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 many social hymenoptera keep sperm alive within their specialized storage organ, the spermatheca, for years, defying the typical trade-off between lifespan and reproduction. However, whether honey bee queens experience a trade-off between reproduction and immunity is unknown, and the biochemical processes underlying sperm viability are poorly understood. Here, we survey quality metrics and viral loads of honey bee queens from nine genetic sources. Queens rated as 'failed' by beekeepers had lower sperm viability, fewer sperm, and higher levels of SBV and BQCV. Quantitative proteomics on N = 123 spermathecal fluid samples shows, after accounting for sperm count, health status, and apiary effects, five spermathecal fluid proteins significantly correlating 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—with sperm viability is consistent with a reproduction vs. immunity trade-off in honey bee 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 some hymenopteran species, much work has been dedicated to sexual selection among males via sperm competition[2-6] and the trade-offs of male and female innate immunity with sperm quality and stored sperm viability.[7-12] However, the molecular processes linked to sperm viability during storage are not well understood. Honey bee queens could serve as an excellent model system to investigate such processes because they are highly amenable to empirical manipulation.

The reproduction versus immunity trade-off hypothesis—also known as the immunocompetence handicap—is a prevailing hypothesis in reproductive biology.[7,13-16] In males of a variety of species, including insects, there is a well-established negative relationship between sperm viability and immune

45 function.[8,10,12,17-21] A similar trade-off appears to exist in female insects that must engage in sperm
46 storage.[9,11,22]

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

59 Both the collateral damage hypothesis and the resource allocation hypothesis predict that
60 immunosuppressed individuals will have higher sperm viability, and likewise, that immune stimulation
61 decreases sperm viability. This phenomenon has been observed in crickets[11] and fruit flies,[9] where
62 female immune stimulation (using peptidoglycan fragments) reduced sperm viability in the female's
63 seminal receptacles. In addition, mating reduces phenoloxidase activity (an immune effector responsible
64 for the melanization cascade) in wood ant and leaf-cutter ant queens.[22,30]

65 In honey bee queens, the relationship between immune protein expression and sperm viability (whether
66 via collateral damage or resource-allocation trade-offs) is, as yet, unexplored. Additionally, there is
67 limited data on how proteins linked to sperm viability change after mating, when the queen must

transition from storing no sperm to maintaining sperm viability for years. Here, we investigated the reproduction versus immunity trade-off hypothesis by performing quantitative proteomics on a large sample of genetically distinct queens, relating protein expression with stored sperm viability, and inspecting protein-protein correlation matrices for functional patterns.

Results and Discussion

Evaluating quality metrics for healthy, failed, and imported queens

For this survey, we initially sampled 125 queens belonging to three major cohorts: healthy queens (n = 52), failed queens (n = 53), and imported queens from commercial suppliers (n = 20, 10 each from California and Hawaii). The sperm count and viability data did not satisfy all the assumptions for an ANOVA, therefore least squares and weighted least squares linear models were used where appropriate (**Table 1**). We also included queen producer as a fixed effect to account for potential genetic or environmental differences between sources. The average sperm viability and sperm counts were nearly identical between healthy queens and imported queens, but failed queens had significantly lower sperm viability ($p = 0.00132$, $t = 3.29$, $df = 118$) and sperm counts ($p = 0.00472$, $t = 4.86$, $df = 117$) compared to healthy queens (**Figure 1a-d**). Ovary masses also differed significantly between groups – imported queens had significantly lower ovary masses compared to either healthy queens ($p = 0.0021$, $t = 3.1$, $df = 134$) or to failed queens ($p = 0.038$, $t = 2.1$, $df = 134$), but ovary mass can be strongly influenced by caging time and worker care. These differences are not detectable when “producer” is included as a fixed effect in the statistical model, since all the imported queens were produced by California and Kona suppliers. Among the local and imported queens, sperm viability was consistently high across all producers and import sources, and sperm counts were statistically similar (**Figure S1**). Sample metadata is available in **Supplementary Table S1**.

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

98 *Viral analysis*

99 To assess patterns of viral abundance in the queen cohorts, we measured deformed wing virus (DWV),
100 sacbrood virus (SBV) and black queen cell virus (BQCV) levels using RT-qPCR. We first analyzed a subset
101 of 45 queens for DWV, SBV, BQCV, as well as acute bee paralysis virus (ABPV), Kashmir bee virus (KBV),
102 and Israeli acute paralysis virus (IAPV), but found no detectable levels of the latter three. We therefore
103 analyzed a further 61 queens for only DWV, SBV, and BQCV (in total, $n = 44$ healthy queens, $n = 13$
104 imported queens, and $n = 49$ failed queens). We found that failed queens had significantly higher copy
105 numbers of SBV and BQCV relative to imported queens and healthy queens but lower copy numbers of
106 DWV (**Figure 1e**). Combining copy numbers of all three viruses into a total viral load, failed queens had
107 significantly higher loads than healthy queens (see **Table 1** for all associated p values). We also identified
108 significant effects of queen source (producer) for all three viruses, indicating that the apiaries from
109 which the queens came had characteristic viral profiles (DWV: $p = 4.66\times10^{-6}$, $F = 5.2$, $df = 11$ and 94; SBV:
110 $p = 3.95\times10^{-8}$, $F = 6.66$, $df = 11$ and 94; BQCV: $p = 0.0359$, $F = 2.01$, $df = 11$ and 94; Total load: $p =$
111 2.44×10^{-8}, $F = 6.83$, $df = 11$ and 94). $N = 94$ queens had both viability and virus data, among which none
112 of the viruses nor total viral load were dependent on sperm viability (DWV: $p = 0.330$, $t = -0.979$; SBV: p
113 $= 0.424$, $t = 0.802$; BQCV: $p = 0.579$, $t = 1.79$; Total: $p = 0.878$, $t = -0.153$).

