SEMINAL FLUID PROTEIN DIVERGENCE AMONG POPULATIONS EXHIBITING POSTMATING PREZYGOTIC REPRODUCTIVE ISOLATION

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ABSTRACT

Despite holding a central role for fertilisation success, reproductive traits often show elevated rates of evolution and diversification. The rapid evolution of seminal fluid proteins (Sfps) within populations is predicted to cause mis-signalling between the male ejaculate and female reproductive tract between populations resulting in postmating prezygotic (PMPZ) isolation. Crosses between populations of *Drosophila montana* show PMPZ isolation in the form of reduced fertilisation success in both noncompetitive and competitive contexts. Here we test whether male ejaculate proteins deriving from either the accessory glands or the ejaculatory bulb differ between populations using liquid chromatography tandem mass spectrometry. We find more than 150 differentially abundant proteins between populations which may contribute to PMPZ isolation. These proteins include a number of proteases and peptidases, and several orthologs of *D. melanogaster* Sfps, all known to mediate fertilisation success and which mimic PMPZ isolation phenotypes. Males of one population typically produced greater quantities of Sfps and the strongest PMPZ isolation occurs in this direction. The accessory glands and ejaculatory bulb have different functions and the ejaculatory bulb contributes more to population differences than the accessory glands. Proteins with a secretory signal, but not Sfps, evolve faster than non-secretory proteins although the conservative criteria used to define Sfps may have impaired the ability to identify rapidly evolving proteins. We take advantage of quantitative proteomics data from three *Drosophila* species to determine shared and unique functional enrichments of Sfps that could be subject to selection between taxa and subsequently mediate PMPZ isolation. Our study provides the first high throughput quantitative proteomic evidence showing divergence of reproductive proteins implicated in the emergence of PMPZ isolation between populations.

IMPACT SUMMARY (word count: 300)

Identifying traits that prevent successful interbreeding is key to understanding early stages of the formation of new species, or speciation. Reproductive isolation arising prior to and during fertilisation frequently involves differences in how the sexes interact. In internally fertilising taxa, such interactions are mediated between the female reproductive tract where fertilisation occurs and the receipt of the ejaculate necessary for fertilisation. Because ejaculate proteins are at least partially responsible for these interactions, differences in male ejaculate protein composition could negatively impact fertilisation success, generating reproductive isolation. While the biological classes of ejaculate proteins are shared across all animal taxa, proteins that are secreted by males tend to show rapid evolution in gene expression and genetic sequence. Thus, reproductive proteins are suggested as prime targets facilitating reproductive isolation that arises after mating but before fertilisation (PostMating PreZygotic or PMPZ isolation). Most research on PMPZ isolation has focussed on differences between species for which it is not possible to determine the causative and temporal order of early speciation processes. Here, we test whether populations that exhibit few genetic differences but show strong PMPZ isolation also exhibit variation in ejaculate composition using quantitative high throughput proteomic analyses. We find a number of proteins are differentially abundant between populations including several known to impact fertilisation success in other species. We show that secreted proteins are evolving at an elevated rate, implicating their potential role in PMPZ isolation. We test divergence in ejaculate composition between species, finding a core set of functions that were conserved across species which last shared a common ancestor more than 40 million years ago along with species-specific investment. This work highlights the divergent evolution of reproductive proteins which may contribute to barriers between populations within a species early during speciation, extendable to similar analyses in other taxa in the future.

INTRODUCTION

For internally fertilising taxa the male ejaculate and female reproductive tract must interact during and after mating to ensure optimal fertility (Pitnick et al. 2009, 2020). In polyandrous species, ejaculate x female reproductive tract interactions are subject to rapid coevolution and diversification due to postcopulatory sexual selection (sperm competition and cryptic female choice) and sexually antagonistic coevolution (Birkhead and Pizzari 2002; Sirot et al. 2015; Firman et al. 2017; Meslin et al. 2017). Thus, despite holding a central role for fertilisation, ejaculate and female reproductive tract traits often show elevated rates of molecular and morphological evolution (Rowe et al. 2015; Ahmed-Braimah et al. 2017; Meslin et al. 2017; VanKuren and Long 2018; Simmons and Fitzpatrick 2019; McGeary and Findlay 2020). Divergence between populations in these traits is predicted to result in the early emergence of reproductive isolation that occurs after mating but before fertilisation (postmating prezygotic; PMPZ) (Lande 1981; Gavrilets 2000; Panhuis et al. 2001). Studies have increasingly documented PMPZ isolation, including prior to any postzygotic isolation (Howard et al. 2009; Bono et al. 2011; Sagga and Civetta 2011; Manier et al. 2013; Jennings et al. 2014; Cramer et al. 2016; Devigili et al. 2018; Garlovsky and Snook 2018; Turissini et al. 2018). In the *Drosophila melanogaster* subgroup, PMPZ isolation accumulates quickly measured by relative rates of evolution of different types of reproductive isolating mechanisms and suggests that PMPZ isolation is important in promoting new species and maintaining species barriers (Turissini et al. 2018). Despite the increasing recognition of the importance of PMPZ isolation, there is little understanding of the molecular basis of ejaculate x female reproductive tract interactions that may generate such barriers (McDonough et al. 2016).

