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Mating-induced patterns of spermathecal fluid protein expression in two eusocial insect species, *Lasius niger* and *Apis mellifera*

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Eusocial insect queens exhibit some of the most extreme durations of sperm storage in the animal kingdom. This extended lifespan of sperm within the queen's storage organ (the spermatheca) after mating is largely sustained by the spermathecal fluid matrix—a rich and proteinaceous secretion that fills the void volume within the spermatheca. Here, we conducted a comparative proteomics study on mating-induced changes in spermathecal fluid of two long-lived hymenopteran species, *Lasius niger* and *Apis mellifera*. We found some similarities between species; for example, enolase and other enzymes responsible for carbohydrate metabolism were among the top differentially expressed proteins in both *A. mellifera* and *L. niger*. Additionally, both species exhibited post-mating upregulation of catalase, glutathione peroxidase, and Mn-conjugated superoxide dismutase (SOD), all of which are important antioxidant enzymes. However, we also identified notable differences, with Cu/Zn-conjugated SODs being consistently downregulated after mating in *L. niger* but upregulated in *A. mellifera*. Likewise, canonical immune effectors (phenoloxidase and lysozyme) showed similar patterns of expression in both species, (with phenoloxidase remaining unchanged and lysozyme increasing after mating), but ferritins, which are multifunctional antioxidant proteins that are also induced by immune challenges, differed, increasing in *L. niger* but decreasing in *A. mellifera*. Herein, we discuss expression patterns of these proteins and additional immune proteins, hexamerins, odorant binding proteins, and a key carbohydrate metabolism enzyme (glyceraldehyde-3-phosphate dehydrogenase) in the context of the differential life histories of these two eusocial insect species.

Reproductive division of labour between queen and worker castes is one of the hallmarks of eusocial Hymenoptera (ants, eusocial bees, and eusocial wasps). Queens are specialized for reproduction, including mating and laying eggs. In contrast, workers usually do not reproduce; they are in many cases sterile or incapable of mating. Instead, they take on a variety of non-reproductive tasks that are essential for the colony's growth and survival. Queens are markedly longer lived than their non-reproductive female nestmates, with lifespans varying greatly among species[1,2]. Queen bumble bees (*Bombus*) for instance, live for one year —— substantially longer than their unmated daughters, who live for several weeks[3]. Queen honey bees (*Apis mellifera*) live comparatively longer, frequently reaching 2–3 years of age[4], with the longest recorded lifespan of 8 years[5]. Queen ants, however, hold the record for being the longest-lived hymenopterans, with *Atta* queens living > 10 years, and *L. niger* and *Pogonomyrmex owyheei* in particular reaching > 20 years old[1]. Incredibly, queens of all these species continue laying fertilized eggs for their entire lifetime using sperm they acquired during just one mating period early in life.

Given that queens may only remain productive as long as they maintain a supply of viable sperm, and that they can not re-mate later in life, their fecundity and lifespan are sperm-limited and effective sperm storage

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(reviewed in Degueldre & Aron[6] is therefore essential for their longevity. To support this function, each queen possesses a spermatheca —— a specialized sperm storage organ —— where their supply of sperm cells are densely maintained[7] in a bath of spermathecal fluid that is secreted into the reservoir[8]. This fluid, produced by the adjoining spermathecal glands, is rich in antioxidant enzymes while also being depleted in oxygen[12,13], which appears to offer a layered mechanism by which damage to the sperm from reactive oxygen species (ROS) can be limited long-term. When stored, sperm may also enter quiescence (lower metabolic activity), which further minimizes sperm senescence by reducing damage by ROS[14,15], although this has not yet been demonstrated in eusocial Hymenoptera.

Studying the protein profiles of spermathecal fluid among species with different sperm storage requirements, such as *A. mellifera* and *L. niger* (which differ in lifespan by approximately an order of magnitude), before and after mating may help determine the molecular systems that support varying degrees of long-term sperm maintenance. While such studies could eventually reveal an evolutionary origin of these critical systems, initial comparisons are lacking. To date, there have been many molecular analyses comparing the profiles of spermathecal fluid of mated to virgin queens in *A. mellifera* and several ant species[9–11,16–25]; however, few investigations remove the sperm cells prior to conducting molecular analysis[9,11,24], and elsewhere it is not clear if sperm removal has been achieved or not[10]. This may bias the interpretation of the data, as the molecular source of the genes and proteins analyzed could be the spermathecal fluid, the sperm, or other cells associated with the spermathecal tissue (e.g., the epithelial cells that make up the spermathecal wall[26]. The spermathecae of mated *A. mellifera* and *L. niger* queens contain several million sperm cells[27–30]; therefore, if included in the sample, their molecular profiles would be expected to contribute greatly to any differences observed between mated and unmated queens.

Among studies that investigated spermathecal fluid devoid of sperm, in *A. mellifera*, one early proteomic investigation identified 122 proteins in spermathecal fluid, confirming the presence of antioxidant enzymes, heat-shock proteins, and enzymes linked to carbohydrate metabolism, among other minor contributors like major royal jelly proteins (MRJPs) and odorant binding proteins (OBPs)[9]. More recently, upregulation of antioxidant enzyme expression and activity was ascertained in spermathecal fluid, as well as abundance of other antioxidant proteins (transferrin and MRJPs, which are multifunctional proteins that can reduce oxidative stress[31]. A key carbohydrate (glyceraldehyde-3-phosphate, or GAP) and catabolic enzyme (GAP dehydrogenase, or GAPDH) have also been identified as critical to support sperm viability by "safely" (non-oxidatively) and efficiently generating ATP[12]. In analyses of *Lasius* spp. (*L. japonicus* and *L. hayashi*), transcriptomic and proteomic studies generally agree with those in *A. mellifera*, with evidence supporting enrichment of antioxidant capabilities, protein chaperones, and carbohydrate metabolism in the spermatheca, but these studies did not specifically deplete sperm cells from the samples[10,21].

As a queen ages, some sperm will inevitably die despite the substantial investment in limiting oxidative stress[32–34], but the fate of non-viable (defined here as membrane-permeable[35] sperm cells is not always clear. In *A. mellifera*, dead sperm accumulate in the spermatheca over time[32,33], suggesting that non-viable cells are not recycled (or at least not completely) and are expelled along with live cells during each fertilization event. This appears to be an inefficient use of space and cellular components, but given that honey bee queens release a fractional volume of spermathecal contents to fertilize each egg (and not a specific number of spermatozoa)[30], with that volume containing anywhere from 2 to 100 cells, depending on the queen's mating success[30,36,37], the presence of some (< 50%) dead sperm in the spermatheca does not have a meaningful impact on fertilization in the queen's first season[38,39]. Ant queens (*Solenopsis invicta* and *A. colombica*) use sperm comparatively judiciously, at ~ 3 spermatozoa per egg for established queens,[40,41] and, contrary to the honey bee, paradoxically more sperm appear to be released as the queen grows older[40]. Interestingly, in the case of *L. niger*, sperm viability also increases as a queen ages[27], implying that dead sperm must be broken down and possibly recycled or otherwise removed by yet unknown mechanisms, and that extremely long-lived ant queens are under selective pressure to maintain successful fertilizations using a minimal number of sperm. The same study also found that immune activation trades off with sperm viability in established *L. niger* queens, suggesting that older queens may invest less in constitutive self-preservation processes and more in sperm maintenance[27].

