Research Article

Stored sperm differs from ejaculated sperm by proteome alterations associated with energy metabolism in the honeybee *Apis mellifera*

Running title: Proteomic differences in ejaculated and stored sperm

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Abstract

Sperm are exposed to substantially different environments during their life history, such as seminal fluid or the female sexual tract, but remarkably little information is currently available about whether and how much sperm composition and function alters in these different environments. Here we used the honeybee *Apis mellifera* and quantified differences in the abundance and activity of sperm proteins sampled either from ejaculates or from the female's sperm storage organ. We find that stored and ejaculated sperm contain the same set of proteins but that the abundance of specific proteins differed substantially between ejaculated and stored sperm. Most proteins with a significant change in abundance are related to sperm energy metabolism. Enzymatic assays performed for a subset of these proteins indicate that specific protein activities differ between stored and ejaculated sperm and are typically higher in ejaculated compared to stored sperm. We provide evidence that the cellular machinery of sperm is plastic and differs between sperm within the ejaculate and within the female's storage organ. Future work will be required to test whether these changes are a consequence of active adaptation or sperm senescence, and whether they alter sperm performance in different chemical environments or impact on the cost of sperm storage by the female. However, these changes can be expected to influence sperm performance and therefore determine sperm viability or sperm competitiveness for storage or egg fertilisation.

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Introduction

25 Sexual reproduction triggered the evolution of anisogamy (Birkhead *et al.*, 2009; Parker
26 *et al.*, 1972) resulting in egg-producing females investing more resources per offspring
27 than sperm evolving males (Simmons, 2001). As selection potentiated gametic
28 dimorphism, sperm became increasingly simplified cells with less of the physiological
29 machinery that is typically found in somatic cells (Birkhead *et al.*, 2009; Parker *et al.*,
30 1972). Indeed sperm cells have a highly reduced cytoplasm and are assumed to be
31 transcriptionally silent (Hecht, 1998). Recent work has generated novel insights into the
32 biology of sperm and has fundamentally changed the earlier notion that sperm are merely
33 propelled pieces of DNA (see review by Ainsworth, 2005). Sperm are now recognized as
34 highly diverse and specialized cells providing much more to paternity than DNA alone.
35 For example, there is increasing evidence that sperm morphology (see for example
36 Fitzpatrick *et al.*, 2009) as well as its molecular content beyond the genome itself (Dorus
37 *et al.*, 2006; Ostermeier *et al.*, 2002) are major determinants of male fitness (Baer,
38 Schmid-Hempel, 2005; Birkhead *et al.*, 2009). However, despite intense work conducted
39 on the biology of sperm we have remarkably little information about sperm plasticity
40 during its life history. For example, it seems obvious that the degree to which sperm
41 might have to respond or adapt to different surroundings, such as the seminal fluid in the
42 ejaculate, the females sexual tract or storage organ, or the surface and cytosol of the egg,
43 might determine paternity success. This is especially true for species where females

44 copulate with multiple males and sperm from different ejaculates compete against each
45 other for egg fertilizations.

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47 Most of what is currently known about sperm plasticity has been observed as changes in
48 sperm physiology and visual attributes of sperm function. For example, sperm
49 capacitation is known to be a crucial process that enables sperm to undergo the acrosome
50 reaction required for egg fertilization (Poiani, 2006). Sperm is also known to become
51 hyper-activated prior to fertilization in various mammal species (Birkhead *et al.*, 2009).
52 Finally sperm senescence has been demonstrated to reduce sperm viability and
53 performance (den Boer *et al.*, 2009a; White *et al.*, 2008; see Pizzari *et al.*, 2008 for a
54 review). All these traits are likely accompanied by molecular changes within the sperm
55 itself but they have not been identified to a great extent, despite their obvious
56 consequences on paternity success and potential use as biomarkers of sperm performance.

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58 Social hymenopteran insects (the bees, ants and wasps) provide an interesting opportunity
59 to study the biology of sperm and the process of sperm adaptation. In these species,
60 females (queens) mate only at the beginning of their life in order to acquire sperm, which
61 is then stored in a specialized organ, the spermatheca (Baer, 2003; Baer, 2005; Baer,
62 2010; Boomsma *et al.*, 2005). These sperm are used throughout the queen's life to
63 fertilize eggs and are never replenished. Sperm storage and use has been taken to
64 spectacular extremes in some social insects where hundreds of million of sperm are
65 initially stored and kept alive over several decades for the production of millions of
66 offspring (den Boer *et al.*, 2009a). In the absence of female remating, selection for the

highest quality sperm has shaped sperm form and function in social insects (Baer *et al.*, 2006).