The ejaculate consists of sperm and male seminal fluid proteins (Sfps) that impact fertilisation (e.g., Avila et al. 2011; South and Lewis 2011). Most Sfps are products of male secretory

101 glands (e.g. in mammals, the prostate gland and seminal vesicles; in arthropods, accessory
102 glands and ejaculate ducts/bulb; for review, see Sirot et al. 2015; Fig. S1). Different secretory
103 organs contribute distinct sets of proteins to the ejaculate allowing increased complexity and
104 modulation or tailoring of the ejaculate (Bayram et al. 2019). The majority of work on insect Sfp
105 evolution has been done on *Drosophila melanogaster* with over 200 Sfps identified (Mueller et
106 al. 2005; Findlay et al. 2008, 2009). However, many of the biochemical classes of Sfps are
107 similar across animals; for example, proteases and protease inhibitors, and those with
108 antimicrobial/immune related functions (Avila et al. 2011; Sirot et al. 2015). Despite conserved
109 protein classes observed in the seminal fluid of all animals, a large fraction of Sfps show rapid
110 molecular evolution and, therefore, even Sfps of the same classes in different species are not
111 orthologous (Avila et al. 2011; Perry et al. 2013; Sirot et al. 2015). Functional confirmation of
112 Sfps, performed mostly in *D. melanogaster*, indicate they aid in sperm transfer and storage,
113 influence the outcome of sperm competition, and/or alter female physiology, behaviour and
114 reproductive tract morphology after mating (Ravi Ram and Wolfner 2007; Wong et al. 2008;
115 Avila and Wolfner 2009; Holman 2009; Wigby et al. 2009; Wolfner 2009; Avila et al. 2011;
116 Fedorka et al. 2011; Mattei et al. 2015). Moreover, some Sfps elicit PMPZ-like phenotypes
117 when genetically manipulated (Ravi Ram and Wolfner 2007; LaFlamme et al. 2012).
118 Differences in Sfp expression between species or abnormal gene expression profiles in the
119 female reproductive tract after mating with hetero- vs. con- specific males are associated with
120 PMPZ isolation (Bono et al. 2011; Ahmed-Braimah et al. 2017). These shared and divergent
121 patterns support Sfps as putative causative agents of PMPZ isolation.
122
123 However, while studies showing divergence in gene expression are associated with disrupted
124 ejaculate x female reproductive tract interactions, changes in gene expression may not
125 correlate with changes in protein abundance (Wang et al. 2019), where the molecular

interactions causing PMPZ isolation take place. Divergence in protein identity or abundance between taxa could disrupt ejaculate x female reproductive tract interactions leading to PMPZ isolation (Goenaga et al. 2015). High-throughput proteomics using liquid chromatography tandem mass spectrometry (LC-MS/MS) has revolutionised identification and quantification of Sfps, revealing that the male ejaculate often contains hundreds of unique proteins (McDonough et al. 2016; Bayram et al. 2019; Karr 2019; Rowe et al. 2019; Whittington et al. 2019). Using LC-MS/MS combined with genomics, Sfps can be predicted by identifying ejaculate proteins with a signal peptide sequence, sometimes called the “secretome”, and those secretome proteins that have an extracellular signal sequence, sometimes called the “exoproteome” (Avila et al. 2011; Ahmed-Braimah et al. 2017; Bayram et al. 2019; Karr et al. 2019; Sepil et al. 2019). Molecular evolution analyses show that proteins with secretory signal and/or are secreted evolve faster than non-secreted ejaculate proteins (Mueller et al. 2005; Wagstaff and Begun 2005; Ramm et al. 2009; Bono et al. 2015; Tsuda et al. 2015; Ahmed-Braimah et al. 2017; Karr et al. 2019)

While these results suggest Sfps are prime candidates for generating PMPZ isolation, no study using high throughput quantitative proteomics has tested the prediction that ejaculate composition will vary between populations exhibiting PMPZ isolation, and that these proteins will more rapidly evolve. Similarly, while previous work has suggested that the different ejaculate secretory organs may perform different functions (Bayram et al. 2019), their potential contribution to PMPZ isolation has not been examined. Here we use LC-MS/MS on the accessory glands and the ejaculatory bulb/duct, followed by molecular evolutionary rates analysis, to test these predictions using the malt fly, *Drosophila montana*. We have focused on two populations (Crested Butte, Colorado; Vancouver, Canada) and found that reciprocal crosses between populations result in PMPZ isolation both after a single mating as males

transfer sperm to females who store them, but many eggs are not fertilised (Jennings et al. 2014; Garlovsky and Snook 2018), and where within- and between- population males compete for fertilisation (frequently referred to as conspecific sperm precedence) (Garlovsky et al. in review). The similar results suggest a shared mechanism generating PMPZ isolation. Genomic analysis found no fixed SNPs between these populations, likely due to a history of gene flow during divergence (Parker et al. 2018; Garlovsky et al. in review), although genes enriched for reproductive function show divergence (Parker et al. 2018). These results support focussing on Sfps as potential causative agents of PMPZ isolation. We also leverage recent high throughput mass spectrophotometry data on ejaculate composition in two other *Drosophila* species (Karr et al. 2019; Sepil et al. 2019) to provide insights into shared and divergent Sfp functions that may contribute to PMPZ isolation.