We conducted a comparative proteomics analysis of *A. mellifera* and *L. niger* spermathecal fluid pre- and post-mating to determine if mating-induced changes in protein abundance reflect the species' differences in sperm maintenance strategies. As queens in both species are relatively long lived, we expected to see many similarities in the types of proteins that were differentially abundant in spermathecae after mating, but we also anticipated some differences. Specifically, we hypothesized that since proteases are abundant in *Lasius* spermathecae[10] and evidence suggests that dead sperm may be eliminated in this species, *L. niger* spermathecal fluid may be enriched for proteolytic enzymes in mated queens, but the same pattern is not expected in *A. mellifera*. Secondly, we hypothesized that *L. niger* queens would invest more heavily in antioxidant systems post-mating than *A. mellifera*, since the latter species can afford to maintain lower sperm viability without immediate consequences for fertilization[39,42]. For the same reason, we thirdly hypothesized that *L. niger* queens may exhibit reduced investment in innate immune defences after mating, whereas *A. mellifera* queens may still afford relatively high constitutive immune effector expression. Finally, since GAP can be efficiently metabolised anaerobically to produce ATP, which is advantageous to both species, we hypothesized that *A. mellifera* and *L. niger* would both exhibit elevated GAPDH levels post-mating.

Methods

Queens

Generation of the queens used in this study has been previously described[43]. Because *L. niger* exhibits fully claustral nest founding, where the queen is solely responsible for rearing the first workers, whereas *A. mellifera* colonies reproduce by fission (and new queens are supplied with an existing workforce), three reproductive time

points were sampled for *L. niger* (virgin, incipient, and established) and two were sampled for *A. mellifera* (virgin and incipient). Briefly, virgin *L. niger* queens were captured as they exited their nest to commence their nuptial flight, incipient (newly mated) *L. niger* queens were collected seven days after mating (indicated by dealation), while established *L. niger* queens were sampled seven days after their first worker progeny emerged (9–10 weeks after mating). Virgin *A. mellifera* were collected 1–2 days after emerging from their pupal cell and incipient *A. mellifera* were collected 10–12 days after emerging (with successful mating unequivocally indicated by active laying).

Sample Preparation

L. niger queens ($n = 20$ virgins, $n = 20$ incipient, and $n = 70$ established queens) were killed by decapitation and spermathecae were dissected in 200 µl of semen diluent (188.3 mM sodium chloride, 5.6 mM glucose, 574.1 nM arginine, 684.0 nM lysine, and 50 mM tris[hydroxymethyl]aminomethane, pH 8.7)[44] under a Leica EZ4 stereomicroscope. Each sperm sample was transferred in an empty 1.5 ml microtube, which was gently inverted until homogenisation. The samples were then stored at -80 °C until shipping to the University of British Columbia (UBC) on dry ice. Although freeze-thaw cycles are sometimes used to lyse cells (which would be undesirable in our case), we routinely freeze sperm samples and conduct cell counting assays at a later date, at which time the sperm cells show no visible morphological differences. At UBC, the samples were thawed and centrifuged for 10 min (10,000 *g*, 4 °C), then the supernatant was removed and again centrifuged for 15 min (18,000 *g*, 4 °C). At both centrifugation steps, ~ 10% of the supernatant was left behind in the tube to minimize possible sperm cell carryover (Figure S1). Because of the low protein content (due to small spermathecal size) of *L. niger* samples, 3–4 samples were pooled to yield final sample sizes of $n = 6$ virgin, $n = 8$ incipient, and $n = 22$ established queen samples.

A. mellifera queens ($n = 7$ virgins and $n = 10$ mated) were dissected by removing the final abdominal tergites and retrieving the spermatheca using fine forceps. The spermatheca was placed on a clean tissue paper and gently rolled to remove the tracheal net. The spermatheca was then placed in a 1.5 mL tube containing 200 µl Tris (100 mM, pH 8.0) and gently ruptured by pressing with a plastic pestle. After suspending the sperm, the samples were centrifuged following the same methods as for *L. niger*.

For both sample sets, protein in the supernatant was precipitated by adding ice-cold acetone to a final concentration of 80%, then incubated overnight at -20 °C. The protein pellet was washed twice with 250 µl ice-cold 80% acetone, discarding the supernatant. Hereon, sample preparation steps (resuspension, reduction, alkylation, digestion, and desalting) were conducted exactly as previously described for hemolymph samples[43], except that for *L. niger*, given the low protein yields (in most cases precipitated pellets were not visible), there was insufficient protein to enable quantification for each sample; therefore, each sample was assumed to be 5 µg for the purposes of reduction, alkylation, and digestion. Briefly, we suspended the precipitated protein in urea buffer (8 M urea, 2 M thiourea, 100 mM Tris, pH 8.0), reduced disulfide bonds using dithiothreitol (1 µg per 50 µg protein), alkylated with iodoacetamide (1 µg per 10 µg protein) and digested with LysC/Trypsin mix (1 µg per 25 µg protein). After four hours of initial digestion, the samples were diluted in 50 mM ammonium bicarbonate and allowed to continue digesting overnight at room temperature. Digested peptides were desalted using in-house made C18 stop and go gel extraction (STAGE) tips[45] and peptides were eluted using 150 µl of 40% acetonitrile, 0.5% formic acid. After evaporating to dryness, peptides were suspended in 11 µl of injection solvent (0.5% acetonitrile, 0.1% formic acid) and 1 µl was used to quantify peptide concentrations based on A205 nm. *A. mellifera* samples were diluted to 10 ng/µl, whereas *L. niger* samples were diluted to 1 ng/µl.

Liquid chromatography and mass spectrometry

Because *L. niger* peptide concentrations were low, they were analyzed on a high-sensitivity mass spectrometry system (timsTOF SCP; Bruker Daltonics, Germany) designed for single-cell proteomics, whereas *A. mellifera* samples were analyzed on a timsTOF Pro2 (Bruker Daltonics, Germany). *L. niger* samples (10 ng each) were randomly injected and analyzed using a NanoElute UHPLC system (Bruker Daltonics) with Aurora Series Gen2 (CSI) analytical column (25 cm x 75 µm 1.6 µm FSC C18, with Gen2 nanoZero and CSI fitting; Ion Opticks, Parkville, Victoria, Australia) heated to 50 °C (by Column toaster M, Bruker Daltonics) and coupled to timsTOF SCP operated in data-independent acquisition parallel accumulation serial fragmentation (DIA-PASEF) mode. The gradient ramped from 2 to 12% buffer B over 15 min, then to 33% buffer B from 15 to 30 min, then to 95% buffer B over 0.5 min, and held for 7.72 min. Before each run, the analytical column was conditioned with 4 column volumes of buffer A. Buffer A consisted of 0.1% aqueous formic acid and 0.5% acetonitrile in water, and buffer B consisted of 0.1% formic acid in 99.4% acetonitrile. The NanoElute thermostat temperature was set at 7 °C. The analysis was performed at 0.3 µL/min flow rate.

