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Ant sperm storage organs do not have phenoloxidase constitutive immune activity

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ABSTRACT

The prophenoloxidase system (proPO-AS) is a primordial constituent of insect innate immunity. Its broad action spectrum, rapid response time, and cytotoxic by-products induced by phenoloxidase (PO) production contribute to the effective clearing of invading pathogens. However, such immune reactions may not be optimal for insect organs that evolved to have mutualistic interactions with non-self-cells. Ant queens are long-lived, but only mate early in adult life and store the sperm in a specialized organ, the spermatheca. They never re-mate so their life-time reproductive success is ultimately sperm-limited, which maintains strong selection for high sperm viability before and after storage. The proPO-AS may therefore be inappropriate for the selective clearing of sexually transmitted infections, as it might also target sperm cells that cannot be replaced.

We measured PO enzymatic activity in the sperm storage organs of three ant species before and after mating. Our data show that no PO is produced in the sperm storage organs, relative to other somatic tissues as controls, and that these negative results are not due to non-detection in small volumes as non-immune-relevant catalase activity in single spermatheca fluid samples of both virgin and mated queens was significant. The lack of PO activity in sperm storage organs across three different ant species may represent an evolutionarily conserved adaptation to life-long sperm storage by ant queens. We expect that PO activity will be similarly suppressed in queen spermathecae of other eusocial Hymenoptera (bees and wasps) and, more generally, of insect females that store sperm for long periods.

1. Introduction

The prophenoloxidase system (proPO-AS) is a major component of the invertebrate innate immune system. It is a constitutive, non-specific and immediate response involved in wound healing and the immobilization and eradication of foreign agents (Sugumaran, 2002; Schmid-Hempel, 2005). The immune response is activated by the recognition of pathogen-associated molecular patterns or cell injury (Schmid-Hempel, 2005), which triggers the conversion of zymogenic prophenoloxidase (proPO) into active phenoloxidase (PO) through enzymatic interactions (Söderhäll and Cerenius, 1998; Laughton and Siva-Jothy, 2010). PO catalyzes the oxidation of phenols into quinones, which results in melanin synthesis and deposition around wounds or pathogens (González-Santoyo and Córdoba-Aguilar, 2011). By-products of this reaction (such as dopamine derivatives and reactive oxygen species (ROS)) are toxic for microbial pathogens, but also for the infected individuals themselves (Cerenius and Söderhäll, 2004; Wilson-Rich et al., 2009). For example, ROS are involved in the oxidation of various types of molecules, including proteins, RNA, DNA, and membrane lipids, and may contribute to aging, carcinogenesis or cell death (Heifetz and Rivlin, 2010). Such damages can be prevented thanks to the activity of antioxidative enzymes, such as catalase, superoxyde dismutase or glutathione-S-transferase (Weirich et al., 2002; Collins et al., 2004; DeJong et al., 2007). To date, several studies of insect immune capacity have shown an increase in the hemolymph PO-level associated with the presence of 'non-self' elements such as lipopolysaccharides injections or nylon inserts (Castella et al., 2009; Baer et al., 2006; McNamara et al., 2013; Schwarzenbach and Ward, 2006).

Females of many insect species store sperm in specialized organs, spermathecae, before using it to fertilize eggs (Shuker and Simmons, 2014). Depending on species or lineage, sperm can be stored for days, weeks or even years making it logical to expect that spermathecae have been under selection to preserve sperm viability when that serves female fitness interests. Recent studies

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have indeed shown that insect females interfere with the metabolic rate of stored sperm reducing the production of harmful ROS (Reinhardt and Ribou, 2013; Ribou and Reinhardt, 2012). Another factor that affects the preservation of stored sperm is the prevalence of sexually transmitted pathogens (Knell and Webberley, 2004; Fievet et al., 2006) that might cause sperm death (Otti et al., 2013). While the female immune system probably plays an important role in eliminating such pathogens, such defences might have collateral damage if they also recognize sperm as non-self elements to be eliminated. The proPO-AS is a generalist immune response that can therefore potentially induce sperm death. Mating has indeed been reported to trigger development of dark reaction masses in *Drosophila* female sexual tract (Patterson, 1946) and to induce melanization of the spermatheca in bumblebees (Ashida and Brey, 1995). Sperm cell melanization has further been observed in the shrimp *Penaeus vannamei* where it induced sperm degeneracy and reduced male fertility (Dougherty and Dougherty, 1989, 1990).