114 *Proteomics analysis on spermathecal fluid*

115 We first took a broad view of proteins linked to sperm viability by correlating protein expression in the
116 spermathecal fluid to the viability of stored sperm (underlying proteomics data and statistics are
117 available in **Supplementary Table S2 and S3**). Of the 2,512 proteins identified and 1,999 quantified
118 (proteins identified in fewer than 10 samples were removed), five specific proteins significantly
119 correlated with sperm viability: Lysozyme, Odorant binding protein (OBP)14, Serpin 88Ea, Artichoke, and
120 Heat-shock protein (HSP)10 (**Figure 2a, Table 2**). Since queen source (producer) was included as a
121 random effect in our statistical model, these differences are unlikely to be a result of source bias.
122 Furthermore, colony health status ('failed,' 'healthy,' and 'imported) was included as a fixed effect in the
123 model, and since queens heading failed colonies also tended to be older and had a higher viral load,
124 these proteins are unlikely to simply be linked to sperm viability indirectly through aging queens or
125 differences in viral titer (queen ages, where known, are listed in **Supplementary Table S1**). To be sure,
126 we checked if these five specific proteins were linked to viral copy numbers (individual viruses as well as
127 total load), and found no significant relationships (**Supplementary Figure S3**). Lysozyme is a well-known
128 immune effector that is negatively related to sperm viability in multiple cricket species[8,12,31]—although
129 not entirely unequivocal, this result is consistent with the notion that reproduction versus immunity
130 trade-offs may exist in honey bee queens.

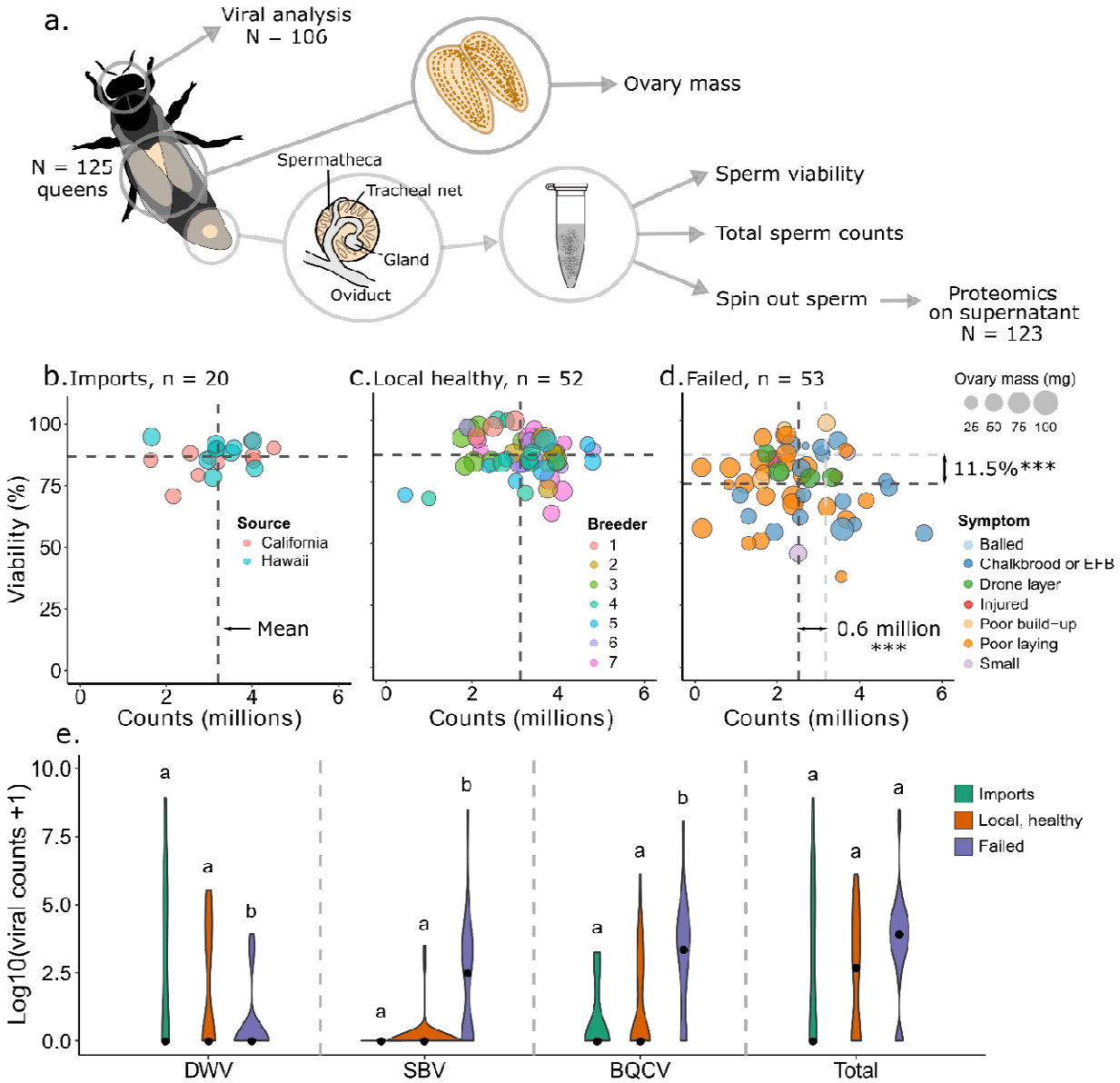


Figure 1. Sperm viability, sperm counts, ovary mass, and viral metrics. See **Table 1** for a complete summary of statistical tests, parameters, and p values. A) Experimental schematic. B) imported, C) healthy, and D) failed queens were surveyed for macroscopic health metrics. Symptoms shown are as reported by donating beekeepers. Sperm viability and sperm count data was acquired for 125 queens, while 123 queens contributed to final proteomics data owing to sample loss during handling. *** indicates $p < 0.005$. E) For a subset of the queens, viral copy numbers were measured using RT-qPCR (n = 44 healthy queens, n = 13 imported queens, and n = 49 failed queens). Lower-case letters indicate statistical significance ($p < 0.05$) within each virus.