The timsTOF SCP was set to PASEF scan mode for DIA acquisition scanning from 100 to 1700 m/z. The capillary voltage was set to 1800 V, drying gas to 3 L/min, and drying temperature to 200 °C. The MS1 scan was followed by 8 consecutive PASEF ramps containing 24 non-overlapping 25 m/z isolation windows, covering 400–1000 m/z. As for the TIMS setting, ion mobility range ($1/k_0$) was set to 0.64–1.4 V·s/cm^2 with a 100 ms ramp time and accumulation time (100% duty cycle), and ramp rate of 9.34 Hz. This resulted in 0.96 s of total cycle time. The collision energy was ramped linearly as a function of mobility from 20 eV at $1/k_0 = 0.6$ V·s/cm^2 to 59 eV at $1/k_0 = 1.6$ V·s/cm^2. Error of mass measurement is typically within 3 ppm and is not allowed to exceed 7 ppm. For calibration of ion mobility dimension, the ions of Agilent ESI-Low Tuning Mix ions were selected (m/z [Th], $1/k_0$ [Th]: 622.0290, 0.9915; 922.0098, 1.1986; 1221.9906, 1.3934).

A. mellifera samples (50 ng each) were analyzed in randomized injection order using the same liquid chromatography system and gradient but coupled to a timsTOF Pro2 mass spectrometer (Bruker Daltonics, Germany). As previously described[43], the Captive Spray ionisation source was operated at 1700 V capillary voltage and 200 °C drying temperature. The MS spectra were collected in positive mode from 100 to 1700 m/z.

The TIMS was operated with equal ramp and accumulation time of 85 ms (100% duty cycle). For each TIMS cycle, seven DIA-PASEF scans were used, each with three to four steps, with a total of 25 DIA-PASEF windows spanning from 299.5 to 1200.5 m/z and from ion mobility range ($1/k_0$) 0.7 V·s/cm^2 to 1.3 V·s/cm^2. Variable isolation width from 36 to 61 m/z was used with an overlap of 1 m/z between two neighbouring windows. The collision energy was ramped linearly as a function of mobility value from 20 eV at $1/k_0$ = 0.6 V·s/cm^2 to 65 eV at $1/k_0$ = 1.6 V·s/cm^2.

Data processing

Raw mass spectrometry data were searched using DIA-NN[46] (1.8.1). Default parameters were used, except that 'FASTA digest for library-free search', 'Deep learning-based spectra, RTs and IMs prediction', and 'MBR' were selected, 'Protein inference' was set to protein names from FASTA, two missed cleavages were allowed and 'Neural network classifier' was set to double-pass mode. The FASTA database for *L. niger* was downloaded from Uniprot on December 5, 2022, and the *A. mellifera* database was downloaded on February 2, 2023. A list of 381 potential protein contaminants were appended to each database[47]. The *A. mellifera* database also contained all viral, bacterial, and microsporidian honey bee pathogen sequences available on Uniprot.

Statistical analysis

Label-free quantitation data was analyzed using the limma package[48] within R (4.3.0)[49]. First, contaminant sequences were removed, data were log2 transformed, then complete data histograms were inspected for approximate normalcy. Proteins with fewer than 25% valid values were removed. Means models with empirical Bayes variance estimation were fit to each dataset to determine significant differences between all pairwise contrasts (3 for *L. niger* and 1 for *A. mellifera*). False discovery rates (FDRs) were controlled to 5% using the Benjamini-Hochberg method. GO term enrichments were assessed using the gene score resampling (GSR) method within ErmineJ[50] (3.1.2; default parameters) for up- and down-regulated proteins separately. The GSR method does not test for enrichment in a hit-list vs. background; rather, it tests for enrichment along the p value continuum and is not reliant on user-defined thresholds (see Lee *et al.*[50] for more details). Enrichment FDRs were controlled to 5% (Benjamini-Hochberg method) in all instances.

Results

Overview and GO enrichment

We identified 2,516 unique proteins across *L. niger* samples, of which 1,447 were considered quantified (present in > 25% of samples) and half (720; 49.8%) of which were differentially expressed (5% FDR, Benjamini-Hochberg method) in at least one pairwise contrast (i.e., virgin vs. incipient, virgin vs. established, and incipient vs. established; Fig. 1A). Notably, more proteins were upregulated than downregulated in incipient and established queens relative to virgins, many of which were putative sperm proteins (structural components of flagella), despite the centrifugation steps taken to remove sperm cells from samples prior to processing (Fig. 1B & C). Interestingly, while both incipient and established queen spermathecae contain sperm, putative sperm proteins were still more abundant in established queens relative to incipient queens (Fig. 1D), though at a smaller magnitude. GO term enrichment analysis on all up- and down-regulated protein lists for each pairwise contrast detected enriched terms only among up-regulated proteins in the virgin-to-incipient comparison (Fig. 1E). Transmembrane transporter activity (GO:0022857) and protein catabolic process (GO:0030163) were the top two most significantly enriched GO terms (adjusted $p = 0.032$ and 0.036, respectively).

In *A. mellifera*, we quantified substantially more proteins (4,223 out of 4,789), 2,796 (58%) of which were differentially expressed (Fig. 2A). Again, putative sperm proteins were among those upregulated in incipient queens (Fig. 2B). Among upregulated proteins, many GO terms were significantly enriched, most of which are related to carbohydrate metabolism, nucleotide metabolism, and transmembrane transporter activity (Figs. 2C and 5% FDR, Benjamini-Hochberg correction). Among downregulated proteins, most of the significantly enriched GO terms were linked to translation, vesicle coat proteins, and protein folding (Fig. 2D).

Top differentially expressed proteins

While many proteins were differentially expressed and yielded enriched GO terms, we also investigated specific groups of proteins, namely, those with the highest magnitude of differential expression as well as key enzymes implicated in successful sperm storage, immune proteins, and a curious pattern of odorant binding protein expression. In *L. niger*, the top five most significant differentially expressed proteins were pyruvate dehydrogenase, vesicular glutamate transporter, outer dense fiber protein (a putative sperm protein), enolase, and receptor-expression enhancing protein, all of which were elevated after mating (Fig. 3A), with the former three also upregulated after mating in *A. mellifera* (Data S1). The top five differentially expressed proteins in *A. mellifera* were cytochrome c (testis specific), disintegrin and metalloproteinase with thrombospondin motifs 12, enolase, restin homolog, and an uncharacterized protein, all of which were upregulated after mating (Fig. 3B). Of these, orthologs of the specific cytochrome c (cytochrome c-2) and enolase were also upregulated after mating in *L. niger*, while the others were either not identified or were not differentially abundant.