Queens of the eusocial Hymenoptera (ants, bees, wasps) are inseminated on a single day early in adult life, store the sperm for the rest of their lives, and never re-mate even though they may live for more than a decade (Boomsma et al., 2005). This extreme partner-commitment implies that lifetime reproductive success is ultimately dependent on the amount of sperm stored and the prudency of sperm use (den Boer et al., 2009). We therefore expect that the proPO-AS should be absent in the long-term sperm storage organs of eusocial queens and we evaluated that expectation using queens of three ant species representing the most abundant subfamilies (Formicinae and Myrmicinae) in terms of species richness (Hölldobler and Wilson, 1990). The ant species also differed in their mating system (Boomsma et al., 2009): queens of *Lasius niger* garden ants (Formicinae) are normally either singly or doubly inseminated (Boomsma and Van Der Have, 1998) whereas queens of *Acromyrmex echinatior* and *Atta colombica* leaf-cutting ants (Myrmicinae) are always multiply inseminated (Villesen et al., 2002).

We measured the proPO and PO activity in the spermatheca (specialized organ for lifetime sperm storage) and the *Bursa copulatrix* (sperm deposition organ during copulation) of virgin and mated queens relative to control tissues (hemolymph or homogenized head and thorax), and we analyzed proPO/PO activity in male seminal vesicles where sperm is stored before copulation. Since most of the PO is stored as inactive proPO, we estimated the putative activity of proPO after its cleavage into PO by a non-specific protease (α-chymotrypsin). This allowed us to use "proPO activity" as a predictor of PO activity based on the transformation of stored proPO. To obtain an independent control for another enzyme in the sperm storage organs that is not expected to be absent, we also measured catalase activity in spermathecae and homogenized head and thorax controls of virgin and mated queens.

2. Materials and methods

Virgin queens and males of *L. niger* were collected from field colonies in Brussels (Belgium) in July 2011, 2 weeks before the mating flight. Mated queens were caught on the day of their flight, placed in laboratory nests with *ad libitum* water, and fed sugar water and mealworms. *A. colombica* males and virgin queens were collected in Gamboa (Panama) just before the mating flight in May 2013 and mated queens were collected after the mating flight. Males and virgin queens of *A. echinatior* were taken from mature laboratory colonies in Copenhagen that were collected at the same Panamanian field sites in earlier years. Mated queens (ca. 4 months old) of *A. echinatior* originated from incipient laboratory colonies kept in Copenhagen and collected in Panama in May 2014. Although the exact age of the harvested sexuals was unknown, males and females of each species sampled at a given time came from the same brood cohort under laboratory or natural conditions.

2.1. ProPO/PO activity

Previous studies have shown that ant queen immune responses may vary considerably before and after mating (Baer et al., 2006), so we measured proPO/PO activity at a number of different times. PO activity and potential proPO activity in virgin queens and males of all three species were measured within 24 h after the ants were collected. ProPO/PO activity for newly mated *L. niger* queens was measured 1 day, 1 week, 2 weeks, and 4 months after mating. We also measured proPO/PO activity in queens of *A. colombica* 1 day after insemination and queens of *A. echinatior* ca. 4 months after their mating flight. The timing of these samples does not fully correspond to the larger range of samples for *L. niger* because we could only collect in Panama in May, whereas sampling of *L. niger* in Brussels was not constrained.

