Trade-offs between sperm viability and immune protein expression in honey bee queens (*Apis mellifera*)

Short title: Reproduction versus immunity trade-offs in honey bee queens

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Abstract

Queens of social insects have the remarkable ability to keep sperm alive within their specialized storage organ, the spermatheca, for years. As the longest-lived individual in the colony as well as the sole reproductive female, the queen defies the typical trade-off between lifespan and reproduction that is observed in other animals. However, whether queens are subject to a trade-off between reproduction and immunity is unknown, and the biochemical processes underlying sperm viability are poorly understood. Here, we survey quality metrics (sperm count, sperm viability, and ovary size) of honey bee queens from nine genetic sources. We then performed quantitative proteomics on spermathecal fluid from N = 123 individual queens in order to investigate the biochemical processes underlying natural variation in sperm viability. Five spermathecal fluid proteins significantly correlated with sperm viability: odorant binding protein (OBP)14, lysozyme, serpin 88Ea, artichoke, and heat-shock protein (HSP)10. The significant negative correlation of lysozyme—a conserved immune effector controlled by Toll signalling—with sperm viability strongly supports the reproduction vs. immunity trade-off in honey bee queens. Protein-protein correlation matrices reveal that numerous pathogen-associated molecular pattern recognition proteins and downstream immune effectors are co-expressed and negatively correlate (though not significantly, as individuals) with sperm viability. Previous research identifying immunosuppressive and viability-supporting effects of JH, combined with our observation that putative JH-binding proteins cluster together and negatively correlate with sperm viability suggest that JH signalling or sequestration may mediate immunosuppression in queens.

Introduction

Long-term sperm storage is a remarkable feature of social insect biology, with hymenopteran queens storing sperm for by far the longest duration of any animal (decades).[1] In most social insect species, much work has been dedicated to sexual conflict between males via sperm competition[2-6] and the trade-

45 offs of male and female innate immunity with sperm quality and stored sperm viability.[7-12] However, the
46 molecular processes linked to sperm viability during storage are not well understood. Honey bee queens
47 are emerging as a model system to investigate such processes because they are highly amenable to
48 empirical manipulation.

49 The reproduction versus immunity trade-off hypothesis—also known as the immunocompetence
50 handicap—is a prevailing ideology in reproductive biology.[7,13-16] In males of a variety of species, including
51 insects, there is a well-established negative relationship between sperm viability and immune
52 function.[8,10,12,17-21] A similar trade-off appears to exist in female insects that must engage in sperm
53 storage.[9,11,22]

54 These trade-offs between reproduction and immunity are thought to be driven either by resource-
55 allocation compromises[23,24] or collateral damage of immune effectors.[9,10] The resource-allocation
56 compromise states that the more biological resources a male or female invests in immune function, the
57 lower the reproductive capacity (*i.e.*, sperm quantity or quality in sperm-producing males,[8,12] sperm
58 storage in females,[9,11,22] or ovum provisioning and production in females[7]). The alternate idea of
59 collateral damage of immune effectors is based on the idea that sperm cells may be inadvertently
60 damaged by innate immune defenses of the female.[9] In particular, collateral damage could occur via
61 innate immune mechanisms that utilize bursts of cytotoxic reactive oxygen and nitrogen species (ROS
62 and RNS).[9,25] Queen honey bees are under particularly strong selective pressure to minimize ROS and
63 RNS in the spermatheca in order to support long-term sperm maintenance. Indeed, the spermatheca is a
64 largely anoxic environment,[26] and mated queens upregulate ROS-mitigating enzymes, like catalase and
65 superoxide dismutase.[27-29]

66 Both the collateral damage hypothesis and the resource allocation hypothesis predict that
67 immunosuppressed individuals will have higher sperm viability, and likewise, that immune stimulation

68 decreases sperm viability. This phenomenon has been observed in crickets[11] and fruit flies,[9] where
69 female immune stimulation (using peptidoglycan fragments) reduced sperm viability in the female's
70 seminal receptacles. In addition, mating reduces phenoloxidase activity (an immune effector responsible
71 for the melanization cascade) in wood ant and leaf-cutter ant queens.[22,30]

72 In honey bee queens, the relationship between immune protein expression and sperm viability (whether
73 via collateral damage or resource-allocation trade-offs) is, as yet, unexplored. Here, we investigated the
74 reproduction versus immunity trade-off dogma by performing quantitative proteomics on a large
75 sample (n = 123 queens) of genetically distinct queens, correlating protein expression with stored sperm
76 viability, and inspecting protein-protein correlation matrices for functional patterns.

77 **Results and Discussion**

78 *Evaluating quality metrics for healthy, failed, and imported queens*

79 For this survey, we sampled 138 queens (of which 125 have data for both sperm viability and sperm
80 count) belonging to three major cohorts: healthy queens (n = 52), failed queens (n = 53), and imported
81 queens (n = 20, 10 each from California and Hawaii). The sperm count and viability data did not satisfy
82 all the assumptions for an ANOVA, therefore least squares and weighted least squares linear regressions
83 were used where appropriate (**Table 1**). The average sperm viability and sperm counts were nearly
84 identical between healthy queens and imported queens, but failed queens had significantly lower sperm
85 viability ($p = 1.1e-7$, $t = 5.6$, $df = 130$) and sperm counts ($p = 0.00050$, $t = 3.47$, $df = 128$) compared to
86 healthy queens (**Figure 1**). Ovary masses also differed significantly between groups – imported queens
87 had significantly lower ovary masses compared to either healthy queens ($p = 0.0021$, $t = 3.1$, $df = 134$) or
88 to failed queens ($p = 0.038$, $t = 2.1$, $df = 134$). Among the local and imported queens, sperm viability was
89 consistently high across all producers and import sources, and sperm counts were statistically similar
90 (**Figure S1**). Sample metadata is available in **Supplementary Table S1**.

Since the healthy and failed queens are not age-matched, we cannot say if the differences in counts and viability that we observed are due to age or quality differences. Furthermore, in preliminary analyses we found that the ovary-mass data only marginally passed Levene's test for equal variance (p = 0.051), which was driven by low variation in imported queen ovary masses. This, on top of imported queens having the smallest ovaries, strongly suggests that the data are not from the same statistical population. In follow-up experiments, we observed that the ovary mass of imported queens is regained after two weeks spent caged inside a colony, and therefore is not likely an intrinsic quality of imported queens (**Figure S2**). Rather, it is likely an artifact of longer caging duration during international transit.