Antioxidant enzymes

Most major antioxidant enzymes, including catalase, superoxide dismutase (SODs), glutathione peroxidase (GPx), peroxidase (Px), and thioredoxin-dependent peroxidase (Trx) were upregulated after mating in both species, but there were notable differences between species for SOD metalloenzymes that conjugate different metals. While Mn-conjugated SOD was elevated after establishment in *L. niger*, Cu/Zn-conjugated SODs were downregulated (Fig. 4A). In *A. mellifera*, however, both Mn- and Cu/Zn-conjugated SODs were upregulated after mating (Fig. 4B). The various peroxidases were generally upregulated in both species after nest establishment

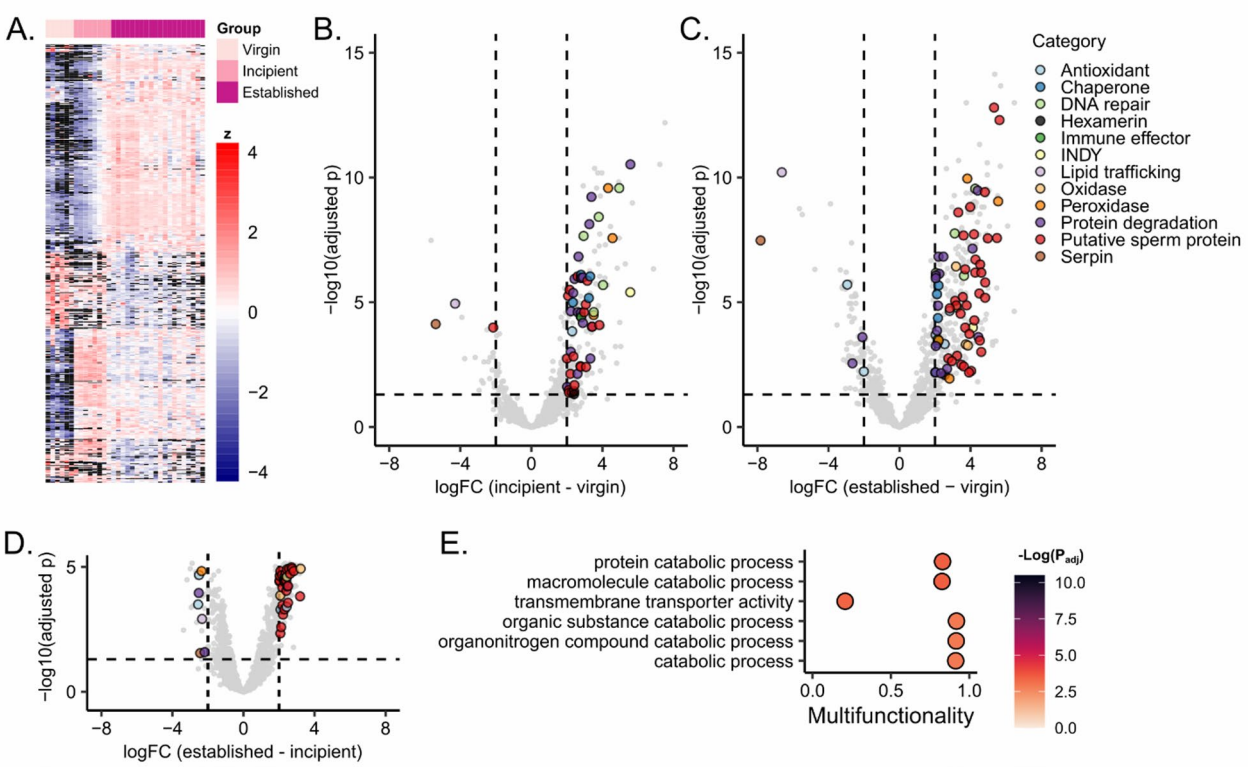

Fig. 1. *L. niger* differential expression and functional enrichment of spermathecal proteins. (**A**) 720 (shown) of 1447 proteins quantified were differentially expressed (5% FDR, Benjamini-Hochberg correction). Black tiles indicate missing values. 274 (**B**), 487 (**C**), and 485 (**D**) proteins were differentially expressed in the virgin-to-incipient transition, the virgin-to-established transition, and the incipient-to-established transition, respectively. Select protein functions of interest are color-coded. FC = fold-change; INDY = protein "i m not dead yet". Structural components of sperm flagella are categorized as "putative sperm proteins." The horizontal dotted lines indicate the 5% FDR threshold, and the vertical dotted lines indicate $\log_2(FC) = 2$. Proteins with adjusted $p < 0.05$ and $\log(FC) > 2$ are considered intensely significant. E) Significantly enriched GO terms (shown; 5% FDR, Benjamini-Hochberg correction) were only identified among up-regulated proteins in one contrast, the virgin-to-incipient transition. Higher multifunctionality scores indicate that enrichments are more likely to occur by chance due to component proteins being tied to many functions.

and mating, respectively, except for one GPx and one Trx, which did not significantly change in *A. mellifera* and *L. niger*, respectively.

Immune-associated proteins

In *L. niger*, prophenoloxidase (PO) and lysozyme (Lys) were the only canonical immune effectors quantified, with PO being unaffected by mating and Lys significantly increasing (Fig. 5A). The same pattern was observed in *A. mellifera*, with immune responsive protein (IRP)30 additionally increasing and hymenoptaecin remaining unchanged (Fig. 5B). Ferritin, a multifunctional protein involved in both mitigating oxidative stress and immunity[51], showed differing patterns of expression in *L. niger* versus *A. mellifera*: While all four quantified ferritin isoforms increased after mating in *L. niger*, all isoforms decreased in *A. mellifera.*

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

GAPDH has been previously implicated as a key enzyme in sperm storage for *A. mellifera*[12]. We quantified two isoforms of the enzyme in *L. niger* (GAPDH and GAPDH-like), with one decreasing and the other increasing after by the time of nest establishment (Fig. 6A). In *A. mellifera*, GAPDH significantly increased after mating (Fig. 6B), consistent with previous data[12].

Odorant binding proteins and hexamerins

The canonical function of odorant binding proteins (OBPs) is to transport odorant molecules in the antennal hemolymph, but their expression in non-chemoreceptive tissues suggests they may have other functions, such as transporting hormones or other small molecules[52–55]. Likewise, hexamerins are complex, multifunctional proteins, with strong caste- and tissue-specific patterns of expression, that have also been implicated as hormone carriers[56,57]. We investigated members of these protein families, and found that while only one OBP was quantified in *L. niger* (and was not differentially expressed), OBPs in *A. mellifera* were abundant and displayed more complex patterns (Fig. 7A & B). In *A. mellifera*, 8 OBPs were quantified, with five significantly increasing,