For the three species sampled, male seminal vesicles and female spermathecae and *bursae copulatrix* were dissected in deionized water at room temperature, carefully opening them in a 1 μl drop of sodium cacodylate buffer (0.01 M Na-Cac, 0.005 M $CaCl_2$, pH 6.5 (Laughton and Siva-Jothy, 2010)) and removing the envelope tissue afterwards. Each 1 μl sample was diluted in 19 μl of sodium cacodylate buffer after which 10 μl was used to measure free PO activity and another 10 μl to measure proPO activity. The spermatheca, *bursa copulatrix* and paired seminal vesicles contain tiny amounts of fluid (ca. 0.2 μl), and a lack of proPO/PO activity in these organs may therefore be due to detection failure. To discard this possibility, we also analyzed proPO/PO activity in a pool of 10 spermathecae from *L. niger* queens 2 weeks after mating (the time at which PO activity is the highest in hemolymph, see Section 3).

Control tissue activities were obtained from the head/thorax tissues or, whenever available in sufficient volumes, hemolymph as we believe that its higher purity would produce clearer results. For *L. niger*, less than 0.5 μl hemolymph per individual could be extracted from males and queens, which was insufficient for accurate testing of proPO/PO activity. To obtain control tissue activities for these samples, head and thorax were immersed in liquid nitrogen and homogenized to release PO and proPO. Then, 50 μl sodium cacodylate buffer was added before centrifuging for 10 min at 13,000 rpm, and recovering 20 μl of the supernatant. For *A. echinatior* and *A. colombica* males and virgin queens, we sampled 3 μl hemolymph in a microcapillary after decapitating the individuals, of which 1 μl was diluted in 19 μl of sodium cacodylate buffer; 10 μl was then used to measure free PO activity and the other 10 μl to measure proPO activity.

PO measurements were carried out following the protocol of Laughton and Siva-Jothy (2010). Ten microliters of each sample were distributed in wells of a 96-well microtiter plate on ice, after which 5 μl dH_2O (for measurement of active PO) or α-chymotrypsin (to artificially activate the proPO) were added. Plates were incubated for 5 min at room temperature to allow α-chymotrypsin activity to be expressed. Standard quantities of 35 μl L-dopa (4 mg/ml) were added to all the wells simultaneously by an injection spectrophotometer (MikroWin 2000 version 4 from Mikrotek Laborsysteme GmbH). Total PO levels were determined by photometrically measuring the dopachrome absorbance synthesized from L-dopa (3,4-dihydroxy-L-phenylalanine) by PO catalysis. Dopachrome absorbance was measured at 492 nm every minute for 90 min at room temperature. PO and proPO activities were quantified from the linear phase of reaction curves using regression analysis. One unit of activity was then calculated as the increase in dopachrome (OD492 + 1) absorbance per minute (Laughton and Siva-Jothy, 2010).

2.2. Catalase activity

Measurements of catalase activity in the spermatheca of virgin and mated queens (2 weeks after their nuptial flight) were used as control experiments to test whether it is possible to detect any enzymatic activity in very small amounts of fluid. Catalase activity was determined with the Catalase Assay Kit (Abcam, Cambridge, UK) using 4 μl of sample solution, as recommended by the manufacturer. Queen spermathecae were dissected in deionized water, opened in 4 μl dH_2O, after which the envelope was removed. For control tissues, the head and thorax of the same individuals were immersed in liquid nitrogen and homogenized to release the catalase, after which 50 μl dH_2O was added before centrifuging for 10 min (13,000 rpm) and recovering 4 μl of the supernatant.