141 **Table 1.** Statistical parameters

Factor	Level	Shapiro p	Levene p	Statistical method	Contrasts		
					Group[a]	p	|t|
Sperm viability[b]	Healthy	0.0534	0.000146	Weighted least squares linear model	H-F	0.00132	3.29
	Failed	0.182			F-I	0.181	1.34
	Imported	0.114			I-H	0.94	0.075
Sperm counts[b]	Healthy	0.160	0.0714	Least squares linear model	H-F	0.00472	4.86
	Failed	0.630			F-I	0.0245	2.28
	Imported	0.250			I-H	0.448	0.762
Ovary mass[b]	Healthy	0.00774	0.0506	Least squares linear model	H-F	0.395	0.855
	Failed	0.3279			F-I	0.36	0.920
	Imported	0.0919			I-H	0.6545	0.449
DWV[c]	Healthy	$2.25x10^{-7}$	0.000436	Weighted least squares linear model	H-F	0.000485	3.61
	Failed	$3.92x10^{-12}$			F-I	0.00824	2.70
	Imported	0.00460			I-H	0.436	0.781
SBV[c]	Healthy	NA	$4.72x10^{-16}$	Weighted least squares linear model	H-F	0.000296	3.75
	Failed	$8.84x10^{-7}$			F-I	0.000107	4.04
	Imported	NA			I-H	0.138	1.50
BQCV[c]	Healthy	$2.70x10^{-8}$	0.149	Least squares linear model	H-F	$1.66x10^{-5}$	4.53
	Failed	0.0019			F-I	$9.3x10^{-5}$	4.08
	Imported	$3.12x10^{-5}$			I-H	0.402	0.826
Total virus[c]	Healthy	$3.81x10^{-5}$	0.0245	Weighted least squares linear model	H-F	0.0769	1.79
	Failed	0.000357			F-I	0.291	1.06
	Imported	0.00459			I-H	0.779	0.282

142 [a]H = Healthy, I = Imported, F = Failed
143 [b]Queen producer was included as a fixed effect
144 [c]Producer could not be included as a fix effect due to singularities
145
146 **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[34,35]	Negative
Artichoke	XP_026295178.1	Essential for cilia and flagella beating in *Drosophila*[41]	Negative
Serine protease inhibitor 88Ea	XP_026298978.1	Negative regulator of Toll[42]	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

147 We cannot exclude that natural infections could be impacting both immune protein expression and
148 quality metrics. The queens did not have appreciable quantities of *Nosema* spores visible in their
149 intestinal tract, and while DWV, SBV, and BQCV were detectable, these viruses were not linked to sperm
150 viability nor expression of the top proteins linked to sperm viability. However, this is not an exhaustive
151 list of potential pathogens. It is also possible that immune proteins could be elevated as a consequence
152 of sperm death, rather than preceding it. However, in other experiments, we have experimentally
153 stressed queens using techniques that are known to reduce stored sperm viability (*i.e.* heat exposure)[29]
154 and we did not observe elevated levels of any of the significant proteins we identified here.

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

167 Although the abundance of OBP14 in semen is low, it is possible that sperm death and subsequent
168 release of proteins could contribute to the abundance patterns we observe (the same is true for Serpin
169 88Ea, which is also present in semen). To check this, we correlated protamine-like protein (a highly
170 abundant sperm nuclear protein)[33] with both sperm viability and absolute number of dead sperm, and

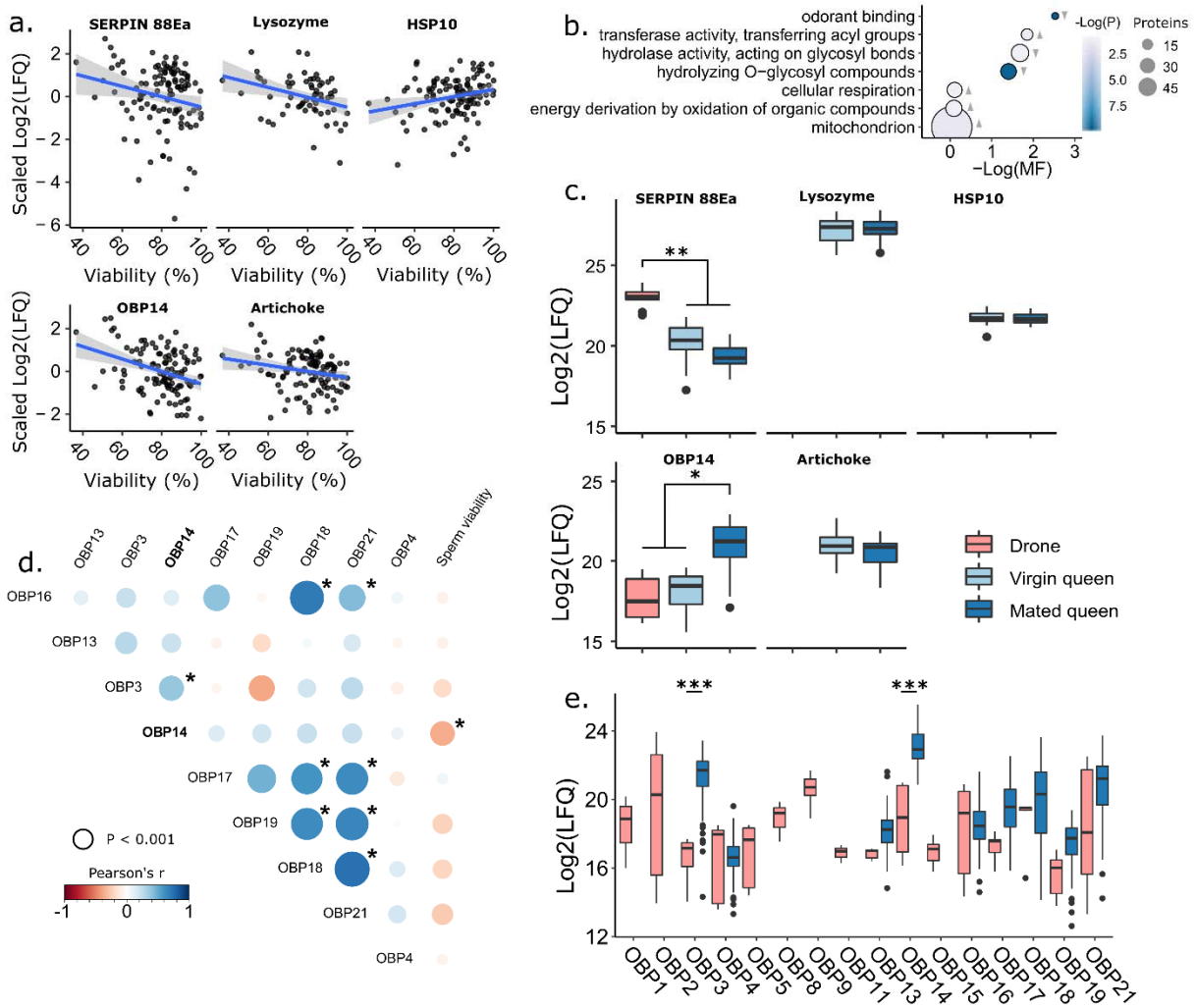
171 found no significant correlations (**Supplementary Figure S4**, Pearson correlation, $p = 0.215$ and $p =$
172 0.321, respectively). The protein was also sparsely identified in only 36 out of 123 samples, and was
173 likely a result of sporadic sperm lysis during sample handling. Therefore, we reason that it is unlikely that
174 the release of sperm proteins upon death can explain the negative correlations we observe for OBP14,
175 Serpin 88Ea, Lysozyme, and Artichoke.