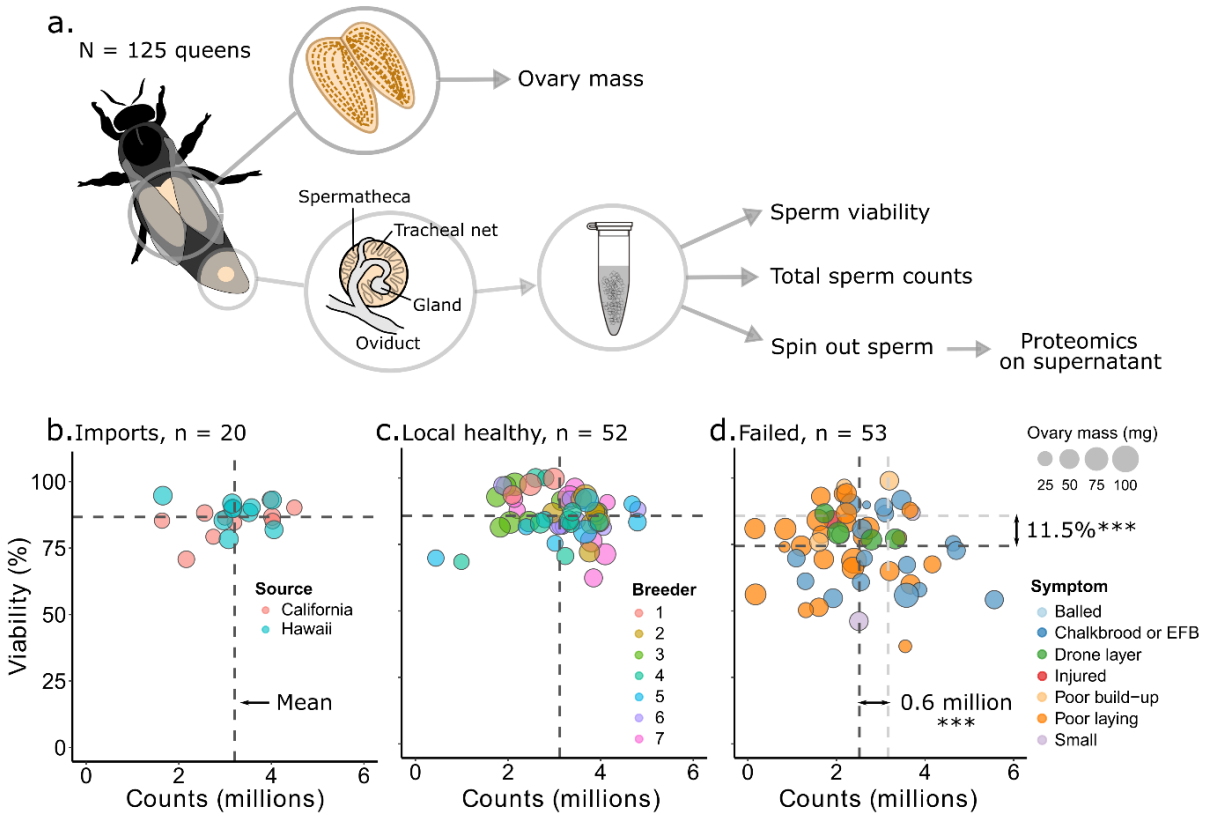

Figure 1. Sperm viability, sperm counts, and ovary mass metrics. A) Experimental schematic. B) imported, C) healthy, and D) failed queens were surveyed for macroscopic health metrics. See **Table 1** for a complete summary of statistical tests and p values. Symptoms shown are as reported by donating beekeepers.

104 *Proteomics analysis on spermathecal fluid*

105 We first took a broad view of proteins linked to sperm viability by correlating protein expression in the
106 spermathecal fluid to the viability of stored sperm. Underlying proteomics data is available in
107 **Supplementary Table S2**. Of the 2,512 proteins identified and 1,999 quantified (proteins identified in
108 fewer than 10 samples were removed), five specific proteins significantly correlated with sperm viability:
109 Lysozyme, Odorant binding protein (OBP)14, Serpin 88Ea, Artichoke, and Heat-shock protein (HSP)10
110 (**Figure 2a, Table 2**). Lysozyme is a well-known immune effector that is negatively related to sperm
111 viability in multiple cricket species,[8,12,31] and supports the notion that reproduction versus immunity
112 trade-offs exist in honey bee queens as well. Results of this correlation analysis are in **Supplementary**
113 **Table S3.**

114 Using the gene score resampling (GSR) approach, several GO terms were significantly enriched among
115 proteins correlating with sperm viability, with odorant binding being one of the most significant after
116 correction for protein multifunctionality and multiple hypothesis testing (**Figure 2b**). Furthermore,
117 OBP14 is strongly upregulated in mated queens relative to virgin queens and has relatively low
118 abundance in semen (**Figure 2c**), suggesting that its upregulation may be controlled by the act of mating
119 or presence of sperm. The strong, significant enrichment of odorant binding is driven by the combined
120 effect of the aforementioned OBP14 correlation, as well as weaker correlations of OBPs that are co-
121 expressed with OBP14 (OBP3, 4, 13, 16, 19, and 21), all of which are negatively (but not significantly)
122 correlated with sperm viability (**Figure 2d**). The strong representation of OBPs in drone ejaculates
123 (**Figure 2e**) is consistent with previous reports of odorant reception regulating sperm motility,[32] but the
124 significantly higher expression of OBP14 in the spermatheca relative to ejaculates, and its negative
125 correlation with viability, suggest an alternate role in the context of sperm storage.

126 Like other OBPs, OBP14 is a soluble, globular protein with a hydrophobic ligand binding pocket. OBP14 is
127 the only honey bee OBP with a crystal structure, and previous work suggests that it preferentially binds
128 terpenoid molecules.[33,34] Juvenile hormone (JH) is a sesquiterpenoid insect hormone with numerous
129 functions related to development, immunity, and reproduction,[35-37] making it an appealing candidate
130 ligand for OBP14. JH has immunosuppressive effects in specific contexts;[38,39] therefore, it has the
131 features of a key mediator for controlling the reproduction-immunity trade-off. We thus speculate that
132 in the spermathecal fluid, OBP14 may be involved in hormonal signalling that regulates queen immunity.