The 4 μl from each sample was diluted in 156 μl catalase assay buffer (final volume). Two aliquots of 78 μl were distributed in two wells of a 96-well microtiter plate on ice. A blank solution was prepared in the first well by adding 10 μl of the stop solution provided in the kit, which contains proteases and inhibits the catalase activity; the content of the second well was used for testing the catalase activity. Twelve microliters H_2O_2 (1 mM) were added both to the blank and the test wells, after which they were incubated for 30 min at 25 °C (catalase consumes the H_2O_2 and produces water and oxygen). Then, 50 μl of the developer mix (containing assay buffer, OxiRed probe and HRP solution) provided in the kit was added to each well. The H_2O_2 not transformed by catalase reacts with the OxiRed and forms a colored product that absorbs at 570 nm. This procedure was repeated for each sample. A standard curve was obtained by exposing different concentrations of H_2O_2 to the OxiRed (30 min at 25 °C) and used to determine the amount of H_2O_2 transformed by the catalase, serving as a measure of enzymatic activity in each sample. One catalase unit corresponded to the amount of catalase that decomposed 1 μmol H_2O_2 per minute, at pH 4.5 at 25 °C.

2.3. Statistical analysis

Deviations from normality and homoscedasticity were tested with Shapiro–Wilk and Levene's tests, respectively. Parametric tests were used when enzymatic activity units were normally distributed and had homogeneous variances. Nonparametric tests were used when transformations did not suffice to normalize data or homogenize variances (Sokal and Rohlf, 1995).

First, in order to show that the enzymatic activity in the controls differs from zero, we compared proPO/PO and catalase activity with a hypothesized level of zero activity using one sample *t*-tests or Mann–Whitney *U* tests. Second, to test for a difference between PO and proPO activities in the controls, we used a one-way ANOVA (*L. niger*) or a Kruskal–Wallis test (*A. colombica* and *A. echinatior*). Third, we compared PO activities between head/thorax homogenates of virgin and mated *L. niger* queens and between virgin queens and males, using a Kruskal–Wallis test or a one-way ANOVA. We performed the same tests to compare proPO levels between these samples.

In order to compare proPO and PO levels between multiple queen samples (virgin, 1 day, 1 week, 2 weeks and 4 months after insemination) the ANOVA or Kruskal–Wallis tests were followed by *post hoc* Tukey–Kramer HSD and Dunn tests, respectively. Finally, to compare hemolymph proPO/PO levels between virgin queens and males and between virgin and mated queens of *A. colombica* and *A. echinatior*, we used either one-way ANOVAs or Kruskal–Wallis tests. When using Kruskal–Wallis tests, *P*-values were obtained from chi-square probability distributions at $(k-1)$ degrees of freedom. For all statistical tests, the significance levels were Bonferroni corrected to account for multiple comparisons. All statistical analyses were carried out using JMP software (version 10, SAS Institute).

3. Results

No prophenoloxidase or phenoloxidase activity was detected in the queen spermathecae and *bursae copulatrix* or in the male seminal vesicles. In contrast, we detected significant proPO and PO activities in the control tissues (one sample *t*-tests or Mann–Whitney *U* tests against the zero activity alternative; *L. niger*: all $P < 0.006$; *A. echinatior* virgin queens: proPO: $P < 0.0001$, PO: $P = 0.002$; *A. colombica* virgin queens: proPO: $P < 0.0001$, PO: $P = 0.07$; *A. colombica* 1 day old mated queen: proPO: $P < 0.001$; *A. colombica* males: proPO: $P < 0.001$, PO: $P = 0.008$) (Figs. 1 and 2). Substantial proPO/PO activity was also detected in control tissues of *A. echinatior* males and queens ca. 4 months after their mating flight, but it was not significantly different from zero (Mann–Whitney *U* test) probably due to the small number of individuals in the sample ($n = 2$ and 3, respectively). No proPO/PO activity was found in the samples of ten pooled spermathecae dissected 2 weeks after the mating flight, *i.e.* when the proPO activity in the control tissues was maximal (Fig. 1), indicating that our inability to find proPO/PO activity in spermathecae of individual queens was not due to lack of detection efficiency. However, significant catalase activity (Fig. 3) was measured in the minute volumes (4 μl) of fluid from single spermathecal samples of virgin and mated queens (one sample *t*-tests against the zero activity alternative; both $P < 0.008$). This activity was not significantly different from that found in the head/thorax homogenate controls (two sample *t*-test; $P > 0.25$ in both virgin and mated queens).