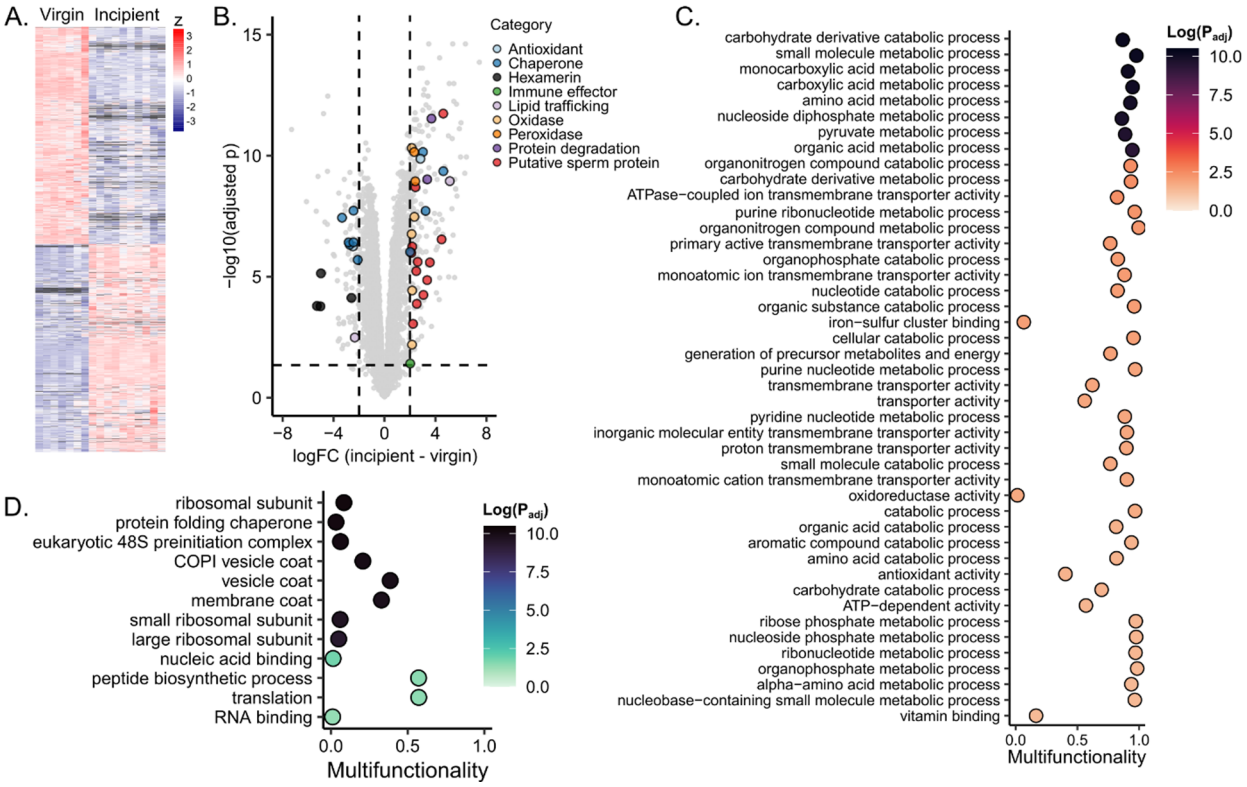

Fig. 2. *A. mellifera* differential and functional enrichment of spermathecal proteins. (**A**) 2796 (shown) of 4138 proteins were differentially expressed among virgin and incipient queens. (**B**) Protein functions of interest are color-coded. The horizontal dotted line indicates the 5% FDR threshold, and vertical dotted lines indicate $\log_2(FC) = 2$. Proteins with adjusted $p < 0.05$ and $\log(FC) > 2$ are considered intensely significant. (**C**, **D**) Significantly enriched GO terms among upregulated and downregulated proteins, respectively (Benjamini-Hochberg correction, 5% FDR). Higher multifunctionality scores indicate that enrichments are more likely to occur by chance due to component proteins being tied to many functions.

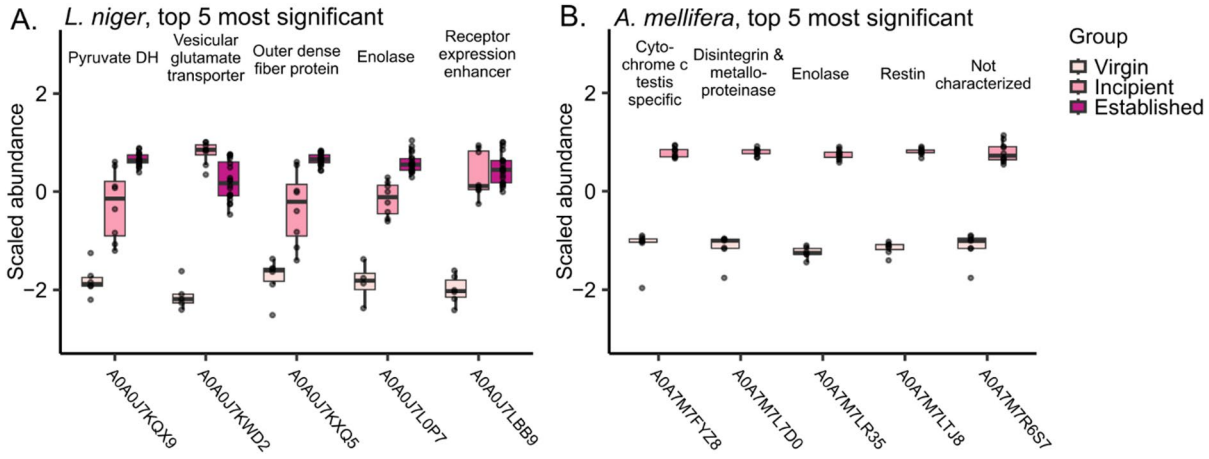

Fig. 3. Top five differentially expressed proteins in *L. niger* and *A. mellifera*. Pyruvate DH = pyruvate dehydrogenase. Boxes represent the interquartile range (IQR) and whiskers span 1.5 IQR. Median bars were removed if they obscured box size and colour. (**A**) *L. niger* proteins. (**B**) *A. mellifera* proteins.

two significantly decreasing, and one remaining unchanged after mating. All hexamerin genes were quantified in the spermathecal fluid of both species (two in *L. niger* and four in *A. mellifera*),and again marked species differences were apparent (Fig. 7C & D). In *L. niger*, one hexamerin was upregulated after mating and the other remained unchanged, while in *A. mellifera*, all four hexamerins were strongly downregulated after mating.

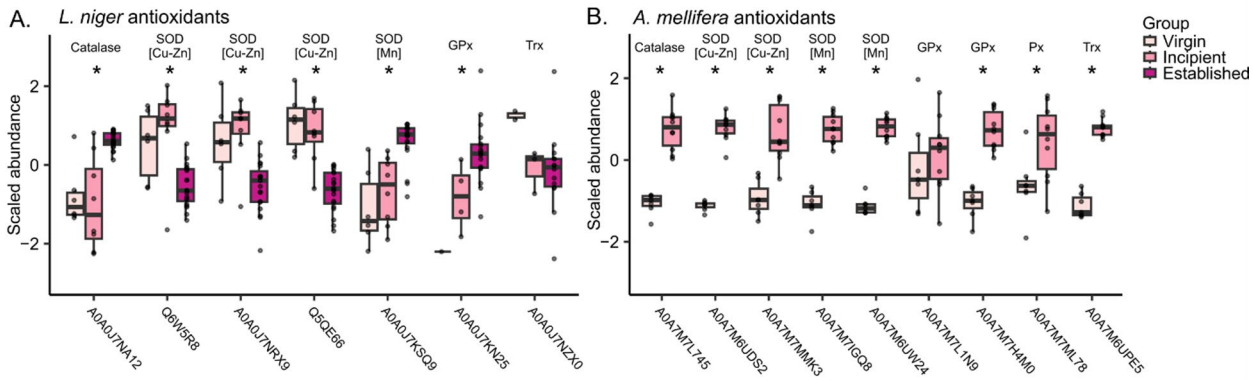

Fig. 4. Antioxidant enzyme expression. SOD = superoxide dismutase; GPx = glutathione peroxidase; Px = peroxidase; Trx = thioredoxin-dependent peroxidase. Boxes represent the interquartile range (IQR) and whiskers span 1.5 IQR. Asterisks indicate that the protein was differentially expressed at 5% FDR (Benjamini-Hochberg correction). Median bars were removed if they obscured box size and colour. (**A**) *L. niger* antioxidant enzymes. (**B**) *A. mellifera* antioxidant enzymes.