Recent work in honeybees has provided new insights into the molecular environment of sperm and its effects on sperm performance (Al-Lawati *et al.*, 2009; Collins *et al.*, 2006). During ejaculation, males provide glandular secretion to sperm in the form of seminal fluid which is of central importance for sperm survival (den Boer *et al.*, 2008; den Boer *et al.*, 2009b) and competitiveness (den Boer *et al.*, 2010) and its main protein constituents have been identified (Baer *et al.*, 2009b). Queen bees provide stored sperm with glandular secretions in the form of spermathecal fluid. Interestingly, the proteomes of honeybee seminal fluid and spermathecal fluid are fundamentally different (Baer *et al.*, 2009a). Whereas the protein profile of seminal fluid contains elements to optimize sperm storage success, spermathecal fluid appears to provide a cytosol-like environment to ensure long-term sperm survival (Baer *et al.*, 2009a). Honeybee sperm can survive in both of these environments but whether and how sperm physiology is adjusted to achieve this remains unknown. As sperm can be sampled from ejaculates as well as from the spermatheca (see methods), honeybees offer an opportunity to quantify differences in the proteome of ejaculated and stored sperm to gain insights into molecular sperm modification and its phenotypic plasticity over sperm life history.

In this study, we quantify differences in the proteomes of freshly ejaculated sperm and stored sperm extracted from the female storage organ. To do this, we first performed a series of experiments to demonstrate that we are able to efficiently separate sperm from

its surrounding environment, the seminal fluid, before identifying the major proteins in honeybee sperm. We then performed a comparative proteomic analysis of purified sperm collected from ejaculates and spermathecae that revealed that all proteins in the ejaculate are also present in stored sperm and *vice versa*, but we found significant quantitative differences in protein abundances. Finally we performed enzymatic tests for a subset of these proteins and show that their catalytic activity is indeed different in stored and ejaculated sperm.

Material and Methods

Animal breeding and sampling

Honeybee males were bred in colonies of *Apis mellifera carnica* kept at the University of Western Australia and used at an age of 2-3 weeks after eclosure to ensure they had reached sexual maturity. Male sperm was collected using a standardized protocol (Baer *et al.*, 2009b), with each male providing approximately 1 µl of ejaculate. A total of three independent biological samples of 100, 118 and 100 µl of ejaculate became available. To collect stored sperm, a sample was extracted from spermathecae of egg laying queens that were between 9 and 24 month of age using a standardized protocol developed earlier (Baer *et al.*, 2009a) with an average yield of about 0.5 µl of spermathecal content per queen. Three independent biological samples of 20 µl, 35 µl and 50 µl of stored sperm became available.

Sperm purification protocol

In order to remove non-sperm components such as seminal or spermathecal fluid from samples collected, we developed the following protocol. Sperm samples collected as outlined above were first diluted 2.5 times in Hayes solution (9.0 g/l NaCl, 0.2 g/l CaCl₂, 0.2 g/l KCl, 0.1 g/l NaHCO₃, pH 8.7), briefly mixed and centrifuged for 20 min at 4°C and 16100g to separate sperm from its surrounding seminal or spermathecal fluid. The supernatant was collected and centrifuged again for 20 min at 16100g and 4°C to remove remaining sperm. The sperm pellet was resuspended in Hayes solution using 5 times the original sample volume and centrifuged for 5 min at 16100 g and 4°C. After removing the supernatant this step was repeated twice although samples were centrifuged for 10 minutes during the last step. After removing the supernatant of the final centrifugation the purified sperm pellet was weighed and re-suspended in Hayes solution to a final concentration of 100 mg/ml. All sperm and supernatant samples were stored at -80°C prior to further analyses.

Gel electrophoresis

Protein profiles of honeybee sperm from purified ejaculate samples were characterized and identified using two-dimensional IEF/SDS-PAGE gels. To do this sperm proteins were acetone precipitated and resolubilized in 8 M Urea, 2% (w/v) CHAPS, 37.3 mM Dithiothreitol and 0.5 % (v/v) 3-11NL IEF buffer. The sample was loaded to pH 3-11NL 24cm strips (Immobiline DryStrip, GE Healthcare) and run on an IPGphor Unit (GE Healthcare) using the following settings: 12 h at 30 V (rehydration step), 1 h at 500 V, 1 h gradient from 500 V to 1000 V, 1 h gradient from 1000 V to 3000 V, 2 h gradient from 3000 V to 8000 V and 5 h at 8000 V.