METHODS

Fly stocks

Adult *Drosophila montana* were collected with malt bait buckets and mouth aspirators in Crested Butte, Colorado, USA (38°49'N, 107°04'W) in 2013 (referred to as Colorado), and Vancouver, British Columbia, Canada (48°55'N, 123°48'W) in 2008 (referred to as Vancouver) (Fig. S1). Stocks were established by combining 20 F3 males and females from 20 isofemale lines (800 flies total per population) and cultured on Lakovaara malt media (Lakovaara 1969) in overlapping generations in constant light at 19ºC. Flies were collected within 3 days of eclosion and housed in groups of between 10-20 single sex individuals in food vials until reproductively mature at 21 days old.

176 *Tissue collection and protein extraction*

177 Twenty-one-day old males were anaesthetised with ether and the accessory glands and
178 ejaculatory duct/bulb separated from nontarget tissues, and from each other. We collected
179 three biological replicates, two of which were separated into technical replicates (Fig. S1).
180 Following protein extraction and purification, we quantified protein concentration to standardize
181 loading 5µg of protein for each sample into the mass spectrometer (see supplementary
182 material; Fig. S2). Samples were reduced with TCEP, alkylated by addition of MMTS, and
183 digested with trypsin, followed by drying to completion using vacuum centrifugation. Samples
184 were resuspended in 20µl 3% v/v acetonitrile, 0.1% v/v trifluoroacetic acid prior to LC-MS/MS
185 analysis.

186

187 *LC-MS/MS analysis*

188 Detailed description of LC-MS/MS data acquisition and processing can be found in the
189 supplementary material.

190 LC-MS/MS was performed by nano-flow liquid chromatography (U3000 RSLCnano, Thermo
191 Fisher™) coupled to a hybrid quadrupole-orbitrap mass spectrometer (QExactive HF, Thermo
192 Scientific™). Peptides were separated on an Easy-Spray C_{18} column (75 µm x 50 cm) using a
193 2-step gradient from 97% solvent A (0.1% formic acid in water) to 10% solvent B (0.08% formic
194 acid in 80% acetonitrile) over 5 min then 10% to 50% B over 75 min at 300 nL/min. The full
195 105-minute MS data dependent acquisition was set up from 375-1500 m/z acquired in the
196 Orbitrap in profile mode, resolution 120,000. Subsequent fragmentation was Top 10 in the HCD
197 cell, with detection of ions in the Orbitrap using centroid mode, resolution 30,000. MS
198 parameters; MS1: Automatic Gain Control (AGC) target 1e6 with a maximum injection time (IT)
199 of 60 ms; MS2: AGC target 1e5, IT of 60 ms and isolation window 2 Da.