133 **Table 1. Statistical parameters**

Factor	Level	Shapiro p	Levene p	Statistical method	Contrasts		
					Group	p	t
Sperm viability	Healthy	0.0534	0.000146	Weighted least squares linear regression	H-F	1.1e-7	5.53
	Failed	0.182			F-I	7.7e-5	-4.08
	Imported	0.114			I-H	0.89	0.14
Sperm counts	Healthy	0.160	0.0714	Least squares linear regression	H-F	7.7e-4	3.59
	Failed	0.630			F-I	3.7e-3	-2.96
	Imported	0.250			I-H	0.74	0.33
Ovary mass	Healthy	0.00774	0.0506	Least squares linear regression	H-F	0.13	1.52
	Failed	0.3279			F-I	0.038	2.09
	Imported	0.0919			I-H	2.1e-3	-3.14

134

135 **Table 2.** Functional description of proteins significantly correlating with sperm viability

Protein description	Accession	General function(s)	Viability correlation
Odorant binding protein 14	NP_001035313.1	Solubilization of semiochemicals, ligand transport, preferential binding to terpenoid molecules[33,34]	Negative
Artichoke	XP_026295178.1	Essential for cilia and flagella beating in *Drosophila*[40]	Negative
Serine protease inhibitor 88Ea	XP_026298978.1	Negative regulator of Toll[41]	Negative
Lysozyme	XP_026300526.1	Antibacterial and antifungal activity, associated with low sperm viability in crickets[8,12,31]	Negative
10 kDa heat-shock protein (HSP10)	XP_624910.1	Constitutive protein chaperone	Positive

136

137 HSP10, which positively correlates with sperm viability, is a protein chaperone expressed in the

138 mitochondria, but it is also released into extracellular fluid.[42] In vertebrates, it is a negative regulator of

139 immunity—indeed, it is also known as the "early pregnancy factor" because its expression facilitates

140 zygote implantation in the uterus via immunosuppression in the mother (likely through interactions with

141 mammalian Toll-like receptors).[43] This idea is analogous to the collateral damage of immune effectors

142 on stored sperm observed in *Drosophila*.[9] An immunosuppression function of HSP10 has not been

143 demonstrated in invertebrates, and its function in insects, apart from its role as a chaperone, has

144 received little attention.[44-46]

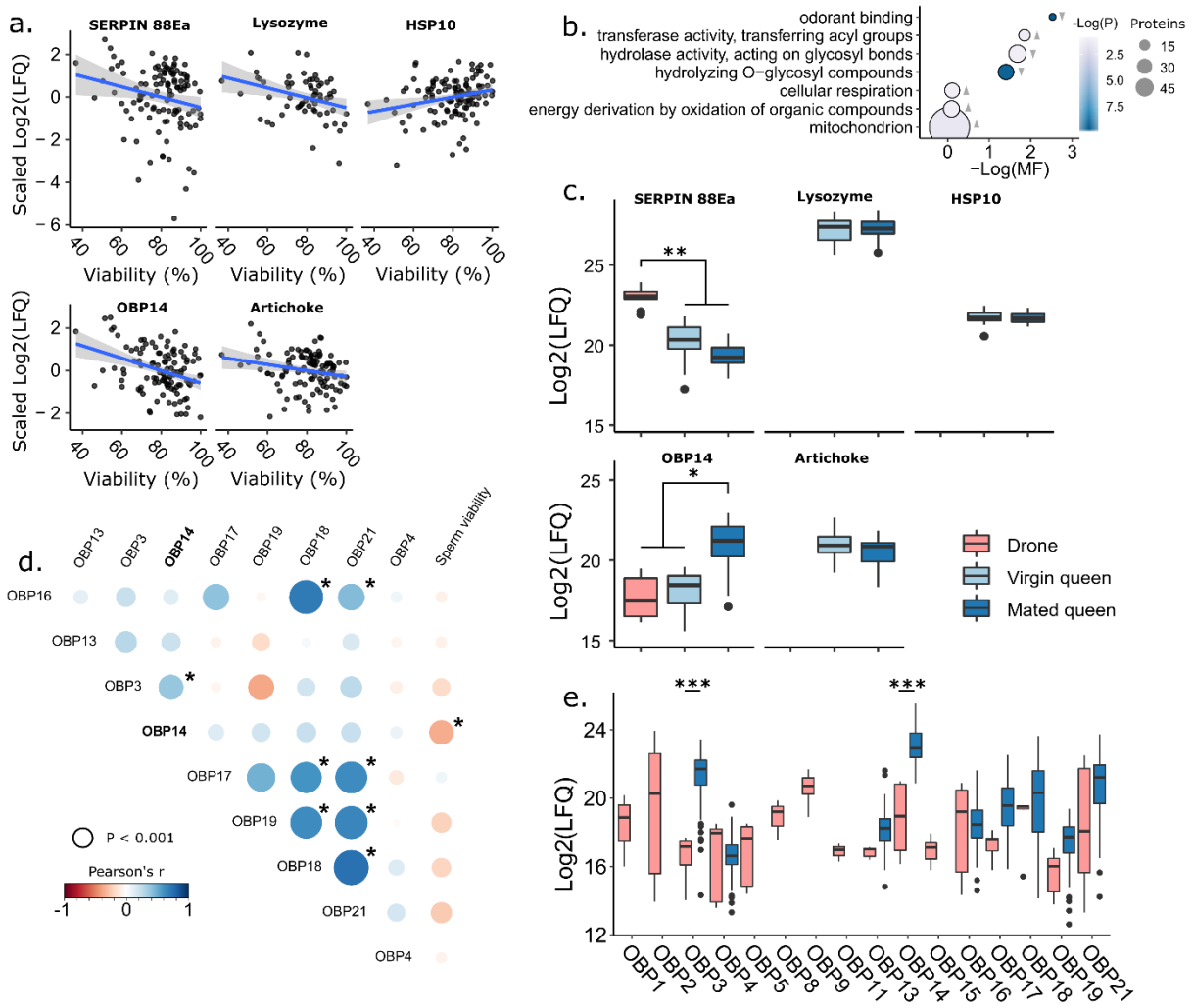

Figure 2. Correlations of spermathecal fluid proteins with sperm viability. a) We analyzed the spermathecal fluid of N = 125 queens by label-free quantitative proteomics. The linear regression model included sperm viability, sperm counts, and cohort (healthy, failed, and imported) as fixed factors, queen producer as a random effect, and the false discovery rate (FDR) was set to 10% (Benjamini-Hochberg method). The y axis depicts mean-centered LFQ intensity data after log2 transformation. Adjusted p values are shown. Statistical parameters can be found in Supplementary Table S3. b) GO terms that are significantly enriched among proteins correlating with sperm viability using the gene score resampling (GSR) method. c) Protein expression in drone ejaculates, virgin spermathecae, and mated spermathecae (each with N = 10). Data were analyzed using an ANOVA followed by Tukey contrasts for those with significant ANOVA results. Serpin 88Ea contrasts: Drone-Virgin $p < 1 \times 10^{-7}$, Drone-Mated $p < 1 \times 10^{-7}$. OBP14 contrasts: Mated-Drone $p = 3.9 \times 10^{-5}$, Mated-Virgin $p = 8.0 \times 10^{-5}$. d) Protein-protein and protein-viability correlation matrix of odorant binding proteins in the spermathecal fluid. Dot size is proportional to significance. Significant correlations are indicated with an asterisk ($\alpha = 0.0011$, Bonferroni correction). e) Odorant binding protein (OBP) expression in drone ejaculates and mated queens (N = 10 each). Data

160 underlying panels c and e were previously published.[29] Data were analyzed by a two-way ANOVA, which
161 indicated an interactive effect between sex and protein (df = 8, F = 8.7, $p < 1.8 \times 10^{-11}$) followed by Tukey
162 contrasts. OBP3 and OBP14 contrasts: $p < 1.0 \times 10^{-7}$. In all cases, boxes represent the bounds between the
163 2nd and 3rd interquartile range (IQR), midlines represent the median, and whiskers are extended by
164 1.5 × IQR.

