previous findings of anti-fungal proteins in virgin honeybee queen spermathecal fluid (Baer et al., 2009a). Interestingly, in *Drosophila* and the medfly *C. capitata*, AMPs are constitutively expressed in the female genital tract (Marchini et al., 1991, 1993, 1995; Charlet et al., 1996; Rosetto et al., 1996; Ferrandon et al., 1998; Tzou et al., 2000), suggesting that the absence of re-mating throughout life, characteristic of all social Hymenoptera (Boomsma et al., 2005a; Boomsma, 2017), has not affected this trait in an appreciable manner.

The recurrent downregulation of *defensin* in virgin queen spermatheca and bursa compared with flight muscles (Fig. 1) indicates that there might be selection for only the most appropriate AMPs to be recruited for immune defence in reproductive organs. An earlier study in *A. echinatior* showed that defensin regulation did not respond to infection with insect pathogenic fungi, whereas abaecin did (Yek et al., 2013), suggesting that queens can mount an immune response in their sperm storage organs that is appropriate given the kind of pathogenic threats they normally experience

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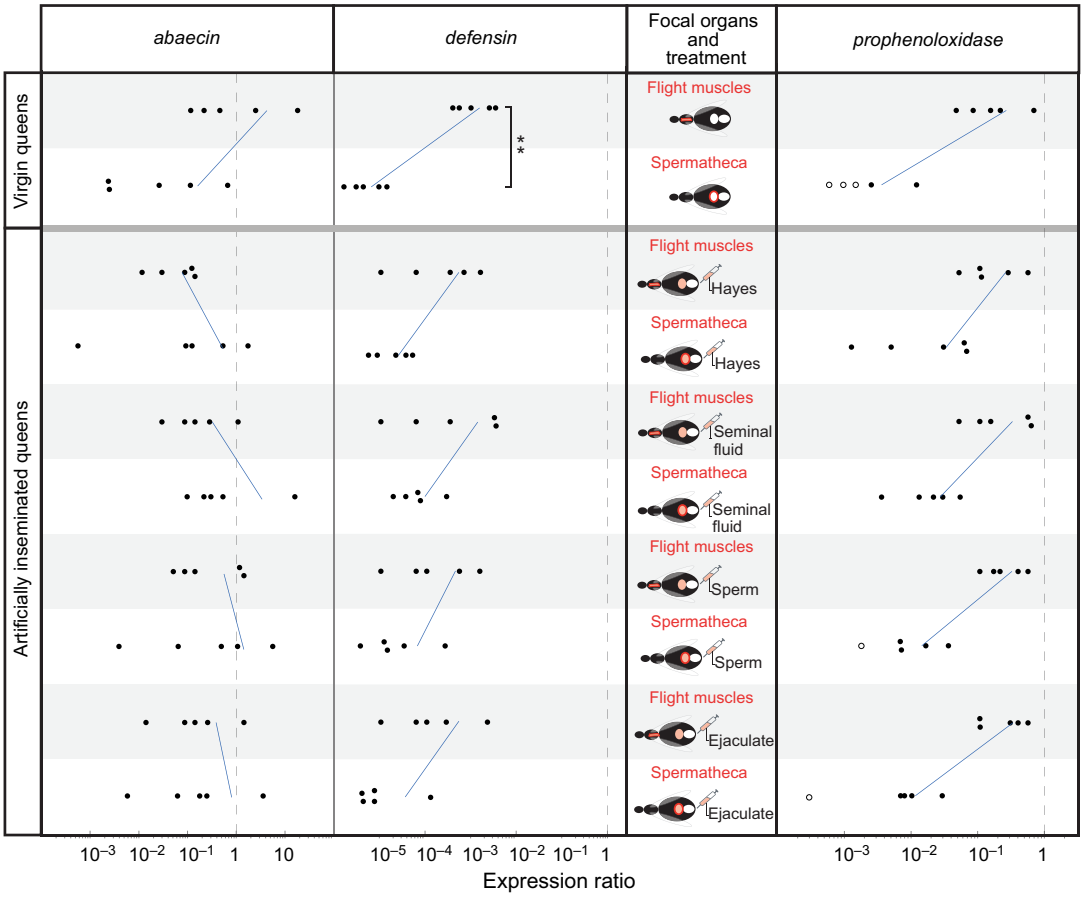