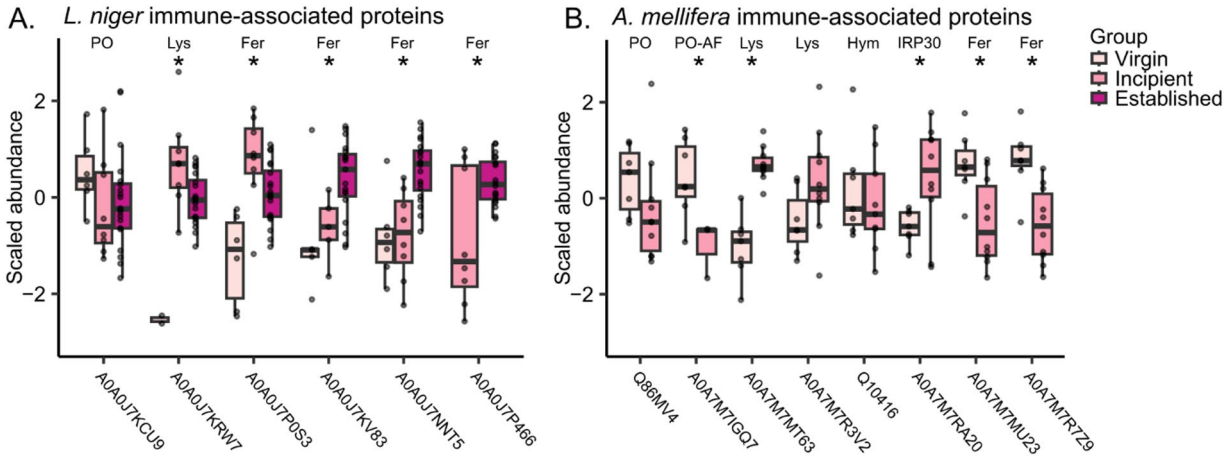

Fig. 5. Immune-associated proteins. PO = prophenoloxidase; PO-AF = prophenoloxidase activating factor; Lys = lysozyme; Fer = ferritin; Hym = hymenoptaecin; IRP30 = immune-responsive protein 30. Boxes represent the interquartile range (IQR) and whiskers span 1.5 IQR. Asterisks indicate that the protein was differentially expressed at 5% FDR (Benjamini-Hochberg correction). (**A**) *L. niger* immune-associated proteins. (**B**) *A. mellifera* immune-associated proteins.

Discussion

Our comparisons of *L. niger* and *A. mellifera* adult queen reproductive stages offers some of the richest proteomics data yet on spermathecal fluid samples that have been depleted of sperm. Although the reproductive stages we analyzed are not precisely congruent (with virgin, incipient, and established queens for *L. niger* and virgin and incipient queens only for *A. mellifera*), we are able to draw several key findings: (1) Our data tentatively support the notion that *L. niger* may have mechanisms to remove dead sperm components from the spermatheca, which could involve protein degradation machinery (e.g. the ubiquitin-proteasome system and other proteases); (2) Despite *A. mellifera* having reduced (relative to *L. niger*) selective pressure on efficient sperm maintenance, incipient honey bee queens appear to invest in elevating expression of a wide range of canonical antioxidant enzymes (catalase, SODs, GPx, Trx, and Px), whereas *L. niger* exhibits more narrow investment; (3) Canonical immune effector expression was similar between species, but *L. niger* consistently increased ferritin production (proteins involved in both immunity and mitigating oxidative stress) after nest establishment, possibly pointing to investment in alternative antioxidant proteins that moonlight as immune effectors; and (4) Our data confirm previous findings that *A. mellifera* upregulates GAPDH in the spermatheca after mating[12] but in *L. niger*, expression patterns of two versions of the enzyme (GAPDH and GAPDH-like) diverge, making it unclear if the enzyme has similar importance in this species. Confidently determining which expression patterns are a result of differences in age-related selective pressure will require additional experiments on species with more diverse life histories and on individuals across a broader range of ages, but these preliminary insights offer a starting point from which to explore these systems more deeply.

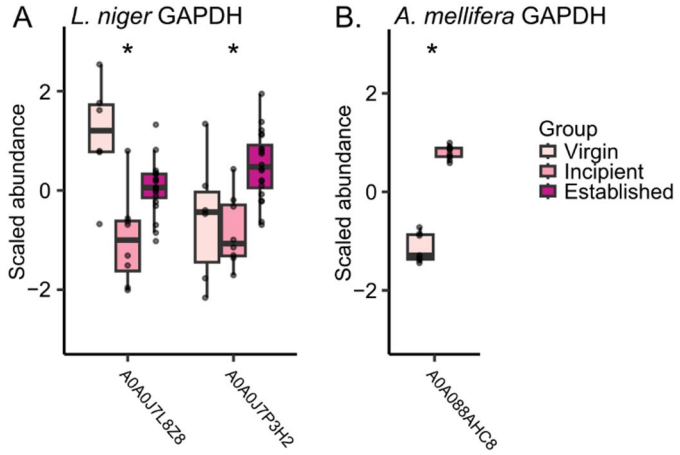

Fig. 6. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Boxes represent the interquartile range (IQR) and whiskers span 1.5 IQR. Asterisks indicate that the protein was differentially expressed at 5% FDR (Benjamini-Hochberg correction). (**A**) Enzyme isoforms in *L. niger.* (**B**) *A. mellifera.*