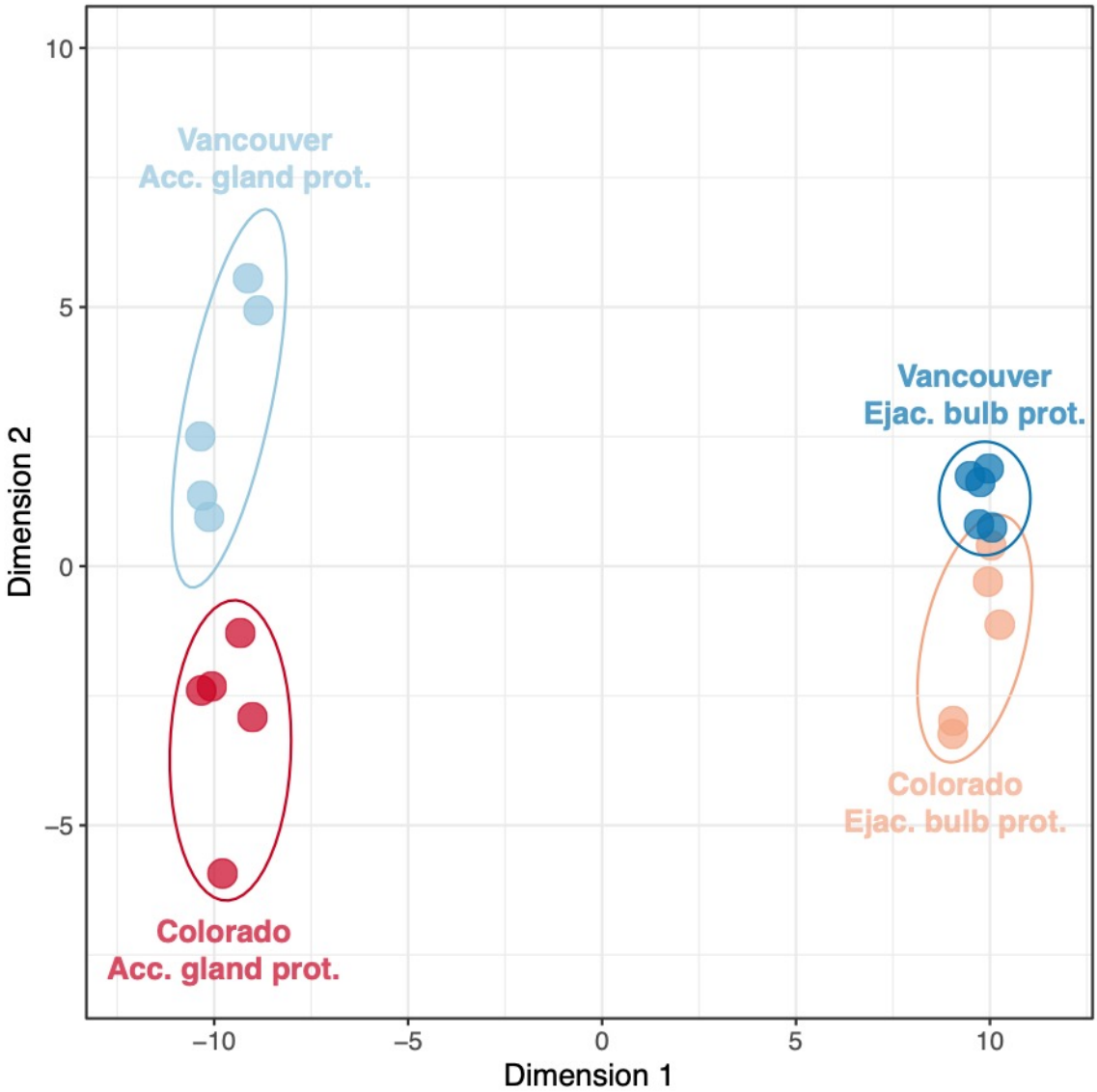
200

201 We performed label free quantitative proteomic analysis using MaxQuant to generate relative
202 peptide and protein intensities (Cox et al. 2014; Tyanova et al. 2016) (see supplementary
203 material). For protein identification we matched mass spectra to the *D. montana* predicted
204 proteome (Parker et al. 2018), generated using gene predictions from the Maker2 pipeline (Holt
205 and Yandell 2011) reciprocally blasted against *D. virilis* proteins (Parker et al. 2018).
206
207 *Gene Ontology (GO) and functional analysis*
208 We performed network analyses and GO enrichment for Biological Processes (BP), Cellular
209 Components (CC) and Molecular Functions (MF) with the ClueGO plugin (Bindea et al. 2009)
210 for Cytoscape (Shannon et al. 2003) using FlyBase gene numbers (FBgns) for *D. virilis*
211 orthologs of *D. montana* genes retrieved from Parker et al. (2018) or *D. melanogaster* orthologs
212 converted via FlyBase.org. Specific settings for network groups are provided in figure and table
213 legends. For GO enrichment we used right-sided hypergeometric tests with Benjamini-
214 Hochberg multiple test correction.
215
216 *Differential abundance analysis between D. montana populations and functional differences*
217 *between tissues*
218 We performed differential abundance analysis of MaxLFQ ion intensities using the 'edgeR'
219 (Robinson et al. 2010) and 'limma' (Ritchie et al. 2015) packages in R (v.3.5.1) (R Core Team
220 2018) (see supplementary material). Proteins were considered differentially abundant based
221 on an adjusted p-value < 0.05. To identify differentially abundant proteins between populations,
222 we analysed the accessory gland proteome and the ejaculatory bulb proteome separately. We
223 only considered proteins that were present in all five replicates of each tissue for both
224 populations. To identify differentially abundant proteins between tissues, we analysed each

225 population separately. Again, we only considered proteins that were present in all five
226 replicates of each population for both tissues. (Table S1).
227
228 *Characterising the male seminal fluid proteome across species*
229 We compared Sfp functions for three *Drosophila* species for which proteomic data generated
230 using LC-MS/MS is available for the male accessory gland and ejaculatory duct and bulb
231 tissues: *D. montana* (this study), *D. melanogaster* (Sepil et al. 2019), and *D. pseudoobscura*
232 (Karr et al. 2019). We retrieved FBgns for *D. melanogaster* genes identified by Sepil et al.
233 (2019) and *D. melanogaster* orthologs for *D. pseudoobscura* genes identified by Karr et al.
234 (2019) and downloaded the corresponding canonical protein sequences from uniprot.org. For
235 proteins we identified in our analysis we retrieved *D. montana* protein sequences from Parker
236 et al. (2018). We submitted protein sequences for each species to *SignalP* (Petersen et al.
237 2011) and *Phobius* (Käll et al. 2004) and combined the resulting lists of proteins containing a
238 signal peptide to generate a list of secretome proteins for each species. For *D. montana* we
239 converted the corresponding *D. virilis* FBgns for each protein to *D. melanogaster* orthologs via
240 FlyBase.org (for 215/245, 88%). To identify Sfps for each species we submitted secretome
241 lists to FlyBase.org to retrieve genes with GO terms containing “extracelluar” (Fig. S3; Table
242 S2). To compare GO enrichment between species we adjusted network settings in ClueGO to
243 reflect the different numbers of proteins identified in each species.
244
245 *Evolutionary rates analysis*
246 To obtain sequence divergence estimates for *D. montana* proteins we used a pipeline
247 developed previously (Wright et al. 2015). We obtained protein coding sequences for *D.*
248 *montana* from Parker et al. (2018) and for *D. pseudoobscura* (r3.04, September 2019) and *D.*
249 *virilis* (r1.07, August 2019) from FlyBase.org. We identified the longest isoform of each gene