Fig. 3. Antimicrobial peptide and *prophenoloxidase* gene expression in focal organs of virgin and artificially inseminated *A. colombica* queens. Focal organs are outlined in red in the third column from right together with insemination treatments. Antimicrobial and *prophenoloxidase* gene expression is shown in the top rows for virgin queens and in the bottom rows for artificially inseminated queens. Data points are organ-specific expression ratios for genes of interest (AMPs: *abaecin* and *defensin*; and *prophenoloxidase*) relative to housekeeping genes (N=5 biological replicates for each organ in the different insemination treatments). The vertical dashed lines at value '1' represent no difference between expression of *abaecin*, *defensin* and *prophenoloxidase* and housekeeping genes. Significant differences in gene expression between organs are marked with asterisks (*P<0.05; **P<0.01; ***P<0.001). For each treatment, blue lines connect the means of expression ratios in the spermathecae and the flight muscles. Open circles represent samples that did not give a PCR product.

shortly after insemination, but that they do not express all possible AMPs. Alternatively, the anti-microbial activity of defensin might be complementary to that of abaecin, so that low levels of defensin are sufficient to allow abaecin to fully function. Such synergistic effects of abaecin, defensin and hymenoptaecin have been demonstrated in the bumblebee (Rahnamaeian et al., 2015; Marxer et al., 2016). Hymenoptaecin can create pores in bacterial membranes and the ensuing influx of abaecin into cells could then restrict proper activity of bacterial chaperones and eventually trigger bacterial cell death (Rahnamaeian et al., 2015). Defensin also seems to be a pore-former (Matsuyama and Natori, 1990; Cociancich et al., 1993; Maget-Dana and Ptak, 1997), suggesting that this AMP might also be involved in promoting abaecin influx into bacterial cytoplasm. The low expression of defensin could also indicate that its interactions with membrane integrity might harm sperm cells, something that needs to be tested in the future. Abaecin, however, interferes directly with bacterial chaperones so it is unlikely that this AMP would damage sperm, which is consistent with it being abundantly expressed. The downregulation of *proPO* and, in certain samples the complete lack of transcript detection, in virgin queen bursae copulatrix and spermathecae (Fig. 2) matched our expectation that the activation of the melanization response should have been selected against in sperm storage organs because it might damage irreplaceable sperm either by direct immune attack or indirectly via the production of cytotoxic by-products. The latter might not only damage sperm but also an individual's own tissue, which explains that arthropod melanin synthesis is normally tightly regulated in space and time. In order to cleave proPO into active PO, the melanization response passes through a cascade of serine proteinases and regulation of the reaction is often based on serine proteinase inhibitors (serpins), which can block melanization before the production of cytotoxic products (Cerenius and Söderhäll, 2004; Cerenius et al., 2008; González-Santoyo and Córdoba-Aguilar, 2012). The low or absent *proPO* expression found in our study, together with the absence of proPO enzymatic activity demonstrated by Dávila et al. (2015), suggests that incapacitation of the melanization response relies on suppressing the transcription of *proPO* mRNA instead of inhibiting serine proteinases, both in *A. echinatior* and *A. colombica* and across the bursa and spermatheca. Such a strategy might be more robust when the expected outcome is to completely shut down melanization rather than just regulate its intensity. In the case of samples showing low levels of, but not completely absent *proPO* mRNA, melanization could also be blocked via mechanisms interfering with translation.