165

166 *Protein-protein co-expression matrices*

167 The proteins we identified as correlating with sperm viability are multifunctional and, in some cases,
168 poorly characterized. We therefore exploited protein correlation matrices and hierarchical clustering to
169 make further inferences about the proteins' potential functions based on proximal associations with
170 other proteins. The guiding principle is that co-expressed proteins are more likely to function in the
171 same biochemical pathway, or be physically interacting as components of a protein complex.[47]

172 We computed Pearson correlation matrices including all 1,999 quantified proteins, then performed
173 hierarchical clustering to group those proteins that are co-expressed (**Figure 3a-d, Supplementary Table**
174 **S4**). We first confirmed that the clusters we defined are biologically meaningful by testing for GO terms
175 enriched within each protein cluster. Of the 79 clusters we defined (to which 1,377 proteins belong,
176 singletons and doubletons removed), 18 of them had enriched GO terms for biological functions or
177 molecular processes. Next, we identified which clusters our five proteins of interest belong to, and
178 found that Serpin 88Ea, Lysozyme, and Artichoke are part of the same cluster (cluster 5), in addition to
179 numerous proteins involved in pathogen-associated molecular pattern (PAMP) recognition and others
180 involved in cellular encapsulation immune reactions (Phenoloxidase, Proclotting enzyme, and
181 Apolipophorin-III; **Figure 3e**). Ten of the 27 proteins belonging to Cluster 5 are clearly linked to innate
182 immunity (twelve, if counting Serpin 88Ea itself and Serpin 27A, both of which are involved in innate
183 immune signalling in *Drosophila*). Unfortunately, we were not able to perform an enrichment test for
184 this function because no proteins in the whole dataset contained the GO terms GO:0008592 (regulation

185 of Toll) nor GO:0002252 (immune effector response), despite identifying proteins that clearly should
186 possess these GO terms. This seems to indicate a deficiency in GO term mapping between *Drosophila*
187 and honey bees, and it is not clear how this can be systematically overcome. All of the 13 innate
188 immune factors are also negatively (but not significantly, individually) correlated with sperm viability. On
189 top of the significant negative correlation of Lysozyme expression with sperm viability, these data
190 support the idea that queens are subject to reproduction-immunity trade-offs when it comes to sperm
191 storage.

192 Very little is known about the artichoke protein's function, but some evidence demonstrated that it is
193 essential for cilia and flagellar function.[40] In the quiescent state of the sperm during storage, flagellar
194 motion is undesirable because it demands large amounts of ATP and is not necessary for sperm storage.
195 This is consistent with the negative relationship we observe between artichoke and viability. However, if
196 this were its primary function, we would expect artichoke to also be present in drone ejaculates, which
197 we did not observe (**Figure 2c**). Artichoke has not been previously linked to immunity, but it is an
198 understudied protein and since it clusters with known immune effectors and regulators here, that is one
199 alternate role that should be explored.

200 While the cluster containing OBP14 did not yield any significant GO terms, two of the other cluster
201 members are Apolipophorin I/II and Hexamerin 70a. Apolipophorins are known to bind JH in other
202 insects,[48,49] suggesting that hormone sequestration or signalling could be playing a role in sperm
203 viability. Hexamerin 70a also binds JH, was part of the same cluster, and is known to physically interact
204 with apolipophorins.[50] These results strongly suggest that hormone trafficking, particularly JH trafficking,
205 is linked to sperm viability, and that OBP14, Apolipophorin I/II, and Hexamerin 70a could be facilitators.
206 Others have shown that JH diet supplementation improves sperm viability,[51] and JH serves as an
207 immunosuppressant in mated females of other insects, which is consistent with the reproduction-
208 immunity trade-off hypothesis.[52]

209 HSP10 assists with folding proteins as they are imported into the mitochondria, in a 1:1 stoichiometric
210 complex with HSP60. In our correlation matrix, we found that these two HSPs were contained within the
211 same cluster along with other mitochondrial proteins, supporting the biological relevance of this
212 clustering method (**Table 3**), and the idea that ATP synthesis and utilization is linked to sperm
213 maintenance, as has been previously suggested.[26] Surprisingly, most of the other proteins in the HSP10-
214 containing cluster are also mitochondrial proteins, which we did not expect because we removed sperm
215 cells from the spermathecal fluid through centrifugation. The presence of so many mitochondrial
216 proteins in the spermathecal fluid is puzzling and unresolved. It is possible that more mitochondrial
217 proteins are released into the extracellular matrix or spermathecal fluid than previously thought, or that
218 these originate from cells loosely associated with the spermathecal structural components that become
219 dislodged or lysed during spermathecal fluid extraction. Whatever the origin, the consistent
220 representation of mitochondrial proteins within this cluster again confirms that correlation matrices
221 enabled us to identify biologically meaningful protein-protein associations.