After focusing, IEF strips were incubated in equilibration buffer (6 M urea, 50 mM Tris, 4% (w/v) SDS, 26% (v/v) glycerol) first containing 65 mM dithiothreitol and then containing 135 mM iodoacetamide. After a brief wash in 1.5 M Tris/HCl, 1% SDS, pH 8.8, the strips were transferred on top of a 12% acrylamide gel where they were covered with 1.2% agarose in 1x-gel buffer (including a trace of bromophenol blue). Second dimension SDS gels were run at 12 mA per gel for 18 hours. For Coomassie blue (G 250) colloidal staining, gels were then incubated in fixing solution (40% methanol, 10% acetic acid) for an hour before being stained overnight. Gels were kept in 0.5% (v/v) orthophosphoric acid at 4°C prior to protein identifications using mass spectrometry.

To compare proteomic differences between seminal fluid and sperm or stored and ejaculated sperm we used two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) using standard lab procedures. For 2D DIGE IEF/SDS-PAGE, all sperm samples were acetone precipitated and resolubilized in rehydration buffer (8 M Urea, 2% (w/v) CHAPS). Proteins from seminal fluid samples were concentrated in rehydration buffer using a 5 KMWCO spin filter column (Millipore). To determine protein concentration, 1µl of each sample was run on 1D SDS-PAGE gels alongside a sample of known concentration in duplicate. Samples were then adjusted to the same concentration based on intensity measurements of lanes from ImageJ 1.40g (<http://rsbweb.nih.gov/ij/>). Each sample containing approximately 50 µg of protein was minimally labelled with 400 µM of either Cy3 or Cy5 dye (GE Healthcare). A pooled sample was created and labelled with Cy2 dye. Samples to be compared were then mixed along with a third of the pooled sample. Labelling was performed according to manufacturer's instructions. Samples were then run on 2D IEF/ SDS PAGE gels as described above.

Proteins were visualised on a Typhoon™ laser scanner (GE Healthcare) in fluorescence mode and scanned at a resolution of 100 microns using a PMT voltage of 505V for the sperm/seminal fluid comparison and 520V for the ejaculate/stored comparison. Overlay images were created using the ImageQuant TL software (GE Healthcare). Quantitative analyses were performed using the DeCyder™ software package, Version 6.5 (GE Healthcare). Each gel was first processed using the differential in-gel analysis tool and spots were filtered based on slope, peak height and volume ratio. All three gels were then combined using the Biological Variance Analysis module. Student's t-tests were used to determine whether individual spots changed significantly in abundance between sperm and seminal fluid or between ejaculated and stored sperm. All spots were visually inspected before inclusion in the final results and only spots that appeared in all three gels were used.

Mass spectrometry

Protein identifications were based on MS/MS runs of trypsin digested gel spots using a MALDI-TOF/TOF and an ESI – Ion trap. Coomassie colloidal blue stained protein spots were cut from gels and destained twice in 10 mM Na₂HCO₃ with 50% (v/v) acetonitrile. Samples were dried at 50°C before being rehydrated with 15 µl of digestion solution (10 mM NH₄CO₃ with 12.5 µg/ml trypsin (Invitrogen) and 0.001% trifluoroacetic acid) and incubated over night at 37°C. Tryptic peptides were extracted from gel plugs using 15 µl of acetonitrile. The supernatant was collected and plugs were washed twice with 15 µl of 50% (v/v) acetonitrile and 0.5 % (v/v) trifluoroacetic acid. These supernatants were

combined with initial supernatant. The pooled extracts were dried by vacuum centrifugation and stored at -20°C before being analysed by mass spectrometry.

Gel spots from 2D DIGE gels were analyzed on an Agilent XCT Ultra Ion Trap mass spectrometer with ESI source equipped with a low flow nebuliser in positive mode and controlled by Chemstation (Rev B.01.03 [204]: Agilent Technologies) and MSD Trap Control software v6.1 (Bruker Daltonik GmbH), as described in (Baer *et al.*, 2009b). MS/MS spectra were exported from the Data Analysis for LC/MSD Trap version 3.3 (Build 149) software package (Bruker Daltonik GmbH) using default parameters for AutoMS(n) and compound Export. The resulting .mgf files, which were then searched as outlined below.