176 Like other OBPs, OBP14 is a soluble, globular protein with a hydrophobic ligand binding pocket. OBP14 is
177 the only honey bee OBP with a crystal structure, and previous work suggests that it preferentially binds
178 terpenoid molecules.[34,35] Juvenile hormone (JH) is a sesquiterpenoid insect hormone with numerous
179 functions related to development, immunity, and reproduction,[36-38] making it an appealing candidate
180 ligand for OBP14. JH has immunosuppressive effects in specific contexts;[39,40] therefore, it has the
181 features of a key mediator for controlling the reproduction-immunity trade-off. We thus speculate that
182 in the spermathecal fluid, OBP14 may be involved in hormonal signalling that regulates queen immunity,
183 and OBP14-mediated JH signalling may influence sperm viability directly or indirectly through immune
184 effects. We reason that if OBP14 were to bind and sequester free JH, JH may be less able to exhibit its
185 immunosuppressive effects, thus lowering sperm viability by tipping the reproduction-immunity trade-
186 off in favour of immunity. Alternatively, the proposed OBP14-JH complex may bind specific receptors
187 and initiate physiological changes through signalling, rather than sequestration. Further experiments will
188 be necessary to determine the specific molecular mechanisms involved.

189 HSP10, which positively correlates with sperm viability, is a protein chaperone expressed in the
190 mitochondria, but it is also released into extracellular fluid.[43] In vertebrates, it is a negative regulator of
191 immunity—indeed, it is also known as the "early pregnancy factor" because its expression facilitates
192 zygote implantation in the uterus via immunosuppression in the mother (likely through interactions with
193 mammalian Toll-like receptors).[44] This idea is analogous to the collateral damage of immune effectors
194 on stored sperm observed in *Drosophila*.[9] An immunosuppression function of HSP10 has not been

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

196 received little attention.[45-47]



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198 **Figure 2.** Proteins associated with sperm viability. a) We analyzed the spermathecal fluid of N = 123
199 queens by label-free quantitative proteomics. The linear model included sperm viability, sperm counts,
200 and cohort (healthy, failed, and imported) as fixed factors, queen producer as a random effect, and the
201 false discovery rate (FDR) was set to 10% (Benjamini-Hochberg method). The y axis depicts mean-
202 centered LFQ intensity data after log2 transformation. Adjusted p values are shown. Statistical
203 parameters can be found in Supplementary Table S3. b) GO terms that are significantly enriched among
204 proteins correlating with sperm viability using the gene score resampling (GSR) method. c) Protein
205 expression in drone ejaculates, virgin spermathecae, and mated spermathecae (each with N = 10). Data
206 were analyzed using an ANOVA followed by Tukey contrasts for those with significant ANOVA results.
207 Serpin 88Ea contrasts: Drone-Virgin $p < 1 \times 10^{-7}$, Drone-Mated $p < 1 \times 10^{-7}$. OBP14 contrasts: Mated-

208 Drone $p = 3.9 \times 10^{-5}$, Mated-Virgin $p = 8.0 \times 10^{-5}$. d) Protein-protein and protein-viability correlation
209 matrix of odorant binding proteins in the spermathecal fluid. Dot size is proportional to significance.
210 Significant correlations are indicated with an asterisk ($\alpha = 0.0011$, Bonferroni correction). e) Odorant
211 binding protein (OBP) expression in drone ejaculates and mated queens (N = 10 each). Data underlying
212 panels c and e were previously published.[29] Data were analyzed by a two-way ANOVA, which indicated
213 an interactive effect between sex and protein ($df = 8$, $F = 8.7$, $p < 1.8 \times 10^{-11}$) followed by Tukey
214 contrasts. OBP3 and OBP14 contrasts: $p < 1.0 \times 10^{-7}$. In all cases, boxes represent the bounds between
215 the 2nd and 3rd interquartile range (IQR), midlines represent the median, and whiskers are extended by
216 1.5 × IQR.