250 for each species and determined orthology with reciprocal BLASTN (Altschul et al. 1990), using
251 a minimum percentage identity of 30% and an E-value cut-off of $1x10^{-10}$. We then identified
252 reciprocal one-to-one orthologs across all three species using the highest BLAST score. We
253 identified open reading frames using BLASTx and aligned orthologs using PRANK (Löytynoja
254 and Goldman 2010). We calculated the ratio of non-synonymous (dN) to synonymous (dS)
255 nucleotide substitutions, omega (ω), using the CODEML package in PAML (Yang 2007) (one-
256 ratio estimates, model 0) with an unrooted phylogeny. Results were filtered to exclude
257 orthologs with branch-specific dS ≥ 2 (due to potential mutational saturation) or where S*dS ≤
258 1.
259
260 We then tested for differences in evolutionary rates between sets of proteins we identified in
261 our LC-MS/MS analysis. We relaxed filtering criteria so that a protein need only be identified
262 in a single replicate in a single population or tissue, but still had to be identified by two or more
263 unique peptides. After filtering, we obtained ω values for 757/1474 (51%) proteins with a
264 reciprocal one-to-one ortholog. We categorised genes as having higher abundance in either
265 the accessory gland proteome or ejaculatory bulb proteome as those proteins showing
266 concordant differential abundance between tissues across populations (Fig. 3). We classified
267 proteins as belonging to the secretome based on presence of a signal peptide and as Sfps as
268 secretome proteins with extracellular annotation (see above) plus *D. melanogaster* Sfps. We
269 classified the remainder of proteins excluding those with higher abundance in the accessory
270 gland proteome, ejaculatory bulb proteome, secretome, or Sfps, as background tissue. Each
271 class consisted of a unique set of proteins, such that accessory gland proteins did not include
272 ejaculatory bulb proteins, secretome, Sfps, or remaining proteins; secretome proteins did not
273 include Sfps, etc. We tested for differences in evolutionary rates using the Kruskal-Wallis rank

274 sum test followed by pairwise Wilcox rank sum tests corrected for multiple testing using the
275 Benjamini-Hochberg method.
276
277 **RESULTS**
278 *The* D. montana *accessory gland proteome and ejaculatory bulb proteome*
279 We identified 1711 proteins, of which 1474 (86%) were identified by two or more unique
280 peptides. The majority of proteins (1013/1474; 69%) were shared across male secretory
281 tissues, while 138 (9%) and 323 (22%) proteins were unique to the accessory glands and
282 ejaculatory bulb, respectively (Fig. S4a). Proteins identified only in the accessory gland
283 proteome showed a 3.2-fold lower mean abundance compared to the remainder of proteins
284 whereas proteins identified only in the ejaculatory bulb proteome showed a 14.9-fold reduction.
285 These proteins likely represent missed rather than truly unique proteins and are not considered
286 further. We identified 79 *D. montana* Sfps, consisting of 38 orthologs of *D. melanogaster* Sfps
287 identified by converting *D. virilis* FBgns on FlyBase.org, plus 55 secretome proteins with
288 extracellular annotations identified by 2 or more unique peptides (14 of which overlapped) (Fig.
289 S4a; Table S3) (Mueller et al. 2005; Findlay et al. 2008, 2009). A multidimensional scaling
290 (MDS) plot of normalised intensities using all proteins (n = 1474) showed a clear separation of
291 samples by tissue type (dimension 1), and separation by population (dimension 2) with clear
292 separation of populations for the accessory gland proteome and marginal overlap between
293 populations in the ejaculatory bulb proteome (Fig. 1).



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295 Figure 1. Multidimensional scaling (MDS) plot of normalised intensities for proteins identified
296 by two or more unique peptides (n = 1474) in each replicate (points). Dimension 1 separates
297 the two tissue types (accessory glands and ejaculatory bulbs). Dimension 2 separates the two
298 populations (Colorado and Vancouver).