Gel spots cuts from the 2D IEF/ SDS PAGE gel were run on a Bruker Ultraflex III MALDI TOF/TOF with a Smartbeam solid-state laser. Samples were spotted onto polished steel plates after resuspension in 2 µl of α -Cyano-4-hydroxy-cinnamic acid matrix and allowed to dry. Before each run calibration was performed using a standard mixture of peptides and the laser power adjusted for optimal signal: noise ratio. In MS mode, 400 shots/ spots were collected in a random walk pattern. Spectra from these were automatically processed by FlexAnalysis (Bruker Daltonics) software. For those spots with an identified peptide mass fingerprint, a maximum of 5 parent ions were selected for MS/MS measurements, while for those with no identified peptide mass fingerprint up to 10 parent ions were selected. Only parent ions with a Goodness factor > 175 were used. For each MS/MS spectrum 250 shots were recorded for the parent and 700 shots recorded

for the fragments following a random walk. Spectra were automatically sent to the BioTools software (Bruker Daltonics) for analysis.

Mass spectra output files were analyzed against the predicted *Apis mellifera* peptide set (PreRelease2, 11069 sequences; 5989390 residues) from BeeBase (<http://www.beebase.org/>). For 2D DIGE gel samples analyzed on the Ion Trap, searches were conducted using the Mascot search engine version 2.2.03 (Matrix Science) utilizing error tolerances of ± 1.2 Da for MS and ± 0.6 Da for MS/MS, 'Max Missed Cleavages' set to 1, the Oxidation (M) and Carboxymethyl (C) variable modifications and the instrument set to ESI-TRAP and peptide charge set at '2+ and 3+'. For reference gel spots run on the MALDI TOF-TOF, searches were conducted using the Mascot search engine version 2.2.03 (Matrix Science) in the BioTools software (Bruker Daltonics) utilizing error tolerances of 100 ppm for MS and ± 0.4 Da for MS/MS, 'Max Missed Cleavages' set to 1 the Oxidation (M) and Carboxymethyl (C) variable modifications, the instrument set to Maldi TOF-TOF and peptide charge set at +1. Results were filtered using 'Standard scoring' with the 'Significance threshold' at $p < 0.05$. To build protein lists we applied conservative approaches to minimize false positives. Protein matches were only claimed if at least two distinct peptides were detected per protein, and MOWSE scores were higher than 50 ($p < 0.05$ significance level is score > 37).

Each identified protein sequence was BLAST searched to identify homologous proteins from insects and other organisms. We used only the protein sequence between (and including) detected peptides in our search so as to minimize any effect from annotation inconsistencies. This process was used to confirm or modify the functional annotation of

the proteins from the honeybee PreRelease2 dataset. Each protein was placed into a functional category (as done previously (Baer *et al.*, 2009b)) according to its annotation and, if necessary, manual literature searches.

3. Sperm reference gel

We created a reference gel of sperm proteins using approximately 1 mg of protein from ejaculated sperm that was run on a 2D IEF/ SDS PAGE gel and analyzed using MS/MS (MALDI TOF-TOF) as described above. Proteins identified in honeybee sperm were compared to those previously found in *D. melanogaster* sperm (Dorus *et al.*, 2006) from downloaded 381 sequences of *D. melanogaster* sperm proteins from FlyBase (<http://flybase.org/>). Sperm proteins identified in honeybees were also compared to the entire in silico proteome of *D. melanogaster* using the dmel-all-translation-r5.3.fasta file downloaded from <ftp://ftp.flybase.net>.

Spectrophotometric enzyme activity assays

To test whether differences in protein abundances between stored and ejaculated sperm reflect changes in the enzymatic activity we performed assays measuring $V_{\max}$ for six different proteins with significantly changed isoform abundances between stored and ejaculated sperm (Supplemental Table 6). To do this sperm samples were prepared for assays by adding Triton X-100 to a concentration of 5%. Samples were left on ice for 10 min and followed by centrifugation for 10 min at 16100g at 4°C. An aliquot of the supernatant (2.5 µl) was run on a 1D SDS-PAGE gel to ensure equal protein

concentration in the ejaculated and stored sperm. The $V_{\max}$ was measured in a U2810 Spectrophotometer (Hitachi).