217

218 *Protein-protein co-expression matrices*

219 The proteins we identified as correlating with sperm viability are multifunctional and, in some cases,
220 poorly characterized. We therefore exploited protein correlation matrices and hierarchical clustering to
221 make further inferences about the proteins' potential functions based on proximal associations with
222 other proteins – an approach that has been widely used in other systems.[48] The guiding principle is that
223 co-expressed proteins are more likely to function in the same biochemical pathway, or be physically
224 interacting as components of a protein complex.[48]

225 We computed Pearson correlation matrices including all 1,999 quantified proteins, then performed
226 hierarchical clustering to group those proteins that are co-expressed (**Figure 3a-d, Supplementary Table**
227 **S4**). We first confirmed that the clusters we defined are biologically meaningful by testing for GO terms
228 enriched within each protein cluster. Of the 79 clusters we defined (to which 1,377 proteins belong,
229 singletons and doubletons removed), 18 of them had enriched GO terms for biological functions or
230 molecular processes, demonstrating that these are likely to be biologically meaningful groupings. Next,
231 we identified the clusters to which our five proteins of interest belong, and found that Serpin 88Ea,
232 Lysozyme, and Artichoke are part of the same cluster (cluster 5), in addition to numerous proteins
233 involved in pathogen-associated molecular pattern (PAMP) recognition and others involved in cellular
234 encapsulation immune reactions (Phenoloxidase, Proclotting enzyme, and Apolipophorin-III; **Figure 3e**

235 and **Supplementary Table S5**). Twelve of the 27 proteins belonging to Cluster 5 are linked to innate
236 immunity. All of these innate immune factors are also negatively (but not significantly, individually)
237 correlated with sperm viability. On top of the significant negative correlation of Lysozyme expression
238 with sperm viability, these data are consistent with the idea that queens are subject to reproduction-
239 immunity trade-offs when it comes to sperm storage.

240 Very little is known about the artichoke protein's function, but some evidence demonstrated that it is
241 essential for cilia and flagellar function.[41] In the quiescent state of the sperm during storage, flagellar
242 motion is undesirable because it demands large amounts of ATP and is not necessary for sperm storage.
243 This is consistent with the negative relationship we observe between artichoke and viability. However, if
244 this were its primary function, we would expect artichoke to also be present in drone ejaculates, which
245 we did not observe (**Figure 2c**). Artichoke has not been previously linked to immunity, but it is an
246 understudied protein; because it clusters with known immune effectors and regulators here, that is one
247 alternate role that should be explored.

248 While the cluster containing OBP14 did not yield any significant GO terms, two of the other cluster
249 members are Apolipophorin I/II and Hexamerin 70a, both of which are also involved in JH binding in
250 other insects,[49-51] suggesting that OBP14, Apolipophorin I/II, and Hexamerin 70a could be facilitating
251 hormone trafficking. Others have shown that JH diet supplementation improves sperm viability,[52] and JH
252 serves as an immunosuppressant in mated females of other insects, which is consistent with the
253 reproduction-immunity trade-off hypothesis.[53] Indeed, Kim *et al.*[54] recently identified a mosquito OBP
254 which binds JH and activates innate immune defenses – a mechanism which, according to the
255 reproduction-immunity trade-off hypothesis, would be consistent with high levels of OBP14 being
256 associated with low sperm viability in our data.

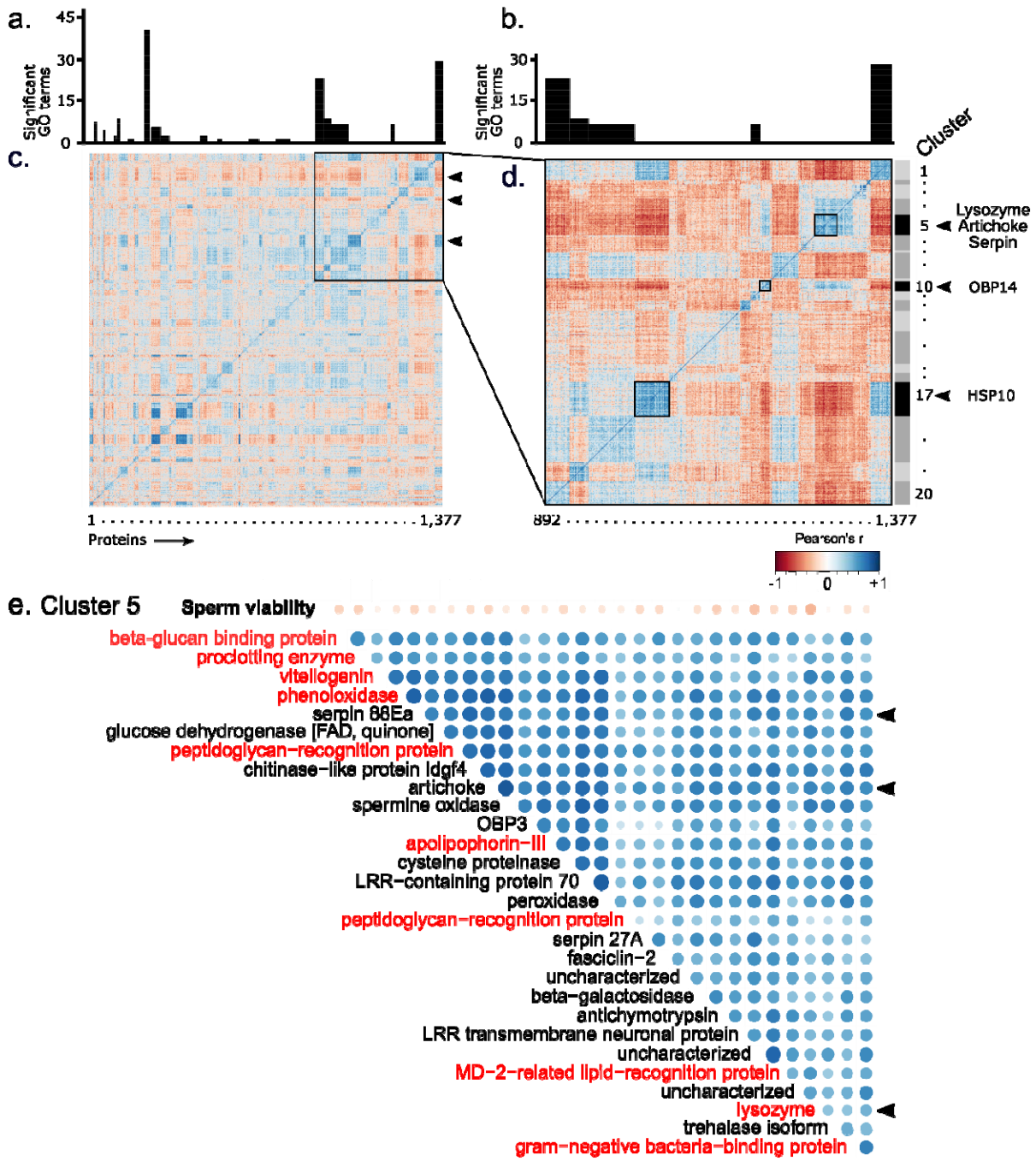

Figure 3. *Protein-protein co-expression matrices.* We computed protein-protein Pearson correlations across n = 123 queen spermathecal fluid samples at a dendrogram cut-off of 666 clusters (one third of the total number of quantified proteins). Singleton clusters were removed before plotting. A) Significantly 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 viability. E) All protein members of cluster 5 and their relationship with sperm viability. Protein names in red indicate proteins directly involved in innate immune signalling.