299

300 *Differential abundance of reproductive proteins between populations*

301 The majority of proteins were identified in both populations (1322/1474; 90%), while 45 (3%)
302 and 107 (7%) were only identified in Colorado, and Vancouver, respectively (Fig. S4b).
303 Proteins only identified in one population showed a 263-fold, and 171-fold, lower mean

304 abundance compared to the rest of proteins in Colorado, and Vancouver, respectively. As
305 above, these low abundance proteins are not considered further. For shared proteins, we then
306 tested for differential abundance. We identified 154 (out of 725) differentially abundant proteins
307 produced in the accessory glands between populations (Fig. 2a), including nine *D.*
308 *melanogaster* Sfps (Table 1). We identified 244 (out of 929) differentially abundant proteins
309 produced in the ejaculatory bulbs (Fig. 2b). Again, this included nine *D. melanogaster* Sfps,
310 two of which overlapped with those identified in the accessory gland proteome (Table 1). In the
311 accessory gland proteome, Sfps and proteins with a predicted secretory signal were not
312 overrepresented in the cohort of proteins showing differential abundance (Chi-squared test, X^2
313 = 1.57, df = 2, p = 0.456) but were overrepresented in the cohort of differentially abundant
314 proteins in the ejaculatory bulb proteome (X^2 = 44.56, df = 2, p < 0.001; Fig. S5). Out of 45
315 proteins that were differentially abundant between populations in both male reproductive
316 tissues, 36 showed higher abundance in one population in both tissues (Fig. S6). Significantly
317 enriched gene ontology (GO) categories for proteins showing differential abundance between
318 populations are in Tables S4-S6.
319

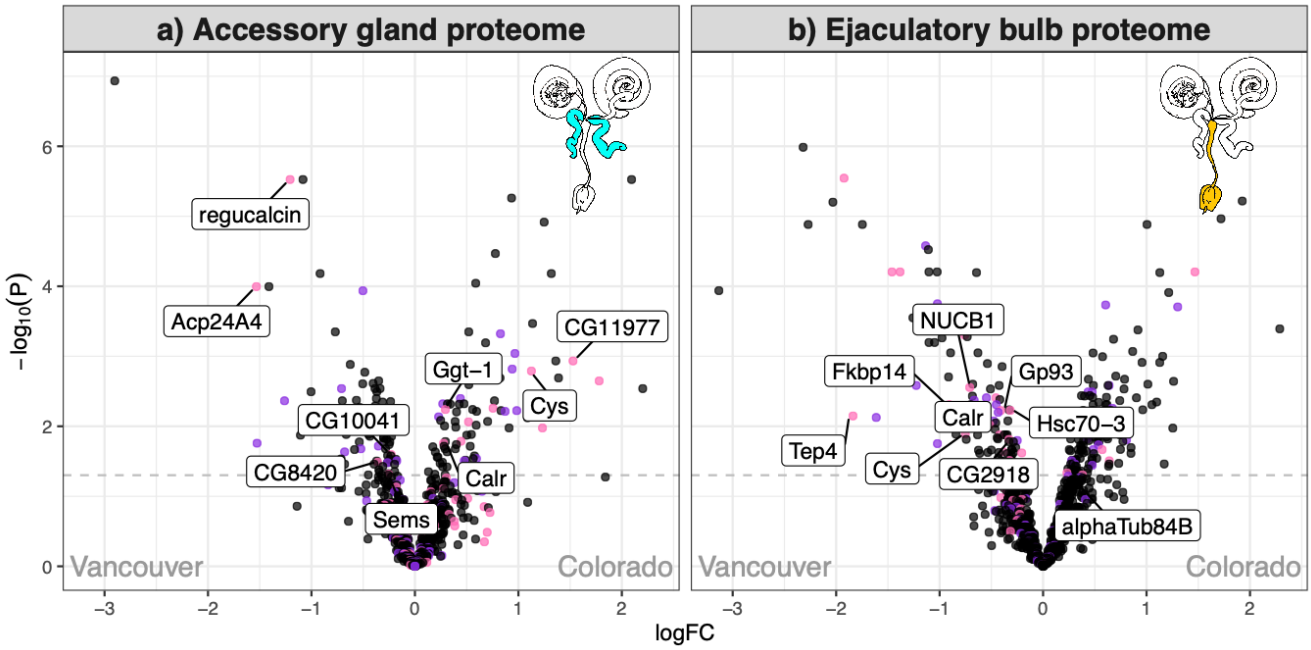


Figure 2. Differentially abundant proteins between Colorado and Vancouver in a) the accessory glands (n = 725) and b) the ejaculatory bulbs (n = 929). Secretome proteins are shown in purple and Sfps in pink. Significantly differentially abundant proteins with a known Sfp ortholog in *D. melanogaster* are labelled.

336 Table 1. Differentially abundant proteins between *D. montana* populations with a known *D.*
337 *melanogaster* seminal fluid protein (Sfp) ortholog. Gene names were retrieved from
338 FlyBase.org using the corresponding *D. virilis* FBgns for *D. montana* proteins we identified via
339 LC-MS/MS. The population for which each protein showed higher abundance is given. Proteins
340 found in both tissue comparisons indicate in which population there was higher abundance.
341 Abbreviations: Acgs, accessory glands; Ebs, ejaculatory bulbs.