The proPO activities were always much higher than the PO activities (*L. niger*: one-way ANOVA; $F_{1,226} = 339.87$, $P < 0.0001$; *A. colombica*: Kruskal–Wallis; $\chi^2 = 32.31$, d.f. = 1, $P < 0.0001$; *A. echinatior*: Kruskal–Wallis; $\chi^2 = 36.89$, d.f. = 1, $P < 0.0001$) (Figs. 1 and 2). For *L. niger* queens (Fig. 1), PO activity in tissue homogenates remained stable and low during the 4 months of the colony founding experiment (Kruskal–Wallis and *post hoc* Dunn test).

Overall, proPO activity in the control tissue homogenates of *L. niger* queens (Fig. 1) varied significantly between just before mating and 4 months afterwards (one-way ANOVA; $F_{4,84} = 19.99$, $P < 0.0001$). ProPO activity was somewhat reduced 1 day after mating (*post hoc* Tukey–Kramer HSD; $P = 0.029$), but increased during the next 2 weeks to become significantly higher than in virgin queens 2 weeks after colony founding (*post hoc* Tukey–Kramer HSD; $P = 0.027$). However, when the first workers had hatched after 4 months, proPO activity had become reduced again to levels lower than those in virgin queens (*post hoc* Tukey–Kramer HSD; $P = 0.0004$).

PO activity in *A. colombica* queens was reduced 1 day after mating (Kruskal–Wallis; $\chi^2 = 4.80$, d.f. = 1, $P = 0.028$), whereas proPO activity had increased (one-way ANOVA; $F_{1,11} = 13.54$, $P = 0.004$). However, *A. echinatior* queens showed no difference in PO levels between virgin and 4 months old queens (Kruskal–Wallis; $\chi^2 = 0.84$, d.f. = 1, $P = 0.36$) and here proPO activity was lower in mated compared to virgin queens (Kruskal–Wallis; $\chi^2 = 4.32$, d.f. = 1, $P = 0.038$).

ProPO control tissue activities in virgin queens of *A. echinatior* (Fig. 2) were higher than in virgin males (one-way ANOVA; $F_{1,26} = 6.34$, $P = 0.018$). In *L. niger*, proPO activities did not differ between the sexes (Kruskal–Wallis; $\chi^2 = 3.34$, d.f. = 1, $P = 0.07$). Whereas, in *A. colombica*, male proPO activities were higher than in queens (one-way ANOVA; $F_{1,16} = 5.50$, $P = 0.032$) (Fig. 2).

As for PO, there were no differences between the sexes for *A. echinatior* hemolymph controls (one-way ANOVA; $F_{1,26} = 0.21$, $P = 0.652$; but note the small sample size for males) (Fig. 2),