Copulation and sperm transfer

Leaf-cutting ant queens mate with several males in quick succession (Villesen et al., 2002; Hughes et al., 2008). Each ejaculate

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transferred to the queen contains sperm from the accessory testes and seminal fluid from the accessory glands (den Boer et al., 2008; Baer, 2010). We showed that both these male organs expressed AMPs (Fig. 1), suggesting that sperm might need an immune protection while it is stored inside the male body before mating or during transfer to the queen. Male AMPs in the accessory glands and testes could also act as a nuptial gift if they are transferred to the queen during copulation. A study in *Drosophila* has shown that males transfer proteins with anti-bacterial properties to females during copulation (Lung et al., 2001), but these flies continue to mate during their adult lives. Social Hymenoptera lack this form of promiscuity (Boomsma et al., 2005a) and it is becoming increasingly evident that males of advanced social insects actively contribute to the maintenance and protection of sperm (Weirich et al., 2002; Collins et al., 2004, 2006; den Boer et al., 2008, 2009b; Baer et al., 2009b; King et al., 2011; Zareie et al., 2013; Gorshkov et al., 2015; Gotoh et al., 2017b) in fine-tuned interactions with the inseminated queens (Weirich et al., 2002; Collins et al., 2004; Baer et al., 2009a; den Boer et al., 2009b; Poland et al., 2011; Malta et al., 2014; Gotoh et al., 2017a; Paynter et al., 2017) in order to provide optimal long-term sperm storage conditions. Honeybee seminal fluid has recently been found to possess anti-microbial defences (Baer et al., 2009b; Peng et al., 2016; Grassl et al., 2017), showing that hymenopteran males can participate in protecting sperm from transmissible pathogens. In *A. colombica*, it appears that abaecin is the most predominant AMP in terms of sperm protection with or without nuptial gift elements because *abaecin* was upregulated in male genitalia, particularly in the accessory testes, compared with flight muscles, whereas *defensin* was downregulated. However, as discussed earlier, low *defensin* expression might also be due to functional synergy across AMPs and/or to AMP-specific differences in potential sperm damage when they interact with cytoplasmic membranes.

In polyandrous ant species such as *A. echinatior* and *A. colombica*, male ejaculates compete for long-term storage in the spermatheca, a process that is known to be mediated by accessory gland secretions (seminal fluid) in both species (den Boer et al., 2010). *proPO* expression in the accessory glands of *Acromyrmex* and *Atta* males was unusually high because levels were similar to those of flight muscles, and was upregulated compared with the accessory testes (Fig. 2). It seems possible, therefore, that *proPO* plays a role in the incapacitation of other males' sperm through melanization. High *proPO* expression in accessory glands could also act as an immune barrier preventing pathogens from reaching the accessory testes where sperm is stored. Grassl et al. (2017) recently showed that honeybee males can change the composition of immune proteins in their seminal fluid, including proteins involved in the proPO cascade, in response to a somatic infection with *Nosema apis*. *proPO* expression in the accessory glands of leaf-cutting ants could be driven by similar mechanisms that would explain the high *proPO* levels detected in our study. However, the lower *proPO* expression in accessory testes may be driven by the strong selective pressure for high sperm viability as sperm cell integrity would probably suffer from contact with the numerous cytotoxic by-products of the melanization reaction, but not from direct immune attack because sperm cells are recognized as self in male tissues. Dávila et al. (2015) found no proPO enzymatic activity in the accessory testes of *A. echinatior* and *A. colombica*. Similar to our inference about females, the suppression of melanization capacity in male accessory testes thus appears to be achieved by inhibiting *proPO* transcription or perhaps translation.

During and after mating, ejaculates are assumed to spend considerably more time in the bursa copulatrix of *A. echinatior* compared with *A. colombica* (Reichardt and Wheeler, 1996; Baer and Boomsma, 2006), suggesting that the bursa has no significant role in the immune protection of ejaculates in *Atta*. In *A. echinatior*, *abaecin* was upregulated in the bursa compared with flight muscles while spermathecal expression values were not enhanced, a pattern that was not observed in *A. colombica* (Fig. 1). This would be consistent with immune defences in the bursa copulatrix of *A. echinatior* queens clearing infections so none of these ever enter the spermatheca when sperm migrate to storage individually, a mechanism unlikely to be relevant in *Atta* where sperm is mass-transferred to permanent storage immediately. *Defensin* was downregulated in the bursa relative to flight muscles in both species (Fig. 1), indicating once more that this AMP is either unnecessary for immune defences in the reproductive tracts of leaf-cutting ants, or that it acts synergistically with other more abundant AMPs, or that its specific mode of action could interfere with sperm membrane integrity.