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.[42] 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,[55] 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. However, we should be able to see the absence or decreased abundance of a peptide, either from the serpin or the protease, if this linkage is occurring. Unfortunately, it would not be possible to see a cleaved spaetzle peptide in our data because spaetzle cleavage occurs C-terminal to an arginine residue (**Figure 4d**), which would be indistinguishable from a cleavage by trypsin, the enzyme used in our sample preparation.

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

297 However, we acknowledge that this is an imperfect analysis, since we do not have a good positive
298 control serpin (one which, under our experimental conditions, is known to appreciably covalently bind a
299 protease). Therefore, we cannot confirm the degree of site peptide decoupling that we should expect if
300 the serpin is acting as an inhibitor. In the future, we aim to conduct experiments involving spiking
301 spermathecal fluid with increasing doses of a serine protease to confirm the expected concomitant
302 decoupling of the site peptide from alternate peptides for an array of predicted inhibitory serpins.

303 Curiously, Dosselli *et al.* recently found that a different Serpin, B10 (along with two serine proteases,
304 easter and snake), became downregulated in the ant *Atta colombica* seminal fluid upon exposure to
305 spermathecal fluid.[56] The authors suggest that the proteases and Serpin B10 are part of a sperm-sperm
306 competition system that becomes quickly deactivated by spermathecal fluid to preserve sperm viability.
307 In our data, Serpin 88Ea along with two other protease inhibitors, Serpin 27A (which targets Easter) and
308 Antichymotrypsin, are all negatively associated with sperm viability. While this is consistent with the
309 overall de-activation of sperm competition favoring viability, it is hard to rationalize how that could be
310 the case here, since honey bee drone sperm and not the seminal fluid migrate through the queen's

311 reproductive tract to the spermatheca. Therefore, if these proteins are the remains of sperm-
312 competition machinery, they would have had to enter the spermatheca by physically associating with
313 the membranes of sperm cells—a highly unlikely scenario, especially given that our statistical model
314 yielded no proteins correlating with sperm counts.

315 Interestingly, in mammals, some serpins actually act as hormone carriers.[57] Although these are typically
316 'non-inhibitory' serpins without an RCL domain and the serpins in our data are ostensibly inhibitory, it is
317 possible that insect serpins have evolved diverse roles. Indeed, despite containing an RCL domain, we
318 found no evidence that Serpin 88Ea is actively serving as a serine protease inhibitor. In addition, the top
319 predicted protein interactor for *Drosophila* Serpin 88Ea, according to STRING (www.string-db.org), is
320 actually an apolipophorin involved in JH transport (FBpp0088252, score: 0.919). Further experiments will
321 be necessary to ascertain whether Serpin 88Ea is associating with hormone carriers, is an immune
322 regulator, is a component of sperm competition machinery, or some combination of different functions
323 in different biological contexts.