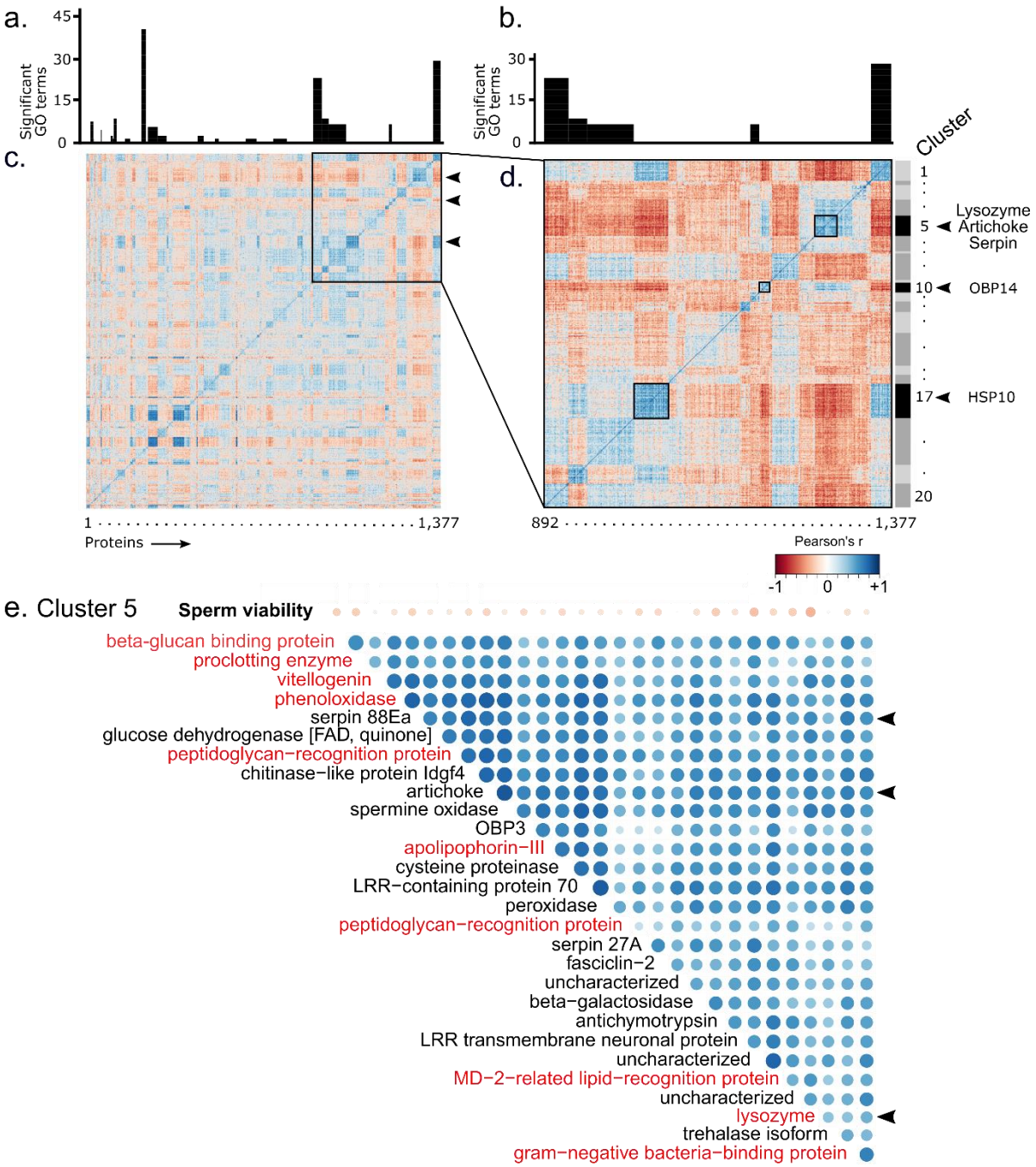

Figure 3. *Protein-protein co-expression matrices.* We computed protein-protein Pearson correlations across n = 123 queen spermathecal fluid samples at a dendrogram cut-off of 666 clusters (one third of the total number of quantified proteins). Singleton clusters were removed before plotting. A) Significantly enriched GO terms within clusters (Fisher exact test, 10% false discovery rate, Benjamini-Hochberg method). B) GO terms of inset clusters. C) All protein clusters (using a cut-off of k = 666 clusters, singletons and doubletons removed). D) Inset protein clusters containing the five proteins correlating with sperm

229 viability. E) All protein members of cluster 5 and their relationship with sperm viability. Protein names in
230 red indicate proteins directly involved in innate immune signalling.

231

232 **Table 3.** Proteins clustering with lysozyme, serpin 88Ea, artichoke, OBP14, and HSP10

Protein accession	Cluster	Description
XP_026300526.1	**5**	**lysozyme**
XP_026298978.1	**5**	**serpin 88Ea**
XP_026295178.1	**5**	**artichoke**
NP_001011578.1	5	vitellogenin
NP_001011627.1	5	phenoloxidase
NP_001107670.1	5	apolipophorin-III
NP_001157186.1	5	gram-negative bacteria-binding protein
NP_001157187.1	5	peptidoglycan-recognition protein
XP_001121565.2	5	beta-galactosidase
XP_001121634.3	5	beta-1,3-glucan-binding protein
XP_001121713.2	5	collagen alpha-1(IV) chain
XP_001121888.2	5	proclotting enzyme
XP_001122067.3	5	serpin 27A
XP_003250675.2	5	uncharacterized
XP_006560184.1	5	fasciclin-2
XP_006564106.1	5	peptidoglycan-recognition protein
XP_006565983.2	5	uncharacterized
XP_016766413.1	5	uncharacterized
XP_016769016.1	5	chitinase-like protein Idgf4
XP_016772427.1	5	LRR-containing protein 70
XP_026296654.1	5	LRR transmembrane neuronal protein
XP_026297659.1	5	peroxidase
XP_026298408.1	5	OBP3
XP_026299604.1	5	antichymotrypsin
XP_392145.3	5	glucose dehydrogenase [FAD, quinone]
XP_392381.3	5	cysteine proteinase
XP_392711.4	5	MD-2-related lipid-recognition protein
XP_393963.3	5	trehalase isoform
XP_396922.2	5	spermine oxidase
NP_001035313.1	**10**	**odorant binding protein 14 isoform X1**
NP_001035349.1	10	alpha-glucosidase precursor
NP_001104234.1	10	hexamerin 70a precursor
XP_016768985.2	10	probable uridine nucleosidase 2
XP_026295419.1	10	leucine-rich repeat-containing protein 40-like
XP_026296673.1	10	uncharacterized protein LOC726286
XP_026296936.1	10	carboxypeptidase D isoform X2
XP_026297227.1	10	hemocyte protein-glutamine gamma-glutamyltransferase