Long-term sperm storage

After mating, queens have to maintain high viability of stored sperm throughout their life, so all processes that might deplete sperm numbers or sperm quality can be expected to have been selected against once sperm has reached definitive storage in the spermatheca (Boomsma et al., 2005a). None of the different artificial insemination treatments significantly affected levels of *abaecin* and *defensin* expression in the spermatheca and the flight muscles of *A. colombica* 12 h after insemination (Fig. 3). This result suggests that AMPs are constitutively expressed in the *A. colombica* spermatheca as the strength of the immune response is unaffected by the nature of the product used for artificial insemination. The pattern of *abaecin* expression did not vary between virgin and inseminated queens, further indicating that expression seems to be constitutive and that levels present in virgin queens are sufficient to clear sexually transmitted pathogens. *Defensin* showed a slight shift in relative expression as it went from being downregulated in virgin queen spermathecae compared with flight muscles to showing similar expression levels across these organs in artificially inseminated queens. This could imply a synergistic interaction between abaecin and defensin provided insemination would allow enhanced abaecin activity by triggering only a slight increase in defensin expression.

Although *proPO* expression was not significantly different between organs and insemination treatments, *proPO* had an overall significantly lower expression in the spermathecae than in flight muscles across the different treatments (Fig. 3). However, *proPO* expression seemed to be downregulated to a lesser extent than in the spermatheca of virgin queens, which seems interesting because Dávila et al. (2015) found no proPO enzymatic activity in the spermathecae of *A. colombica* inseminated queens. Insemination might thus trigger an increase in *proPO* transcription, which could then be countered by inhibition of mRNA translation, thus explaining the absence of proPO enzymatic activity. Overall, these results suggest that ant queens, which only mate on a single occasion early in adult life, continue to supress non-self generalist immune recognition in their spermatheca after insemination; this contrasts with the apparent melanization of the bumblebee queen spermathecae after mating (Greeff and Schmid-Hempel, 2008). However, this could possibly be explained by bumblebee queens only using a subset of the sperm stored in their spermatheca to complete their short, one-year reproductive cycle (Röseler, 1973; Baer and Schmid-Hempel,

2000), so they do not face as strong selective pressure for sperm preservation as long-lived ant queens do.

Conclusions

We began by hypothesizing that non-self sperm harboured in sperm storage organs of leaf-cutting ant queens should be protected from sexually transmitted pathogens through pathogen-specific immune effectors rather than non-specific responses that might damage irreplaceable sperm. However, male reproductive organs that harbour self-sperm may express a wider array of immune responses providing these do not produce secondary substances that have a negative impact on sperm quality. Fine-tuned immune regulation mechanisms should therefore have evolved in both sexes to optimally protect ejaculates during their journey from storage in the male accessory testes to the female spermatheca where they survive for up to several decades. We found that AMPs, pathogen-specific immune effectors, were expressed in both male and queen reproductive organs prior to mating and that artificial insemination did not significantly affect AMP levels in the spermathecae of *Atta* queens, suggesting that their expression is constitutive in the queen spermatheca. As expected, the expression of prophenoloxidase, a critical enzyme mediating the non-specific melanization response, was mostly downregulated in sperm storage organs. These findings suggest that leaf-cutting ants, and possibly many other Hymenoptera with longer-term sperm storage, may have evolved sophisticated organ-specific responses to handle the peculiar immune defence challenges posed by the prolonged storage of non-self sperm cells inside the female body.

Acknowledgements

We thank the Smithsonian Tropical Research Institute in Panama for facilities and logistic support, and the Autoridad Nacional de Ambiente for issuing collection and export permits for the leaf-cutting ants used in this study.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.C., B.B., M.S., J.J.B.; Formal analysis: S.C.; Investigation: S.C., B.B.; Writing - original draft: S.C., M.S., J.J.B.; Writing - review & editing: S.C., B.B., M.S., J.J.B.; Visualization: S.C., M.S., J.J.B.; Supervision: M.S., J.J.B.; Funding acquisition: J.J.B.

Funding

This work was supported by a Danmarks Grundforskningsfond grant (DNRF57) and a European Research Council Advanced Grant (323085) to J.J.B.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.173435.supplemental

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