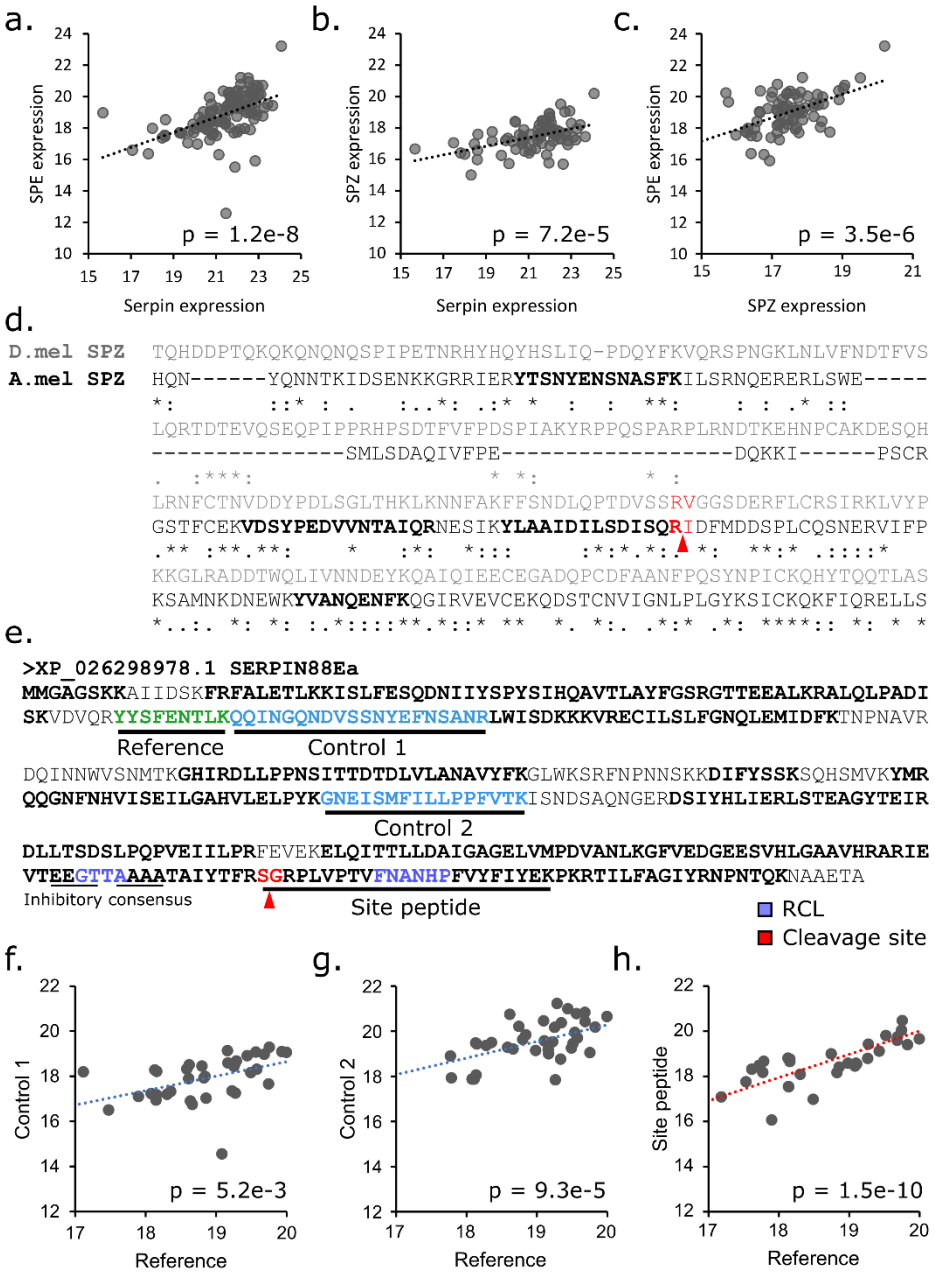

Figure 4. An investigation into Serpin 88Ea, Spaetzle (Spz), and Spaetzle-processing enzyme (SPE). The three proteins are all positively, significantly correlated with each other (linear model on log2 transformed LFQ intensities). a) SPZ-Serpin: N = 114, degrees of freedom (df): 113, F = 37.9, p = 1.2 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). The site peptide contains an internal arginine residue (R); however, it is followed by a proline residue and therefore is not predicted to be cleaved by trypsin. (f-h) LFQ

335 intensities of the site peptide and control peptides are compared to the reference peptide. f) N = 35, df
336 = 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 =
337 81.7.

338

339 **Methods**

340 *Queens*

341 Seven queen producers throughout BC (located in Grand Forks, Merrit, Armstrong, Abbotsford, Telkwa,
342 Surrey, and Powell River) donated healthy queens for this study in the summer of 2019. All queens were
343 approximately two months old and rated as "good quality" by the donors based on having a consistent,
344 contiguous laying pattern. The sperm viability metrics for failed and healthy queens are the same results
345 as described in McAfee *et al*;[29] however, all sperm count, ovary mass, and imported queen data are
346 novel. The queens were part of a regional survey of participating operations: Queens from different
347 operations were handled similarly and not exposed to environmental stressors in the laboratory. Two of
348 the same producers in Grand Forks also donated the majority of failed queens, but other donors located
349 in Squamish, Abbotsford, Cranbrook, Lillooet, and Vancouver also contributed. In most cases, the exact
350 age of the failed queens was unknown. Queen years and approximate ages (in months) are listed in
351 **Supplementary Table S1**. The queens arrived via overnight ground transportation (ACE Courier or via
352 post) to the University of British Columbia in Vancouver and were sacrificed for analysis immediately
353 upon arrival. Imported queens were shipped from producers in Hawaii and California to Edmonton,
354 Alberta, then shipped together to Vancouver within hours of arrival. The queens arrived at 10:30 pm
355 and were dissected and analyzed at the University of British Columbia on the following morning. Queens
356 across all sources were tested for *Nosema* spores using standard microscopy methods.[58] See
357 **Supplementary Table S1** for complete sample metadata.

358 *Sperm viability, sperm counts, ovary masses and viral analysis*

359 We conducted sperm viability assays exactly as previously described,[29] with the original method
360 published by Collins *et al*.[59] Briefly, we dissected spermathecae and lysed them in tubes containing 100
361 µl of room-temperature Buffer D. We transferred 10 µl of the solution to a new tube and stained it with
362 propidium iodide and Sybr green fluorescent dyes, which differentially stains dead sperm red and live
363 sperm green. After incubating for 15 minutes, we acquired images by fluorescent microscopy (three
364 fields of view per queen) and sperm belonging to red and green channels were automatically counted
365 using ImageJ. Sperm that stained both green and red were counted as live, as they were likely in the
366 process of dying as a result of dissection or associated extraneous variables. We averaged the percent
367 viability across the three technical replicates prior to performing protein correlations.

368 We counted total sperm using the methods essentially as described by Baer *et al*.[60] Briefly, we mixed the
369 sperm suspended in Buffer D solution by gently flicking the tube several times until homogeneous, then
370 pipetted three 1 µl spots on to a glass slide, allowing to air dry for 20 min. We stained the spots with
371 DAPI, then imaged the entire area of each spot with a fluorescent microscope (images were taken at
372 200x in a tiling array, then automatically stitched together in the Zeiss image processing software, ZEN).
373 The number of sperm nuclei in each 1 µl spot were then counted using ImageJ and averaged across the
374 three spots. We arrived at the total number of sperm by extrapolation (multiplying the average value by
375 a factor of 100). Finally, we dissected ovaries from the queens using forceps and wet weights were
376 determined using an analytical balance, subtracting the exact mass of the tube. The remaining queen
377 tissue was stored at -70 °C until further analysis.

378 For viral analysis, we submitted N = 106 queen heads to the National Bee Diagnostic Center (NBDC) at
379 Grand Prairie Regional College for analysis through their fee-for-service portal. Viral copy numbers were
380 analyzed by RT-qPCR according to the ΔΔCt method. We first shipped a subset of queen heads (N = 45
381 queens) to the NBDC on dry ice for analysis of DWV, SBV, BQCV, IAPV, KBV, and ABPV. No IAPV, KBV, nor

382 ABPV was detectable in the samples; therefore, we submitted a further N = 61 samples for analysis of
383 only DWV, SBV, and BQCV.