XP_026298016.1	10	sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 isoform X2
XP_026298285.1	10	apolipophorins
XP_026300168.1	10	dipeptidase 1 isoform X3
XP_026301410.1	10	fibrillin-2
XP_392602.4	10	aminopeptidase N
XP_624977.1	10	GILT-like protein 1
XP_624910.1	**17**	**10 kDa heat shock protein, mitochondrial**
XP_392899.2	17	60 kDa heat shock protein, mitochondrial
NP_001153520.1	17	heat shock 70 kDa protein cognate 5
XP_026294937.1	17	protein lethal(2)essential for life
NP_001171495.1	17	Thioredoxin-dependent peroxide reductase, mitochondrial
NP_001229442.1	17	pyruvate dehydrogenase E1 component subunit beta, mitochondrial
XP_001120329.1	17	iron-sulfur cluster assembly 2 homolog, mitochondrial
XP_001120471.1	17	3-hydroxyacyl-CoA dehydrogenase type-2
XP_001122230.1	17	mitochondrial import inner membrane translocase subunit Tim8
XP_006560252.1	17	probable aconitate hydratase, mitochondrial
XP_006561099.1	17	grpE protein homolog, mitochondrial
XP_006562042.1	17	mitochondrial transcription rescue factor 1
XP_006562528.1	17	lon protease homolog, mitochondrial isoform X1
XP_006562944.1	17	mitochondrial import inner membrane translocase subunit Tim9
XP_006562957.1	17	persulfide dioxygenase ETHE1, mitochondrial
XP_006564183.2	17	probable isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial
XP_006564486.1	17	bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase, mitochondrial
XP_006564809.1	17	polyribonucleotide nucleotidyltransferase 1, mitochondrial
XP_006565801.1	17	ATP synthase subunit d, mitochondrial
XP_006566401.1	17	elongation factor Ts, mitochondrial
XP_006572159.1	17	probable pyruvate dehydrogenase E1 component subunit alpha, mitochondrial isoform X2
XP_016766950.1	17	acyl carrier protein, mitochondrial
XP_016767678.1	17	mitochondrial intermembrane space import and assembly protein 40-B
XP_016769522.1	17	complex III assembly factor LYRM7 isoform X1
XP_026295141.1	17	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial
XP_026301996.1	17	delta-1-pyrroline-5-carboxylate synthase isoform X1
XP_391843.1	17	3-ketoacyl-CoA thiolase, mitochondrial
XP_391880.2	17	elongation factor Tu, mitochondrial
XP_392478.2	17	malate dehydrogenase, mitochondrial
XP_392760.2	17	ATP synthase subunit O, mitochondrial
XP_393324.2	17	hemK methyltransferase family member 1
XP_393509.2	17	mitochondrial-processing peptidase subunit beta
XP_393789.2	17	electron transfer flavoprotein subunit beta
XP_393806.2	17	trifunctional enzyme subunit alpha, mitochondrial
XP_393876.1	17	isocitrate dehydrogenase [NAD] subunit gamma, mitochondrial
XP_393900.2	17	isochorismatase domain-containing protein 2-like

XP_394112.2	17	fumarylacetoacetate hydrolase domain-containing protein 2A isoform X2
XP_394885.1	17	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial
XP_395130.4	17	enoyl-[acyl-carrier-protein] reductase, mitochondrial
XP_395766.4	17	succinate-semialdehyde dehydrogenase, mitochondrial
XP_395826.2	17	NFU1 iron-sulfur cluster scaffold homolog, mitochondrial-like
XP_623084.1	17	aldehyde dehydrogenase, mitochondrial
XP_623725.1	17	voltage-dependent anion-selective channel
XP_624102.1	17	electron transfer flavoprotein subunit alpha, mitochondrial
XP_624164.1	17	trifunctional enzyme subunit beta, mitochondrial
XP_624249.1	17	ATP synthase subunit e, mitochondrial
XP_624330.3	17	prohibitin-2 isoform X1
XP_624354.1	17	serine protease HTRA2, mitochondrial
XP_624561.1	17	cob(I)yrinic acid a,c-diamide adenosyltransferase, mitochondrial

No evidence for Serpin 88Ea inhibitory activity

In *Drosophila*, Serpin 88Ea is a negative regulator of Toll immune signalling, and here it clusters with other proteins linked to innate immunity. However, the negative correlation with sperm viability and positive correlation with downstream immune effectors is not consistent with the reproduction-immunity trade-off hypothesis, nor this immune regulatory role. If the reproduction-immunity trade-off applies here and Serpin 88Ea functions in honey bees as it does in fruit flies, Serpin 88Ea should be positively correlated with sperm viability and inhibit expression of immune effectors. In *Drosophila,* Serpin 88Ea regulates Toll signalling by blocking proteolytic cleavage of spaetzle (Spz) by spaetzle-processing enzyme (SPE), which is a necessary step for Toll activation.[41] Despite Serpin 88Ea levels positively correlating with both Spz and SPE (**Figure 4a-c)**, we find no support that Serpin 88Ea is actually inhibiting SPE in our data.

Serpins inhibit proteases by forming a covalent bond with the protease at its active site and inducing a conformational change,[53] so if Serpin 88Ea is predominantly functioning as a protease inhibitor here, this should be confirmed in the mass spectrometry data. It would be unlikely that we would have identified the serpin-protease linkage because such bridged peptides fragment unpredictably in the mass spectrometer and non-canonical covalent bonds are not accounted for in the protein search database.

250 However, we should be able to see the absence or decreased abundance of a peptide, either from the
251 serpin or the protease, if this linkage is occurring. Unfortunately, it would not be possible to see a
252 cleaved spaetzle peptide in our data because spaetzle cleavage occurs C-terminal to an arginine residue
253 (**Figure 4d**), which would be indistinguishable from a cleavage by trypsin, the enzyme used in our sample
254 preparation.

255 The honey bee homolog of Serpin 88Ea has not been characterized, so we used BLAST to identify the
256 conserved reactive center loop (RCL) region and confirmed that the protein contains the consensus
257 sequence characteristic of inhibitory serpins (**Figure 4e**). In *Drosophila*, the site targeted for nucleophilic
258 attack by the protease occurs between residues 386 and 387 (TYR**S/A**RPV) in the RCL. Therefore, the
259 predicted cleavage site in honey bee Serpin 88Ea is between residues 369 and 370 (TFR**S/G**RPL), which is
260 contained in the tryptic peptide SGRPLVPTVFNANHPFVYFIYEK (the 'site peptide'). We evaluated LFQ
261 intensities of two control peptides (distant from the nucleophilic attack site) and the site peptide
262 relative to a fourth reference peptide. All peptides were tightly correlated to the reference peptide at
263 approximately the same slope (**Figure 4f-g)**, suggesting that LFQ intensities of the site peptide were not
264 decoupled and that in this biological context Serpin 88Ea is not actively inhibiting proteases.

265 Curiously, Dosselli *et al.* recently found that a different Serpin, B10 (along with two serine proteases,
266 easter and snake), became downregulated in the ant *Atta colombica* seminal fluid upon exposure to
267 spermathecal fluid.[54] The authors suggest that the proteases and Serpin B10 are part of a sperm-sperm
268 competition system that becomes quickly deactivated by spermathecal fluid to preserve sperm viability.
269 In our data, Serpin 88Ea along with two other protease inhibitors, Serpin 27A (which targets Easter) and
270 Antichymotrypsin, are all negatively associated with sperm viability. While this is consistent with the
271 overall de-activation of sperm competition favoring viability, it is hard to rationalize how that could be
272 the case here, since honey bee drone sperm and not the seminal fluid migrate through the queen's
273 reproductive tract to the spermatheca. Therefore, if these proteins are the remains of sperm-

274 competition machinery, they would have had to enter the spermatheca by physically associating with
275 the membranes of sperm cells—a highly unlikely scenario, especially given that our statistical model
276 yielded no proteins correlating with sperm counts.