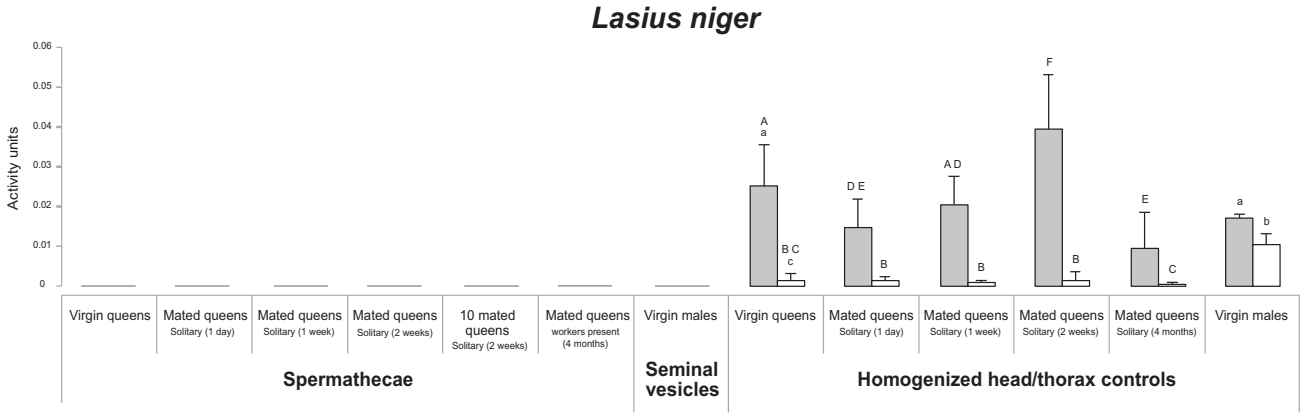

Fig. 1. Prophenoloxidase and phenoloxidase activity in the garden ant *Lasius niger*. Prophenoloxidase (proPO; gray bars) and phenoloxidase (PO; white bars) activity (mean ± SD) in reproductive organs and homogenized head/thorax control tissues. Capital letters above error bars refer to significant differences (or not, when letters are the same) between proPO/PO activity for comparisons between virgin and mated queens while lower case letters refer to differences between virgin males and virgin queens. Sample sizes (always equal for spermathecae/seminal vesicles and homogenized head/thorax controls): virgin queens $n = 10$; mated queens (1 day) $n = 26$; mated queens (1 week) $n = 27$; mated queens (2 weeks) $n = 15$; 10 pooled spermathecae of mated queens (2 weeks) $n = 2$; mated queens (4 months) $n = 15$; virgin males $n = 25$.