384 We used R to perform all statistical analyses. The sperm viability, sperm count, ovary mass, and viral
385 data were first evaluated for normality and equal variance using a Shapiro and Levene test, respectively
386 (viral data were first log transformed to bring copy numbers to a sensible scale, using $x = \log_{10}$(copy
387 number + 1)). If the data passed both tests, or failed for normality and passed for equal variance, a
388 classical least-squares linear model was used. If the data failed for equal variance, a weighted least-
389 squares analysis, using the inverse of the fitted data from a first pass unweighted model as the weights.
390 Queen status and queen producer were included as a fixed effects in viability, count, and ovary mass
391 statistical models. For ovary masses, differences between failed, healthy, and imported queens was
392 tested with and without producer as a fixed effect, and results from both models are reported. For the
393 viral analysis, we performed statistical tests for each virus separately using queen status (levels: failed,
394 healthy, imported) and sperm viability as fixed factors, as well as the combined viral load (copy numbers
395 from all three viruses were summed prior to log transformation). We were not able to include queen
396 producer as a fixed effect for the viral analysis due to singularities, so we evaluated the effect of
397 producer using a separate statistical model.

398 *Proteomics sample preparation*

399 For each queen, we used the remaining ~87 µl of spermathecal solution that was not consumed by the
400 viability and count assays for shot-gun proteomics. We removed spermathecal wall debris and sperm
401 cells by centrifugation (1000 g, 5 min), transferring the supernatant to a new tube. The proteomics
402 samples, therefore, were composed only of spermathecal fluid. We diluted the samples 1:1 with distilled
403 water, then precipitated the proteins by adding four volumes of ice cold 100% acetone and incubating at

404 -20 °C overnight. The final sample count for proteomics was 123 out of 125 initial queens owing to
405 sample losses during handling.

406 We performed all further proteomics sample preparation steps exactly as previously described.[61] Briefly,
407 we used urea digestion buffer to solubilize the protein, then reduced (dithiothreitol), alkylated
408 (iodoacetamide), diluted with four volumes of 50 mM ammonium bicarbonate, digested (Lys-C for 3 h,
409 then trypsin overnight), and desalted peptides using C18 STAGE tips made in-house. We suspended the
410 desalted, dried peptides in Buffer A, then estimated peptide concentration by the absorbance at 280
411 nanometers (Nanodrop). For each sample, 2 µg of peptides were injected into the chromatography
412 system (Easy-nLC 1000, Thermo), which was directly coupled to a Bruker Impact II time-of-flight mass
413 spectrometer, as a single shot unfractionated sample.

414 *Mass spectrometry data processing*

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

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

427 source (producer) as a random effect. P values were adjusted using the Benjamini-Hochberg method
428 (10% FDR). The limma output for viability correlations is available in **Supplementary Table S3**. We
429 verified that all five significant proteins did not correlate with DWV, SBV, BQCV, nor total viral load using
430 a linear least squares model, including sperm viability, status (levels: healthy, failed, imported), and the
431 viral variables as fixed factors.

432 *Protein-protein correlations and GO enrichments*

433 We computed the Pearson protein-protein correlation coefficients and set a cut-off of 666 clusters for
434 hierarchical clustering. This yielded 79 protein clusters containing three or more proteins (clustering
435 results are available in **Supplementary Table S4**, and clusters containing OBP14, Serpin 88Ea, Lysozyme,
436 HSP10, and Artichoke, specifically, are in **Supplementary Table S5**). GO terms were then retrieved using
437 BLAST2GO (v4.0), which yielded 1,773 of 1,999 proteins with GO terms (the GO term association table is
438 available in **Supplementary Table S6**). To identify significantly enriched GO terms within clusters, we
439 performed an over-representation analysis (ORA) using ErmineJ,[62] where proteins within each cluster
440 were considered the 'hit list' and the quantified proteome (1,999 proteins) as the background. To find
441 GO terms that were significantly enriched among proteins correlating with sperm viability, we used the
442 gene-score resampling approach, also using ErmineJ. This approach utilizes p values as a continuous
443 variable, and detects GO terms that are over-represented among proteins with low p values. Further
444 information can be found at https://erminej.msl.ubc.ca/help/tutorials/running-an-analysis-resampling/.
445 In both types of enrichment analyses, within-test multiple hypothesis testing was corrected to 10% FDR
446 using the Benjamini-Hochberg method.

447 **Data Availability**

448 All novel proteomics raw data, search results, and search parameters are available on MassIVE
449 (www.massive.ucsd.edu, accession MSV000085428). Figures associated with these data are Figs 2a, 2b,

450 2d, 3, 4a-c, and 4f-h. Tables associated with these data are Table 2 and 3. The mass spectrometry data
451 comparing virgins, mated queens, and drone semen has been previously published[29] and is publicly
452 available at www.proteomexchange.org (accession: PXD013728). Sample metadata underlying Fig 1 are
453 available in **Supplementary Table S1**. Global protein abundances and P values for the correlation
454 between sperm viability and spermatheca protein expression are available in **Supplementary Table S2**.
455 Hierarchical clustering results of protein-protein correlation coefficients are available in **Supplementary**
456 **Table S4.** Any other data that support the findings of this study are available from the corresponding
457 author on request.

458 **Code availability**

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

460 **Acknowledgements**

461 We would like to acknowledge Kettle Valley Queens, Nicola Valley Honey, Wild Antho, Campbells Gold
462 Honey, Heather Meadows Honey Farm, Six Legs Good Apiaries, Wildwood Queens, Cariboo Honey and
463 Worker Bee Honey Company for donating failed and healthy queens for this research, and Jordan Tam
464 for assisting with the BLAST2GO analysis. This work was supported by Natural Sciences and Engineering
465 Research Council of Canada Discovery grant no. 311654-11 and grants from Genome Canada and
466 Genome British Columbia awarded to L.J.F.; a Project Apis m grant awarded to A.M. and L.J.F, a grant
467 from the Boone Hodgson Wilkinson Trust to A.M., and a USDA-NIFA grant no. 2016-07962 awarded to
468 J.S.P. and D.R.T.

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