277 Interestingly, in mammals, some serpins actually act as hormone carriers.[55] Although these are typically
278 'non-inhibitory' serpins without an RCL domain and the serpins in our data are ostensibly inhibitory, it is
279 possible that insect serpins have evolved diverse roles. Indeed, despite containing an RCL domain, we
280 found no evidence that Serpin 88Ea is actively serving as a serine protease inhibitor. In addition, the top
281 predicted protein interactor for *Drosophila* Serpin 88Ea, according to STRING (www.string-db.org), is
282 actually an apolipophorin involved in JH transport (FBpp0088252, score: 0.919). Further experiments will
283 be necessary to ascertain whether Serpin 88Ea is associating with hormone carriers, is an immune
284 regulator, is a component of sperm competition machinery, or some combination of different functions
285 in different biological contexts.

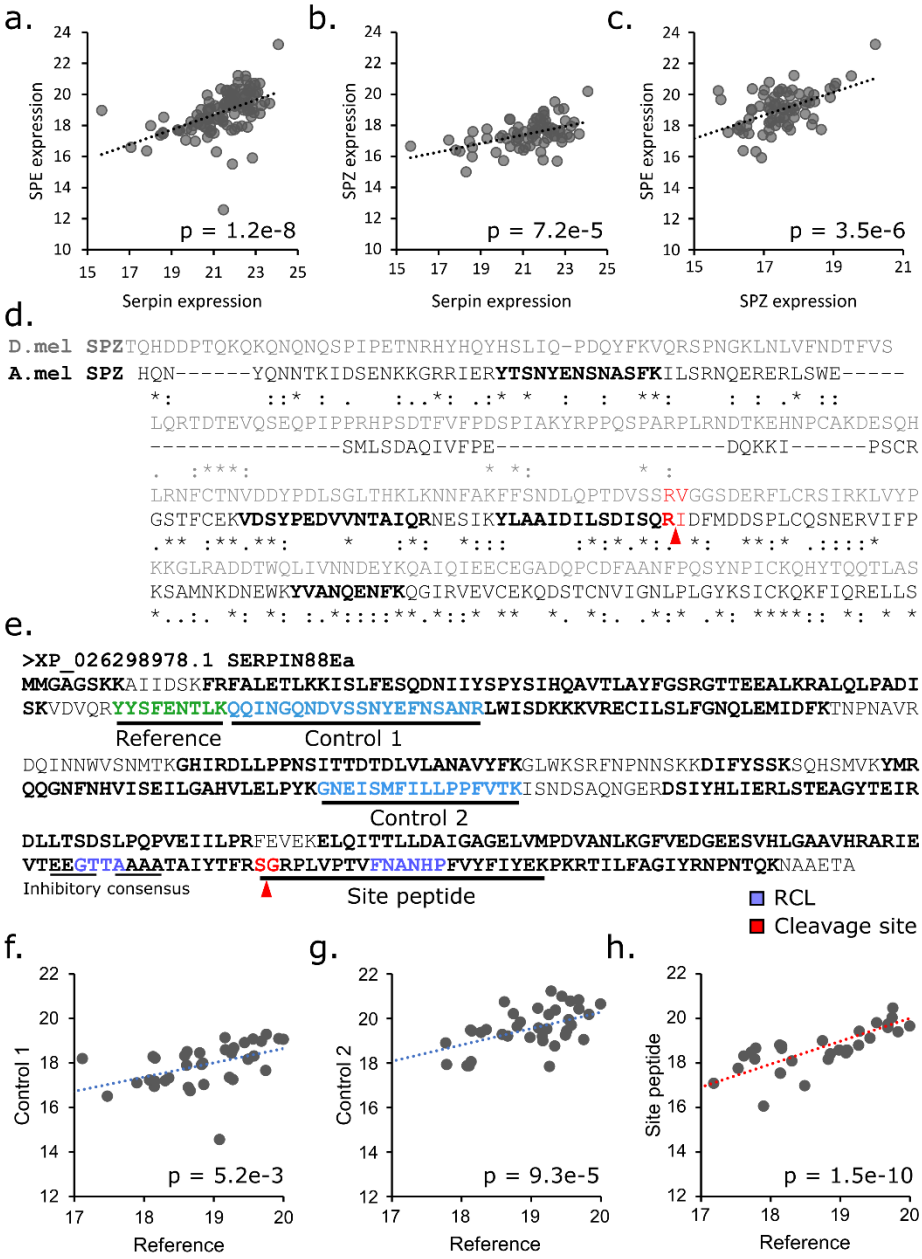

Figure 4. An investigation into Serpin 88Ea, Spaetzle (Spz), and Spaetzle-processing enzyme (SPE). The three proteins are all positively, significantly correlated with each other (linear regression on log2 LFQ intensities). a) SPZ-Serpin: N = 114, degrees of freedom (df): 113, F = 37.9, p = 1.2 x 10^{-8}. b) N = 75, df = 74, F = 17.8, p = 7.2 x 10^{-5}. c) N = 75, df = 74, F = 25.2, p = 3.5 x 10^{-6}. d) Sequence alignment between *Drosophila* (D.mel) Spz and honey bee (A.mel) Spz (* = perfect homology, : = good homology, . = partial homology, based on BLOSUM matrices). Peptides identified by mass spectrometry are bold. The predicted Spaetzle cleavage site is marked in red. e) Annotation of honey bee Serpin 88Ea sequence with predicted cleavage site (red), reactive center loop (RCL, purple), and inhibitory consensus sequence (thin underline). (f-h) LFQ intensities of the site peptide and control peptides are compared to the reference peptide. f) N = 35, df = 34, p = 0.0052; F = 8.9. g) N = 37, df = 36, p = 0.0093; F = 18.9. g) N = 29, df = 28, p = 1.5 x 10^{-10}; F = 81.7.

297

298 **Methods**

299 *Queens*

300 Seven queen producers throughout BC (located in Grand Forks, Merrit, Armstrong, Abbotsford, Telkwa,
301 Surrey, and Powell River) donated healthy queens for this study in the summer of 2019. All queens were
302 approximately two months old and rated as "good quality" by the donors based on having a consistent,
303 contiguous laying pattern. The sperm viability metrics for failed and healthy queens are the same results
304 as described in McAfee *et al*;[29] however, all sperm count, ovary mass, and imported queen data are
305 novel. Two of the same producers in Grand Forks also donated the majority of failed queens, but other
306 donors located in Squamish, Abbotsford, Cranbrook, Lillooet, and Vancouver also contributed. In most
307 cases, the age of the failed queens was unknown. The queens arrived via overnight ground
308 transportation (ACE Courier or via post) to the University of British Columbia in Vancouver and we
309 sacrificed them for analysis immediately upon arrival. See **Supplementary Table S1** for complete sample
310 metadata.