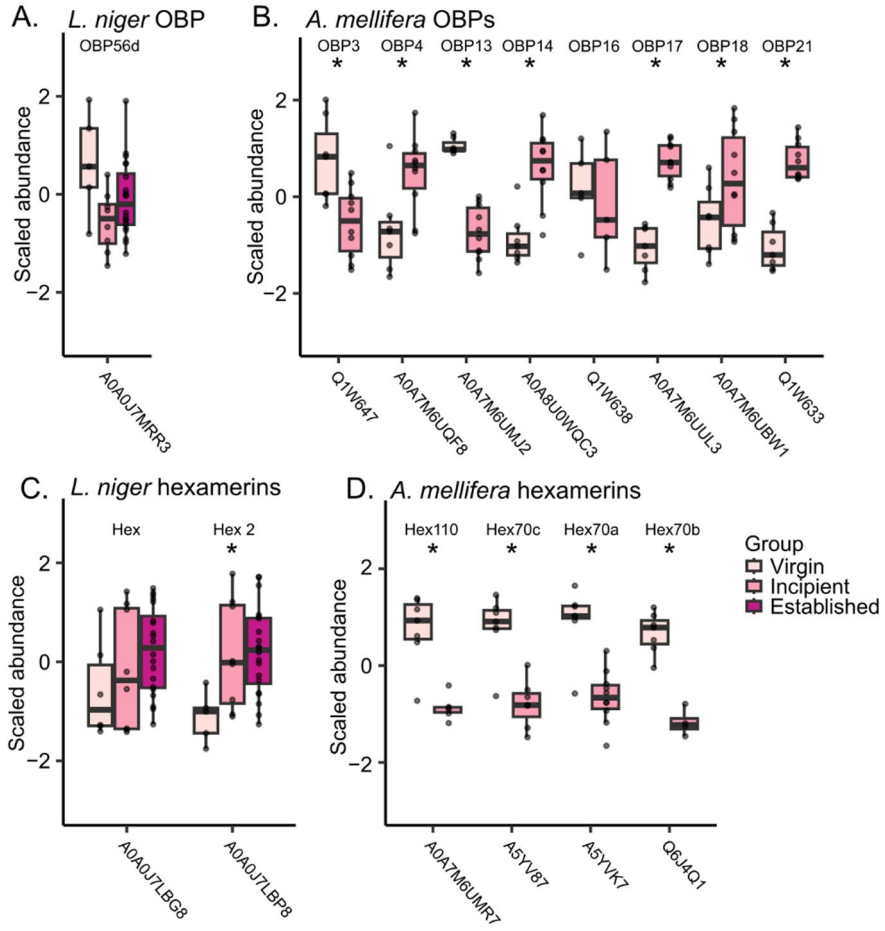

Fig. 7. Odorant binding protein and hexamerin expression. OBP = odorant binding protein; Hex = hexamerin. Boxes represent the interquartile range (IQR) and whiskers span 1.5 IQR. Asterisks indicate that the protein was differentially expressed at 5% FDR (Benjamini-Hochberg correction). (**A**) *L. niger* OBP (only one was quantified in the dataset). (**B**) *A. mellifera* OBPs. (**C**) *L. niger* hexamerins.

In both *L. niger* and *A. mellifera*, a large fraction of the spermathecal fluid proteome changed upon mating, with a subset of those appearing to have been derived from sperm (i.e., major components of flagella). This effect was more apparent in *L. niger*, with 21/172 (12.2%) putative sperm flagellar proteins among those intensely significant (adjusted $p < 0.05$ and fold-change > 4) in the virgin-to-incipient transition, 35/325 (10.8%) in the virgin-to-established transition, and 29/143 (20.3%) in the incipient-to-established transition. By contrast, in *A. mellifera*, only 12/301 (4.0%) were putative sperm proteins among those intensely significant in the virgin-to-incipient transition. Combined with (1) the significant enrichment of protein degradation machinery among *L. niger* proteins upregulated after mating (Fig. 1E), but not in *A. mellifera* (Fig. 2C), (2) the prior suggestion of a sperm degradation or removal system in *L. niger* queens[27], and (3) the persistence of higher abundances of putative sperm proteins in established relative to incipient mated queens (when spermathecae from both of which contain abundant sperm) (Fig. 1D), we suggest that this is a biologically relevant result and not simply a failure to remove sperm contaminants via centrifugation.

Together, these data support our first hypothesis, that *L. niger* may possess a mechanism for removing dead sperm, but the reason for this process being present in *L. niger* but absent in *A. mellifera* is unclear. Differences in mating frequency cannot explain the observed pattern. Sperm from males of highly polyandrous species compete to incapacitate each other[58], which may necessitate a recycling system of incapacitated sperm. However, *L. niger* queens are only facultatively polyandrous (normally mating with only one male, in which case the opportunity for sperm competition would be absent) while honey bees are highly polyandrous. Mating frequency and sperm competition are therefore not likely to be driving the need for a sperm recycling system.

While the underlying reason is elusive, the possibility of eliminating dead sperm from spermathecae is not a novel idea. Da Cruz-Landim found that, in stingless bees (*Melipona bicolor*), sperm appeared to be endocytosed by cells in the spermathecal epithelium[59]. However, the possibility of sperm endocytosis has been investigated intensively in *L. niger* and found not to occur[60]. The present data suggest that the cells could instead be degraded enzymatically prior to transport or recycling of their molecular residues, but without more targeted experimental data, this remains a speculation. No such sperm removal or recycling mechanism has been suggested in *A. mellifera* —— instead, dead sperm tend to accumulate in the spermatheca as a queen ages —— and indeed we see > 3-fold lower proportional representation of putative sperm proteins in the mated honey bee samples relative to *L. niger*, along with no enrichment for protein degradation machinery among differentially expressed proteins. The presence of fewer, but still some, putative sperm proteins in the mated honey bee queen samples is consistent with the observed build-up of dead sperm cells as queens of this species age, and could indicate passive or inefficient degradation as opposed to an active recycling process.

Our investigation of antioxidant enzymes did not match our prediction that *L. niger* would invest more heavily in ROS protection via antioxidant enzyme expression compared to *A. mellifera*. On the contrary, every antioxidant enzyme quantified in *A. mellifera* (catalase, SODs ——both Mn- and Cu/Zn-conjugated versions —— Px, GPx, Trx) was upregulated in incipient queens shortly after mating (Fig. 4B). In *L. niger*, for which we had an additional group of established queens sampled 9–10 weeks after mating, some enzymes were upregulated in this group (catalase, Mn-conjugated SOD, and GPx), while some were downregulated (Cu/Zn-conjugated SODs), and none significantly differed between incipient mated queens and virgins (Fig. 4A).

The marked difference in regulation of the different SOD metalloenzymes is intriguing but the functional relevance of this remains elusive, since all SODs catalyze the same reaction (converting superoxide radicals to hydrogen peroxide and molecular oxygen). Interestingly, previous data shows that Cu and Zn ion concentrations are elevated in the spermathecal fluid of older *L. niger* queens relative to virgins[60]. Since the SODs using these cofactors were conversely downregulated after nest establishment, this implies that the metal ions are serving a different purpose, which is yet to be determined. Regardless, the fact that only one SOD was elevated in established *L. niger* implies that these queens may have a reduced need for removal of superoxide radicals. Ant and honey bee spermathecae are reportedly similarly anoxic[12,13], but some data show that ROS and H_2O_2 are both elevated in honey bee spermathecal fluid after mating[11], which may point to an increased need for antioxidant capabilities in this species. Whole-body Cu/Zn-conjugated SOD is expressed at lower levels in *L. niger* queens relative to drones and, to a lesser degree, workers[61], despite drones having lifespans that are more than two orders of magnitude shorter than the queens. While absolute quantitation or analysis of enzyme activity levels may be more enlightening than relative quantitation between reproductive stages, together, these data suggest that Cu/Zn-conjugated SODs may be less essential when it comes to both sperm and individual longevity. Perhaps the queens have other mechanisms of limiting ROS generation at the source, rendering extensive antioxidant systems unnecessary.

We expected that *L. niger* may exhibit reduced investment in innate immune defenses after mating, owing to the greater pressure on efficient sperm maintenance in this species, but this is generally not what we found. Among canonical immune effectors (i.e. antimicrobial peptides, lysozymes, and phenoloxidase), only phenoloxidase and lysozyme were quantified in *L. niger*, which displayed consistent patterns of expression with the same proteins in *A. mellifera* (Fig. 5). However, ferritins were consistently elevated after mating in *L. niger* but reduced after mating in *A. mellifera*. This is noteworthy because ferritins appear to be non-canonical immune proteins[51,62–64], as well as antioxidant proteins (due to iron sequestration, preventing the formation of hydroxyl radicals via the Fenton reaction)[65]. We speculate that *L. niger* could be achieving efficient antioxidant protection (and immunity) by investing in multifunctional proteins such as these instead of the typical antioxidant enzymes, like *A. mellifera*.