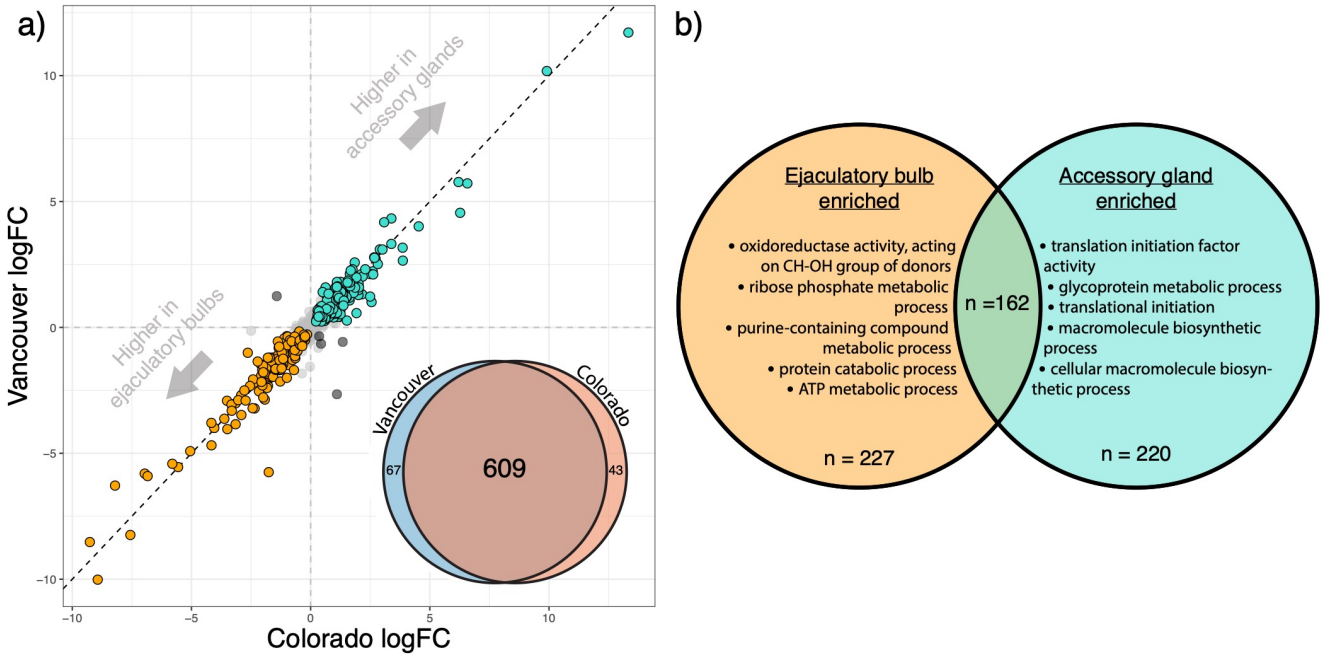
Tissue comparison	Gene name	Higher abundance
Accessory glands	CG11977	Colorado
	gamma-glutamyl transpeptidase	Colorado
	Acp24A4	Vancouver
	CG10041	Vancouver
	CG8420	Vancouver
	Regucalcin	Vancouver
	Seminase	Vancouver
Ejaculatory bulbs	alpha-Tubulin at 84B	Colorado
	CG2918	Vancouver
	FK506-binding protein 14	Vancouver
	Glycoprotein 93	Vancouver
	Heat shock 70-kDa protein cognate 3	Vancouver
	NUCB1	Vancouver
	Thioester-containing protein 4	Vancouver
Both	Calreticulin	Colorado^{Acgs}/Vancouver^{Ebs}
	Cystatin-like	Vancouver^{Acgs}/Colorado^{Ebs}

342

343 *The accessory gland and ejaculatory bulb proteomes differ in function*

344 To test whether the accessory glands and ejaculatory bulb provide different functions we
345 performed differential abundance analysis between tissues for Colorado and Vancouver
346 separately. We found 524 (out of 652) differentially abundant proteins between tissues in
347 Colorado. Similarly, in Vancouver we found 557 (out of 676) differentially abundant proteins.
348 The majority of these proteins were found in both populations (609 proteins). To identify
349 consistently differentially abundant proteins between tissues, we compared the log2-fold
350 change in abundance in each population of these 609 proteins. Proteins with higher abundance