311 *Sperm viability, sperm counts, and ovary masses*

312 We conducted sperm viability assays exactly as previously described,[29] with the original method
313 published by Collins *et al*.[56] Briefly, we dissected spermathecae and lysed them in tubes containing 100
314 µl of room-temperature Buffer D. We transferred 10 µl of the solution to a new tube and stained it with
315 propidium iodide and Sybr green fluorescent dyes, which differentially stains dead sperm red and live
316 sperm green. After incubating for 15 minutes, we acquired images by fluorescent microscopy (three
317 fields of view per queen) and sperm belonging to red and green channels were automatically counted
318 using ImageJ. Sperm that stained both green and red were counted as live, as they were likely in the

319 process of dying as a result of dissection or associated extraneous variables. We averaged the percent
320 viability across the three technical replicates prior to performing protein correlations.

321 We counted total sperm using the methods essentially as described by Baer *et al*.[57] Briefly, we mixed the
322 sperm suspended in Buffer D solution by gently flicking the tube several times until homogeneous, then
323 pipetted three 1 µl spots on to a glass slide, allowing to air dry for 20 min. We stained the spots with
324 DAPI, then imaged the entire area of each spot with a fluorescent microscope (images were taken at
325 200x in a tiling array, then automatically stitched together in the Zeiss image processing software, ZEN).
326 The number of sperm nuclei in each 1 µl spot were then counted using ImageJ and averaged across the
327 three spots. We arrived at the total number of sperm by extrapolation (multiplying the average value by
328 a factor of 100). Finally, we dissected ovaries from the queens using forceps and wet weights were
329 determined using an analytical balance, subtracting the exact mass of the tube.

330 We used R to perform all statistical analyses. The sperm viability, sperm count, and ovary mass data
331 were first evaluated for normality and equal variance using a Shapiro and Levene test, respectively. If
332 the data passed both tests, or failed for normality and passed for equal variance, a classical least squares
333 linear regression model was used. If the data failed for equal variance, a weighted least squares
334 regression analysis, using the inverse of the fitted data from a first pass unweighted regression as the
335 weights.

336 *Proteomics sample preparation*

337 We used the remaining ~87 µl of spermathecal solution that was not consumed by the viability and
338 count assays for shot-gun proteomics. We removed spermathecal wall debris and sperm cells by
339 centrifugation (1000 g, 5 min), transferring the supernatant to a new tube. The proteomics samples,
340 therefore, were composed only of spermathecal fluid. We diluted the samples 1:1 with distilled water,

341 then precipitated the proteins by adding four volumes of ice cold 100% acetone and incubating at -20 °C
342 overnight.

343 We performed all further proteomics sample preparation steps exactly as previously described.[58] Briefly,
344 we used urea digestion buffer to solubilize the protein, then reduced (dithiothreitol), alkylated
345 (iodoacetamide), diluted with four volumes of 50 mM ammonium bicarbonate, digested (Lys-C for 3 h,
346 then trypsin overnight), and desalted peptides using C18 STAGE tips made in-house. We suspended the
347 desalted, dried peptides in Buffer A, then estimated peptide concentration by the absorbance at 280
348 nanometers (Nanodrop). For each sample, 2 µg of peptides were injected into the chromatography
349 system (Easy-nLC 1000, Thermo), which was directly coupled to a Bruker Impact II time-of-flight mass
350 spectrometer.

351 *Mass spectrometry data processing*

352 We searched the mass spectrometry data using MaxQuant (v1.6.8.0). All samples for the queen survey
353 were searched together (123 data files; two samples of the 125 depicted in **Figure 1** were compromised
354 during handling), ensuring a global identification false discovery rate of 1% for both proteins and
355 peptides. We used default search settings, except that label-free quantification was enabled, the
356 minimum number of peptide ratios for quantification was set to 1, and match between runs was
357 enabled. The FASTA database for the search was the newest *A. mellifera* proteome available on NCBI
358 (HAv3.1) along with all honey bee virus sequences.

359 We performed differential protein expression analysis using the limma package for R. First, we removed
360 all protein groups that were reverse hits, contaminants, or only identified by site, followed by proteins
361 identified in fewer than 10 samples. LFQ intensities were log2 transformed prior to analysis (available in
362 **Supplementary Table S2**). The statistical models evaluating protein correlations with sperm viability also
363 included sperm counts, ovary mass, and queen status (failed, healthy, or imported) as fixed effects and

364 source (producer) as a random effect. P values were adjusted using the Benjamini-Hochberg method
365 (10% FDR). The limma output for viability correlations is available in **Supplementary Table S3**.

366 *Protein-protein correlations and GO enrichments*

367 We computed the Pearson protein-protein correlation coefficients and set a cut-off of 666 clusters for
368 hierarchical clustering. This yielded 79 protein clusters containing three or more proteins (clustering
369 results are available in **Supplementary Table S4**). GO terms were then retrieved using BLAST2GO (v4.0),
370 which yielded 1,773 of 1,999 proteins with GO terms (the GO term association table is available in
371 **Supplementary Table S5**). To identify significantly enriched GO terms within clusters, we performed an
372 over-representation analysis (ORA) using ErmineJ,[59] where proteins within each cluster were considered
373 the 'hit list' and the quantified proteome (1,999 proteins) as the background. To find GO terms that
374 were significantly enriched among proteins correlating with sperm viability, we used the gene-score
375 resampling approach, also using ErmineJ. This approach utilizes p values as a continuous variable, and
376 detects GO terms that are over-represented among proteins with low p values. Further information can
377 be found at https://erminej.msl.ubc.ca/help/tutorials/running-an-analysis-resampling/. In both types of
378 enrichment analyses, within-test multiple hypothesis testing was corrected to 10% FDR using the
379 Benjamini-Hochberg method.

380 **Data Availability**

381 All novel proteomics raw data, search results, and search parameters are available on MassIVE
382 (www.massive.ucsd.edu, accession MSV000085428, **reviewer password: SPF123**). Figures associated
383 with these data are Figs 2a, 2b, 2d, 3, 4a-c, and 4f-h. Tables associated with these data are Table 2 and 3.
384 The mass spectrometry data comparing virgins, mated queens, and drone semen has been previously
385 published[29] and is publicly available at www.proteomexchange.org (accession: PXD013728). Sample
386 metadata underlying Fig 1 are available in **Supplementary Table S1**. Global protein abundances and P

387 values for the correlation between sperm viability and spermatheca protein expression are available in
388 **Supplementary Table S2**. Hierarchical clustering results of protein-protein correlation coefficients are
389 available in **Supplementary Table S4.** Any other data that support the findings of this study are available
390 from the corresponding author on request.

391 **Code availability**

392 R code for standard statistical analyses and figure generation will be provided upon request.

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