Reaction mixtures were in a final volume of 500 μ l in quartz cuvettes and 16.7 μ l of sample was added to start the reaction. The following concentration of reagents were used: i) Aldolase – 2.3 mM hydrazine sulphate, 4.0 mM fructose -1,6-bisphosphate pH 7.5, with the formation of hydrazone measured at 240 nm (Drechsler *et al.*, 1959) ii) TPI – 220 mM triethanolamine HCl, pH 7.6, 3.3 mM DL-glyceraldehyde-3- P, 0.27 mM NADH, 1.7 U/ml glycerol-3-phosphate dehydrogenase, with the oxidation of NADH measured at 340 nm (Bergmeyer, 1974) iii) GAPDH – 83 mM triethanolamine, pH 7.6, 6.7 mM 3-phosphoglyceric acid, 3 mM L-Cysteine, 2 mM magnesium sulphate, 0.1 mM NADH, 1.1 mM ATP, 10 U 3-phosphoglyceric phosphokinase, with the oxidation of NADH measured at 340 nm (Bergmeyer, 1974) iv) Enolase – 81 mM triethanolamine, pH 7.4, 1.9 mM 2-phosphoglyceric acid, 0.12 mM NADH, 25 mM magnesium sulphate, 100 mM potassium chloride, 1.3 mM ADP, 7 U pyruvate kinase, 10 U L-lactic dehydrogenase, with the oxidation of NADH measured at 340 nm (Bergmeyer, 1974), v) Dihydrolipoamide dehydrogenase – 50 mM sodium phosphate pH 7.2, 1 mM EDTA, 40 μ M 2,6-dichlorophenolindophenol, 0.2 mM NADH, with the reduction in 2,6-dichlorophenolindophenol measured at 600 nm (Patel *et al.*, 1995), vi) Ornithine aminotransferase – 163 mM Tris-HCl, pH 8.0, 5 mM L-ornithine, 10 mM α -ketoglutaric acid, 0.25 mM NADH, with the oxidation of NADH measured at 340 nm (Charest, Phan, 1990).

Results

1. Separation of sperm from seminal fluid

Gel spot differences on DIGE gels and MS/MS identifications

A 2D DIGE analysis comparing protein abundances between sperm and seminal fluid isolated using our methods revealed little overlap between the two samples (Figure 1a). A quantitative analysis of 385 protein spots matched across all the samples revealed that 85% significantly differed in abundance between ejaculated sperm and seminal fluid (average ratio ≥ 2 or ≤ -2 , $p < 0.05$). A total of 137 protein spots were found to have higher abundance in seminal fluid (range 2.0 – 194.7, mean = 21.2) and 190 protein spots were significantly more abundant in sperm (range 2.0 – 62.5, mean = 14.2). This indicated that the protein composition of sperm and seminal fluid are substantially different from each other. The 82 gel spots that could be matched from the 2D-DIGE analysis to the same gels, when they were Coomassie-stained (Figure 1b) were analyzed by MS/MS. A subset of 74 of these 82 protein spots were positively matched to honeybee proteins, but some protein spots contained matches to the same proteins, while seven spots contained matches to two different proteins (Supplemental Table 1). The final result was a list of 50 non-redundantly identified proteins. Within this set of 50, 13 and 24 proteins could be classified as proteins for either seminal fluid or sperm, respectively, while 13 could not be classified statistically into one group or the other (Supplemental Table 1).

MS/MS analysis of reference gel spots

Proteins from the purified ejaculate sperm sample reference gel separated by 2D IEF/SDS PAGE were then used to identify other protein spots by tandem mass spectrometry (MS/MS) (Figure 2, Supplemental Tables 2, 3 & 4). From the 228 spots investigated 46 revealed no significant hits to any honeybee proteins (Supplementary Table 4). The analysis of the remaining spots resulted in a list of only 52 different proteins, indicating that many spots represent modified versions of the same proteins. These are shown redundantly in Supplementary Table 4, and are grouped together by Am Locus in Supplementary Table 2. The identified proteins formed six functional groups; the allocation of proteins to these groups is shown in Supplementary Table 2, while the total numbers in each group are shown in Supplementary Table 5. We found that 16 proteins were previously identified in our preliminary analysis of sperm proteins (Baer *et al.*, 2009b) while 30 were identified in an independent study of semen and seminal vesicles from honeybee (Collins *et al.*, 2006). These comparisons are shown in detail in Supplementary Table 2, and are counted across functional groups in Supplementary Table 5. Sequence comparison of the sperm proteins with the known set of *D. melanogaster* sperm proteins and the total *D. melanogaster* proteome was performed using BLASTP (Supplementary Table 3). For 42 (81%) of the sperm proteins we found homologs in the *D. melanogaster* proteome and 32 (62%) of these homologs are known *D. melanogaster* sperm proteins, with most proteins being involved in energy metabolism or cellular structure. Details are shown in Supplementary Table 3, and the functional grouping of these homologs are shown in Supplementary Table 5.