Previous work in *A. mellifera* has shown that metabolism of GAP is an efficient way to produce ATP anaerobically, and generating energy in this way supports sperm viability[12]. Since this system would theoretically be advantageous in both species, we therefore expected GAPDH to become elevated in both *L. niger* and *A. mellifera* after mating. Our data confirm that GAPDH was strongly elevated after mating in *A. mellifera*, but the results are less clear for *L. niger* (Fig. 6). Because *L. niger* has two isoforms of this enzyme, and they show opposite patterns of expression (with GAPDH-like increasing upon nest establishment and GAPDH decreasing),

further experimentation is necessary to determine how these enzymes' properties differ and whether GAPDH-like expression is sufficient to support the similar ATP generation efficiency as seen in *A. mellifera*. Enolase (also known as phosphopyruvate hydratase) however, was among the top 5 most strongly differentially expressed proteins in both species and, like GAPDH, is a key enzyme in anaerobic glycolysis. Pyruvate dehydrogenase (which is normally inhibited under anaerobic conditions) was additionally among the same group of top proteins upregulated after mating in *L. niger*; a puzzling finding, given the anoxic environment of the spermatheca.

In addition to the above-discussed *a priori* hypotheses, here we highlight some additional and surprising patterns of expression of OBPs and hexamerins (Fig. 7). OBPs are typically thought to function as soluble odorant transporters, carrying odorants from the antennal sensilla, through the sensillum lymph fluid, to odorant receptors on the odorant receptor neurons[52–54,66]. However, they are widely expressed among non-olfactory tissues[55] and therefore likely carry out multiple functions, as suggested by Pelosi *et al.*[53]. We have previously quantified a plethora of OBPs in *A. mellifera* ejaculates[67] and found that OBP14 was significantly linked to sperm viability in spermathecal fluid (among many other OBPs identified)[68], in addition to quantifying eight distinct OBPs in *A. mellifera* here. The fact that seven of these OBPs were differentially expressed after mating suggests that they are serving an important function in this tissue, possibly as carriers of other small molecules, such as hormones or other hydrophobic signalling ligands. Only one OBP was quantified in *L. niger*, and it was not differentially expressed, but proteome coverage was generally low in this species and a deeper proteome will be necessary to further explore this topic. Hexamerins, while typically being described as a source of amino acid residues during metamorphosis, have also been implicated as hormone transporters (analogous to albumins in vertebrates)[57]. Here, hexamerins also showed striking patterns of expression in *A. mellifera*, with all four proteins becoming dramatically downregulated after mating, whereas in *L. niger*, one of two hexamerins changed, modestly increasing by the time of nest establishment. These data point to divergent roles in these two species' spermathecae, but those functions remain to be clarified.

A further insight gleaned from these data is that the product of spermathecal gland secretions appears to be temporally regulated. If the spermatheca of a virgin already contains the necessary elements to support sperm storage, one might expect the queen-derived spermathecal fluid fraction (as opposed to putative sperm-derived proteins) to remain stable through the virgin-to-mated transition. However, that is not what we observed; rather, even among proteins that are unlikely to be sperm remnants, both up-regulated and down-regulated shifts occurred upon mating in both species. This may be because, early in life, it would be advantageous for virgins to invest resources in expressing proteins in other tissues that support imminently critical tasks (feeding, flying, navigation, etc.), creating a resource deficit for the spermathecal glands. The spermathecal fluid in virgin queens may also be adapted to serve a different function —— reception of metabolically active sperm and transitioning them to quiescence —— than mated queens, which need only maintain sperm viability. Sperm can take > 1 day (~ 40 h for honey bees[69] to enter the spermatheca after mating, during which time the glandular secretion may switch to investing more heavily in producing proteins that are more critical for sperm maintenance. These speculations require further testing by evaluating finer-scale age distributions of both virgins and mated queens, but it provides a possible framework in which adaptive regulation of spermathecal gland products appears plausible.

These data cumulatively provide a rich view into mating-induced changes in the proteins present within queen spermathecae; however, our study does come with several additional limitations. First, despite *L. niger* and *A. mellifera* queens being capable of extraordinary long lifespans (> 20 years for *L. niger*[1] and up to 8 years for *A. mellifera*[5], the queens we sampled were comparatively young (up to 9–10 weeks after mating for *L. niger* and ~ 1 week after mating for *A. mellifera*). We did not sample older queens here, but such samples would add substantial value to the dataset by providing insight into whether additional species differences arise at an older age, potentially helping to explain their differential lifespans. If such samples are generated in the future, care must be taken to eliminate, reduce, or at least quantify extraneous variables that tend to pose a greater influence on sample variation over time (differences in infection status, environment, nutrition, etc.). Second, a more well-rounded experimental design would include spermathecal fluid samples from queens with a shorter lifespan, such as those within the *Bombus* genus. A larger diversity of representative queen lifespans may clarify some of the proposed life history-associated differences described here. Future work addressing both of these points, while possibly conducting a more targeted evaluation of sperm recycling mechanisms, would offer a significant advancement in our understanding of sperm storage processes.

Conclusion

Although sperm maintenance is a common feature among all eusocial hymenopterans, different species likely have evolved different strategies for the task, given their disparate lifespans, fertilization efficiencies, and possible sperm recycling mechanisms. We indeed found similarities but also marked differences in the spermathecal fluid proteomes of *L. niger* and *A. mellifera* before and after mating. Most notably, we identified preliminary support for the notion that *L. niger* may have the capacity to break down sperm internally with the help of proteolytic enzymes, but further experimentation is necessary to fully ascertain this idea. Despite *L. niger* having intensified maintenance requirements (in terms of supporting high-efficiency fertilization and an extreme duration of storage), this species appears to narrowly invest in canonical antioxidant enzymes compared to *A. mellifera*, with all three Cu/Zn-conjugated SODs counterintuitively declining with nest establishment. However, this deficiency could be partly compensated by elevated production of antioxidant ferritins, which may double as immune proteins, and diversify the types of radicals that can be suppressed or neutralized. Future work should focus on studying additional species and measuring absolute values of enzyme activity and ROS concentrations to enable direct species comparisons, which would offer clearer data on whether long-lived hymenopterans are under more intense selection for sperm preservation, as we expect.

Data availability

The raw mass spectrometry data, search results, FASTA databases, and sample metadata are all publicly available on the MassIVE proteomics data repository (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp) under the accession MSV000092460 for *L. niger* and MSV000096180 for *A. mellifera.* All label-free protein quantification data, sample metadata, and statistical outputs are additionally available in Data S1.

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Author contributions

AM and SA conceptualized the experiment. AM conducted the proteomics analysis, generated figures, performed statistical tests, and wrote the first draft of the manuscript. FD and SEH supplied biological material. SA and LJF provided supervision and resources. All authors edited and approved of the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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