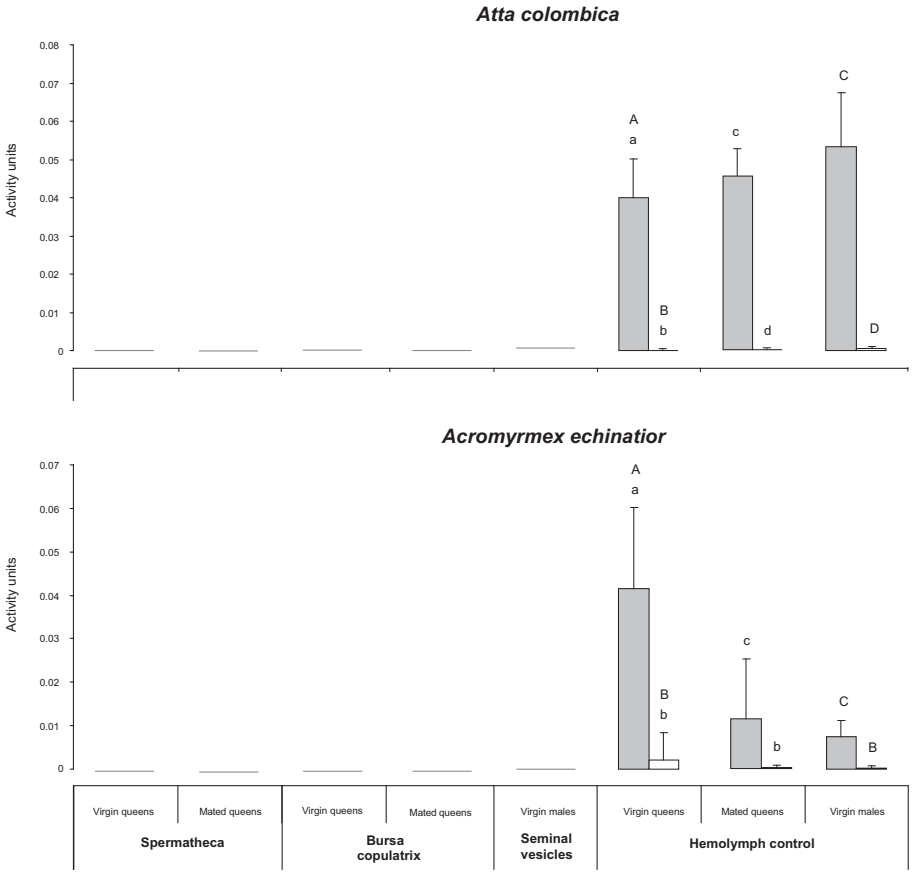

Fig. 2. Prophenoloxidase and phenoloxidase activity in the leaf-cutting ants *Acromyrmex echinatior* and *Atta colombica*. Prophenoloxidase (proPO; gray bars) and phenoloxidase (PO; white bars) activity (mean ± SD) in *Acromyrmex echinatior* and *Atta colombica* virgin and mated queen spermathecae and *bursae copulatrix* and virgin male seminal vesicles, compared to control samples of hemolymph of virgin and mated queens and males. Letters above error bars indicate significances as in Fig. 1. Sample sizes for *Atta colombica*: $n = 9$ for all samples except for mated queen spermathecae and hemolymph controls 1 day after mating where $n = 4$; for *Acromyrmex echinatior*: virgin queen spermathecae $n = 19$; virgin queen *bursae copulatrix* $n = 9$; 4 months old mated queen spermathecae $n = 3$; virgin male seminal vesicles $n = 4$; virgin queen hemolymph controls $n = 26$; 4 months old mated queen hemolymph controls $n = 3$; virgin male hemolymph controls $n = 2$.

whereas male PO activities were higher than those of queens for *A. colombica* (one-way ANOVA; $F_{1,15} = 9.14$, $P = 0.008$) (Fig. 2). The difference in adult PO level was even more pronounced for virgin males of *L. niger* relative to virgin queens (one-way ANOVA; $F_{1,33} = 99.33$, $P < 0.0001$) (Fig. 1).

4. Discussion

Our results show a complete absence of proPO/PO activity in ant sperm storage organs relative to control tissues across three different ant species. This lack of proPO/PO activity is unlikely to be due

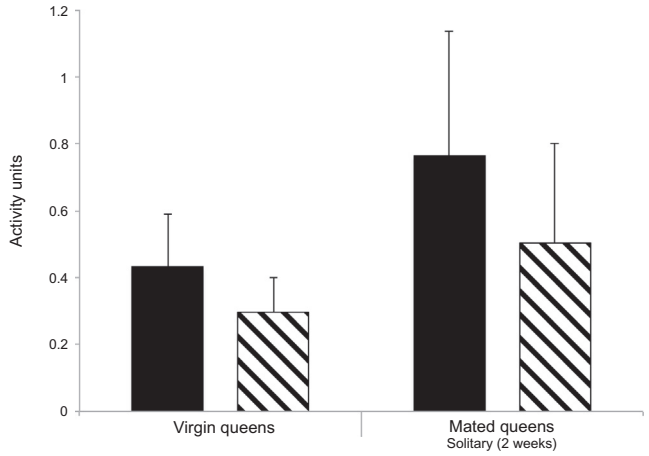

Fig. 3. Catalase activity in the garden ant *Lasius niger*. Catalase activity (mean ± SD) in spermathecae (black bars) and homogenized head/thorax control tissues (hatched bars) of *Lasius niger* virgin queens and mated queens 2 weeks after the nuptial flight. Differences were not significant for either category of queens (see text for details). Sample sizes: virgin queen spermathecae and homogenized head/thorax controls $n = 9$; mated queens (2 weeks) spermathecae and homogenized head/thorax controls $n = 7$.

to detection failure: no proPO/PO activity was found, even when we pooled ten mated queen spermathecae; furthermore, we were able to detect significant catalase activity in the same minute volumes of spermatheca fluid of single virgin or mated queens. We hypothesize that the absence of proPO/PO activity in the spermathecae and *bursae copulatrix* represents an evolutionarily conserved adaptation to sperm storage by female insects, because the effects of the proPO/PO cascade may damage sperm thereby lowering both male and female reproductive success. Such an adaptation seems particularly crucial when females are inseminated during a unique mating event, as seen in social insects where queens are characterized by life-long sperm storage and complete absence of re-mating later in life (Boomsma et al., 2005).