These results led us to conclude that the methods used are a reliable way to efficiently separate proteins from external secretions away from sperm, and that peptide MS/MS

analysis identified a core set of abundant honeybee sperm proteins. This finding is in accordance with our previous work, where fluorescence and light microscopic work indicated that our centrifugation protocol efficiently separates sperm from its surrounding seminal fluid (Baer *et al.*, 2009, King, Eubel, Millar & Baer, submitted and unpublished data).

2. Proteomic differences between stored and ejaculated sperm

Gel spot differences on DIGE gels

Three sets of biological replicates were used to statistically analyze the differences between the proteomes of ejaculated and stored sperm. A total of 388 gel spots could be matched across all three DIGE gels. This set of protein spots could be quantified across all 6 samples and the combined internal standard on each gel. While the overall protein profiles did not significantly differ between ejaculated and stored sperm, we found significant differences in the abundance of specific protein spots (Figure 3a). Protein abundances of 185 spots (48%), changed at least 1.5 times (range 1.5 – 55.4) between stored and ejaculated sperm ($p < 0.05$). In 77 cases, spot abundances were higher in ejaculated sperm and 108 spots had a significantly higher abundance in stored sperm.

Identification of proteins differing in abundance

Protein spots with altered abundance were identified by excising spots and undertaking peptide mass spectrometry. The low abundance of proteins on DIGE gels and differing intensity of Cy-dyes and Coomassie staining meant that only 39 Coomassie-stained DIGE gels spots and 17 spots from reference 2D IEF/SDS PAGE gels were used for protein

identification. The mass spectrometric analysis successfully identified 18 different proteins from 38 spots (Figure 3 b, Supplemental Tables 4 & 6). As three of these proteins, found more abundantly in ejaculates, were detected earlier within the seminal fluid (GB17780-PA, GB16552-PA, GB10133-PA (Baer *et al.*, 2009a; Baer *et al.*, 2009b)) we removed them as possible contaminants from further analyses. This resulted in a list of 15 different proteins that displayed changes in abundances between stored and ejaculated sperm (Table 1, Supplemental Table 6). From these, 9 are crucial for the energy supply of cells and are well known enzymes of carbohydrate, glycolytic or respiratory metabolism. We also detected four protein spots for aldose reductase with a significantly decreased abundance in stored sperm suggesting degradation and/or modification of this enzyme during storage. Aldose reductase is an NADPH-dependent enzyme of carbohydrate metabolism that reduces glucose to sorbitol, that can then be converted to fructose without the use of ATP. Fructose is an abundant component of seminal fluid (Blum *et al.*, 1962) and is known as a metabolite used by sperm for energy production (Williams, Ford, 2001).

Catalytic activity of proteins with differed abundances between stored and ejaculated sperm

To test whether changes in protein abundance are linked to differences in protein activity in purified sperm samples, we measured the maximal catalytic activity of four glycolytic enzymes where we detected significantly higher abundance of isoforms in stored compared to ejaculated sperm: aldolase, triosephosphate isomerase, GAPDH and enolase

(Supplemental Table 8). Interestingly, all of these proteins had a lower mean activity in stored sperm (see Figure 4a-d and Supplemental Table 8 for statistical details).

Ornithine aminotransferase (OAT), a mitochondrial matrix enzyme showed significant abundance changes in eight different spots on the DIGE gels. We noted a decrease in abundance of intact forms (see spots 10C, 11C in Figure 3) and an increase in truncated or degraded forms, which had lower molecular mass (12C-17C, Figure 3). However, measurement of OAT activity did not differ significantly between stored and ejaculated sperm (Figure 4f). A protein spot containing aspartate aminotransferase (AAT) increased in abundance in stored sperm although the spot analyzed is very likely a breakdown product of this enzyme based on the low molecular mass of this protein spot compared to the major AAT isoforms noted in the sperm proteome analysis (Supplementary Tables 1 & 2). Finally, two isoforms of the mitochondrial TCA cycle enzyme dihydrolipoamide dehydrogenase are less abundant in stored sperm (Supplemental Table 8) and its enzyme activity was significantly reduced compared to ejaculated sperm (Figure 4e).

Discussion

We provide a first global insight into honeybee sperm proteome plasticity by quantifying changes in protein profiles between stored and ejaculated sperm and show a significant number of proteins differ in their abundance. Our enzymatic assays indicate that the differences in protein abundance between young ejaculated and old, stored sperm alter sperm physiology thereby influencing sperm competitiveness or the cost of storage to the

female due to altered metabolic rate. If sperm life history traits differ between sperm found within the ejaculate and those exposed and maintained by the female, future work is now required to test how these changes affect sperm traits under natural as well as sexual selection. The finding that ejaculated sperm is physiologically different from fertilizing sperm offers exciting new opportunities to understand observed variation in paternity success.

