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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).