The lack of proPO/PO activity in queen sperm storage organs does not exclude the possibility that other immune defences might be used to fight possible infections transmitted during mating. In the fruit fly *Drosophila melanogaster*, the seminal fluid of males was shown to contain antibacterial proteins from the accessory glands and ejaculatory duct (Lung et al., 2001). Given that the seminal fluid in fruit flies is delivered to females before the sperm, it has been suggested that these antibacterial proteins may also protect sperm from infections. Moreover, expression of several antimicrobial peptides was reported in the reproductive tract of *Drosophila* females (Tzou et al., 2000). A lysozyme-like immune activity involved in the degradation of bacteria and subsequent sperm protection against microbial damage was also documented in ejaculates of the common bedbug *Cimex lectularius* (Otti et al., 2009, 2013).

The control (head/thorax homogenates and hemolymph) measurements showed that PO levels in adult queens and males were always very low compared to proPO levels. At least two explanations may account for this result: negligible immune challenges during our experiments; or active PO enzyme being produced but rapidly degraded because its by-products are damaging (Cerenius and Söderhäll, 2004; Wilson-Rich et al., 2009). Such possible damage might be a particularly acute problem for queens that potentially have a long reproductive life ahead of them. Our data do not allow distinguishing between these two hypotheses.

Our comparisons between male and female proPO/PO activities across species showed no consistent trends. Based on previous studies in social Hymenoptera, we would have expected females to have a higher immune ability than males (Gerloff et al., 2007; Vainio et al., 2004; Baer et al., 2005; Stürup et al., 2014). Because males die on the day of mating, there is no trade-off between present and future reproductive success, so one would not expect that males need much immune protection for the few hours of their lives that remain after leaving the largely aseptic environment of their natal nests (Cremer et al., 2007). On the other hand, queens are very long lived and face immune challenges particularly during the months of solitary colony founding (Baer et al., 2006).

The increase in proPO activity in *L. niger* queens in the weeks after mating when they founded colonies appears logical, with a slight decrease immediately after the immense efforts of completing the mating flight followed by a gradual increase over the following weeks when queens are still on their own to nurse their first brood. This is a very demanding time in terms of resource allocation, which will only be relieved when the first workers hatch and start their foraging and grooming services, explaining the decrease in proPO activity after 4 months (when their workers had hatched). These results are consistent with those previously reported in two other ant species where queen immune activity was shown to vary around the mating period. Virgin queens of *Formica paralugubris* had higher proPO levels than freshly mated queens (Castella et al., 2009) and *A. colombica* queens up-regulated their melanization immune response a few days after mating (Baer et al., 2006). Our limited sample availability did not allow us to corroborate this variation in proPO/PO activity over time in *A. colombica* and *A. echinatior*, but it seems likely that the activation of the proPO system within a few days after mating is an adaptive response to pathogen exposure during colony founding.

5. Conclusions

The results that we obtained are consistent with our expectation of no proPO/PO activity in sperm storage organs combined with significant somatic proPO/PO activity in the same queens showing that their constitutive immune defences are otherwise of general importance. This implies that the protection of stored sperm against pathogens must be based on more specific and less destructive immune defences such as antimicrobial peptides (Baer et al., 2009), but more studies to test this are needed. The consistent lack of proPO/PO activity in the sperm storage organs of ant queens suggests that similar studies in other eusocial Hymenoptera can be expected to give similar results. These clear-cut expectations are based on the complete lack of remating later in life that characterizes all eusocial Hymenoptera. Whether spermathecae of insects where females do remate at specific intervals show similar, but possibly less extreme, reductions of proPO/PO activity in sperm storage organs remains to be seen, but deserves to be addressed.

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