Of specific interest are the observed changes in the amount of enzymes involved in sperm energy metabolism. These differences point towards either a diminished demand for energy in the stored sperm, a reorientation of energy production during this stage in sperm life cycle or a shift in control of metabolism in storage from the sperm to the female. Three proteins of the glycolytic pathway that have been identified to be altered in the DIGE analysis were aldolase, TPI and GAPDH. These three enzymes form part of the glycolytic pathway and catalyze the reversible transformation of fructose 1,6-bisphosphate into 1,3-bisphosphoglycerate, which also culminates in the reduction of NAD^+ to yield NADH. The modified forms of these and other glycolytic enzymes that appear to have lower enzymatic activity were of higher abundance in the stored sperm. Interestingly, with the exception of aldolase, all these proteins that make up the glycolytic pathway are also present in the spermathecal fluid of both virgin and mated queens (Baer *et al.*, 2009a). This implies an active role of the spermathecal lumen in the maintenance and nutrition of the stored sperm through glycolysis as previously proposed (Klenk *et al.*, 2004). Consequently, the trend toward lower activities of glycolytic enzymes in the sperm cells themselves may be compensated for by the enzymatic activity of the

spermathecal fluid. The need to control glycolysis and thus the availability of substrates for respiration is intrinsically linked with the rate of production of reactive oxygen species from the electron transport chain in cells and the potential damage to proteins through oxidative reactions (Pardini, 1995). There are several reports of the high level of antioxidant enzymes in the spermatheca of honeybees (Collins *et al.*, 2004), and we have identified a network of antioxidant proteins in the spermatheca proteome (Baer *et al.*, 2009a). Balancing and/or controlling energy generation for sperm survival and the consequent production of harmful reactive oxygen species (ROS) to minimize oxidative damage to transcriptionally silent sperm cells is a molecular challenge for the female in order to achieve long-term sperm survival. Our data show key aspects of the molecular framework through which this balance could be maintained.

We provide two possible explanations for what causes the differences in the proteomes of stored and ejaculated sperm, namely sperm adaptation and/or sperm senescence. Each have different causes and consequences for sperm.

Sperm adaptation:

Sperm experience substantial changes in their external environment during their life history, as indicated by the substantial proteome differences between seminal and spermathecal fluid (Baer *et al.*, 2009a; Baer *et al.*, 2009b). Chemical modifications of proteins and specialized changes in protein degradation are a common feature of cellular

regulation in the face of changing external conditions (Ravid, Hochstrasse, 2008; Cenci *et al.*, 2008).

Our data suggest that the changes we reported here could be of functional significance for sperm performance and thus the aim to maximise fertilization/paternity success, and could be responsible for previously reported costs of sperm storage (Baer *et al.*, 2006; Baer, Schmid-Hempel, 2005). Protein modifications in sperm are known from mammals where they are important for sperm maturation for example to enable capacitation, a process enabling sperm to become fertilizable (Baker *et al.*, 2005; Vadnais *et al.*, 2007). A metabolic adjustment in sperm such as a degradation or deactivation of those enzymes critical for a successful transfer of sperm into the female storage organ is consistent with the data we have obtained. Obviously, future work is required to understand the consequences of these changes on male fertility and sperm competitiveness.

Sperm senescence:

Alternatively, the differences in protein abundances between stored and ejaculated sperm may be a consequence of sperm senescence, if proteins undergo either targeted modifications or degrade by a non-selective process of sperm aging. This idea is supported by the fact that most of the changes we detected are decreases in the abundance of mature proteins in stored compared to ejaculated sperm. Furthermore we found a tendency of a loss of enzymatic activity in the stored sperm samples (Figure 4). If senescence is taking place, sperm would be an interesting model system to study protein senescence and turn over on a molecular scale because sperm are assumed to be transcriptionally silent and consequently unable to synthesize new proteins (Hecht, 1998).

Indeed we found no new proteins in stored sperm, and the changes in abundance of specific proteins did not indicate they were products of new synthesis, providing some evidence that honeybee sperm do not use transcriptional processes to alter their proteomes during storage. Our list of proteins with changed abundances (Table 1) thus provides valuable biomarkers of sperm age for future studies. Obviously more work is needed to understand the root cause of the proteome differences between stored and ejaculate sperm and whether a subpopulation of dying or dead sperm is specifically targeted for degradation. Honeybees are a promising model system for such future work because their sperm can survive for months in glass capillaries (Collins, 2005), thereby offering opportunities to study proteomics changes either continuously over time or in sperm of the same age that is or is not exposed to storage by the female.

In summary, our experiments provide evidence that protein content of sperm changes during sperm life history. These changes can be expected to affect sperm physiology and functioning and could thus have significant consequences for sperm performance and fitness. Sperm form and functioning thus needs to be separately investigated for sperm in an ejaculate and sperm that has been exposed to and stored by the female. Future work should therefore aim to understand the proximate and ultimate reasons for these changes and link them to male and female reproductive success.

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Acknowledgements

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474 We were supported by the Australian Research Council (ARC) Discovery Program
475 (Queen Elizabeth II Fellowship to BB, an Australian Post-Doctoral Fellowship to HE, an
476 Australian Professorial Fellowship to AHM), an ARC Discovery Project to BB and HE
477 and the ARC Centre of Excellence in Plant Energy Biology. We thank the honeybee
478 keepers of Western Australia, especially Better Bees of Western Australia for providing
479 the necessary honeybee material for this study.

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Figure legends

Figure 1

(a) DIGE 2D IEF/SDS-PAGE of ejaculated sperm (labelled with Cy3, shown in green) and seminal fluid (labelled with Cy5, shown in red). The two samples were electronically overlaid, with yellow spots representing proteins that are of similar abundance, while green spots indicate a higher abundance in ejaculated sperm and red spots indicate a higher abundance in seminal fluid. (b) Colloidal coomassie blue stained DIGE 2D IEF/SDS-PAGE of the same gel as shown in (a). Gel spots picked for MS/MS analysis are labelled (for MS/MS results see Supplemental Table 1).

Figure 2

Reference gel of the honeybee sperm proteome separated by 2D IEF/SDS PAGE and stained with Colloidal coomassie blue. Gel spots picked for MS/MS analysis are labelled, for MS/MS results see Supplemental Tables 2 & 4).

Figure 3

a) DIGE 2D IEF/SDS-PAGE of ejaculated sperm (labelled with Cy3, shown in green) and stored sperm (labelled with Cy5, shown in red). The two samples were electronically overlaid, with yellow spots representing proteins that are of similar abundance, while green spots indicate a higher abundance in ejaculated sperm and red spots indicate a higher abundance in stored sperm. **b)** 2D- DIGE gels were coomassie stained and spots picked from the gel (numbers followed by C) or matched to the reference gel (numbers followed by B). Identified protein spots and the corresponding fluorescent ratios are shown in Supplemental Tables 2 & 4.

Figure 4

Differences in protein activity between 6 different proteins where we found significant abundance differences between stored and ejaculated sperm. Enzyme activities were always lower in stored (open bars) compared to ejaculated sperm (black bars) for (a) aldolase, (b) triose phosphate isomerase (c) glyceraldehyde 3-phosphate dehydrogenase, (d) enolase (e) dihydrolipoic dehydrogenase and (f) ornithine amino transferase. Shown are means and one standard error of mean, significant differences based on p-values of one-sided Wilcoxon signed rank tests given above bars. For statistical details see Supplemental Table 8.

Table 1: The final list of 15 different major sperm proteins with significant changes in protein abundance between stored and ejaculated sperm. For more information see Supplemental Tables 6&7.

Spots in Figure 3	Bee Genome locus	Protein name	Function
1C	GB16546-PA	Dihydrolipoamide dehydrogenase 2	Energy
2C, 3C, 4C	GB17626-PA	Dihydrolipoamide dehydrogenase 1	Energy
5C	GB17473-PA	Triosephosphate isomerase	Energy
6C	GB15039-PA	Enolase	Energy
7C, 8C	GB15463-PA	Fructose 1,6-bisphosphate aldolase	Energy
9C	GB14798-PA	Glyceraldehyde-3-phosphate dehydrogenase 2	Energy
10C – 17C	GB15171-PA	Ornithine aminotransferase	Energy
18C	GB10467-PA	Aspartate aminotransferase 2	Energy
19C	GB12453-PA	Actin 5c	Structural
28B	GB14311-PA	Sperm tail fiber protein	Structural
20C – 22C	GB14501-PA	Tektin	Structural
23C – 25C	GB18109-PA	Aldose reductase	Energy
23B-25B	GB12546	Venome allergen acid phosphatase	-
27C	GB16286-PA	ATPase associated protein	Energy
28C	GB11987-PA	Unknown Protein	unknown

Sperm

Overlaid

Seminal Fluid

a)

b)

Ejaculated Sperm

Stored Sperm

Overlaid

a)

b)

Figure 4