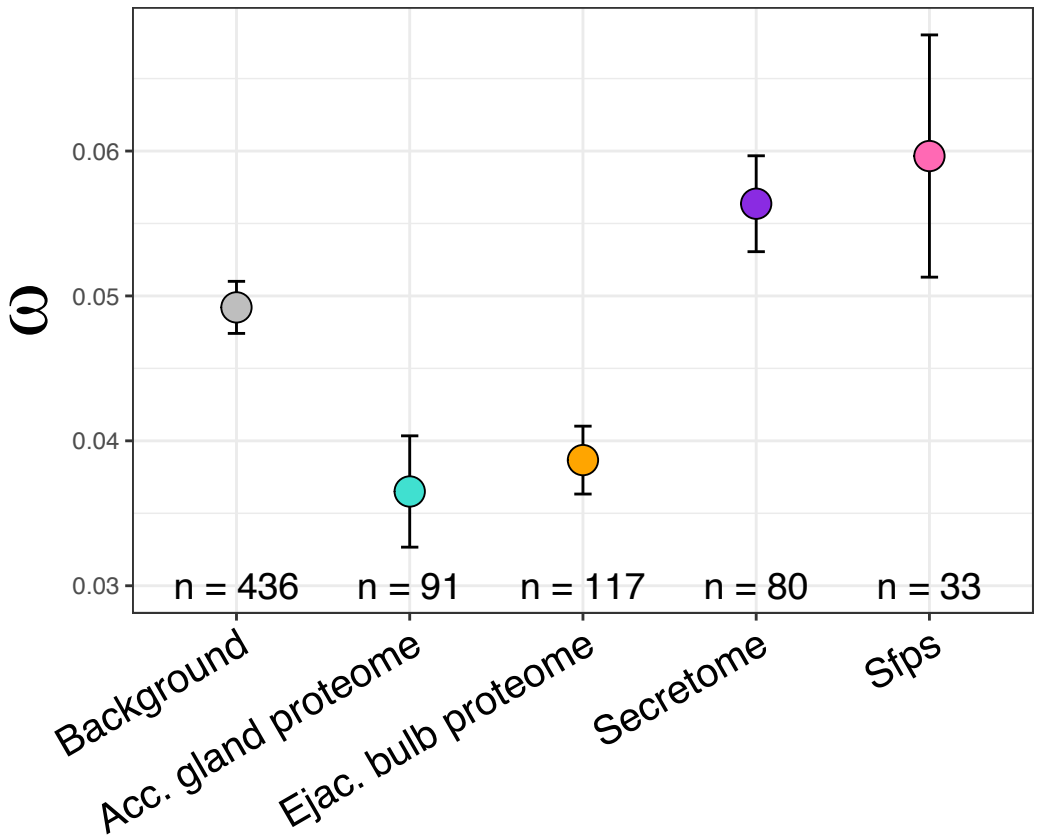
351 in the accessory gland proteome or ejaculatory bulb proteome in Colorado generally also
352 showed higher abundance in Vancouver (Spearman's rank correlation, $\rho = 0.945$, $p < 0.001$, n
353 = 609) (Fig. 3a). GO analyses identified both tissues as having functions expected for highly
354 metabolically active secretory organs (Table S7). Different GO terms were enriched in each
355 tissue highlighting that the two secretory organs provide distinct reproductive functions to the
356 ejaculate (Fig. 3b; Table S7).
357



358

359 Figure 3. Populations show consistent differential abundance between secretory organs. a)
360 Concordance between populations in log2-fold change in abundance of proteins found in either
361 the accessory gland proteome or ejaculatory bulb proteome. Positive values indicate proteins
362 with higher abundance in the accessory glands in both populations (top right), negative values
363 indicate proteins with higher abundance in the ejaculatory bulb (bottom left). Proteins are
364 coloured based on whether they showed a concordant pattern of significantly higher
365 abundance in the accessory gland proteome (turquoise, n = 220), the ejaculatory bulb
366 proteome (orange, n = 227), were discordant (black, n = 5), or were not significantly
367 differentially abundant between tissues (grey). Dashed black line shows 1:1. Inset: Venn

368 Diagram showing numbers of proteins included in separate differential abundance analysis
369 between tissues in each population and overlap. b) Top 5 significantly enriched Biological
370 Process GO terms ranked by percent identity of proteins to each tissue (see Table S7 for full
371 list).
372
373 *Evolutionary rates analysis*
374 We tested whether genes with higher protein abundance in either the accessory gland
375 proteome, ejaculatory bulb proteome (excluding the secretome and Sfps), secretome
376 (excluding Sfps), or Sfps (secretome proteins with extracellular annotation), were evolving at
377 different rates compared to background proteins, defined as those proteins that do not differ in
378 protein abundance between the accessory glands and ejaculatory bulb and excluding
379 secretome and Sfps. There was a significant difference between protein groups in evolutionary
380 rates (Kruskal-Wallis test, X^2 = 40.51, df = 4, $p < 0.001$) (Fig. 4). The secretome and Sfps were
381 evolving at similar rates (pairwise Wilcox rank sum test with Benjamini-Hochberg adjustment,
382 $p = 0.668$). The secretome was evolving faster than proteins with higher abundance in the
383 accessory gland proteome, ejaculatory bulb proteome, or background (all $p < 0.007$). Sfps
384 were also evolving faster than proteins with higher abundance in the accessory gland proteome
385 ($p = 0.001$) and ejaculatory bulb proteome ($p = 0.007$) but at a similar rate to background ($p =$
386 0.171). Proteins with higher abundance in the accessory gland proteome and ejaculatory bulb
387 proteome were evolving at similar rates ($p = 0.089$), and slower than the remaining background
388 proteome (accessory gland proteome vs. background, $p < 0.001$; ejaculatory bulb proteome
389 vs. background, $p = 0.011$).
390



391

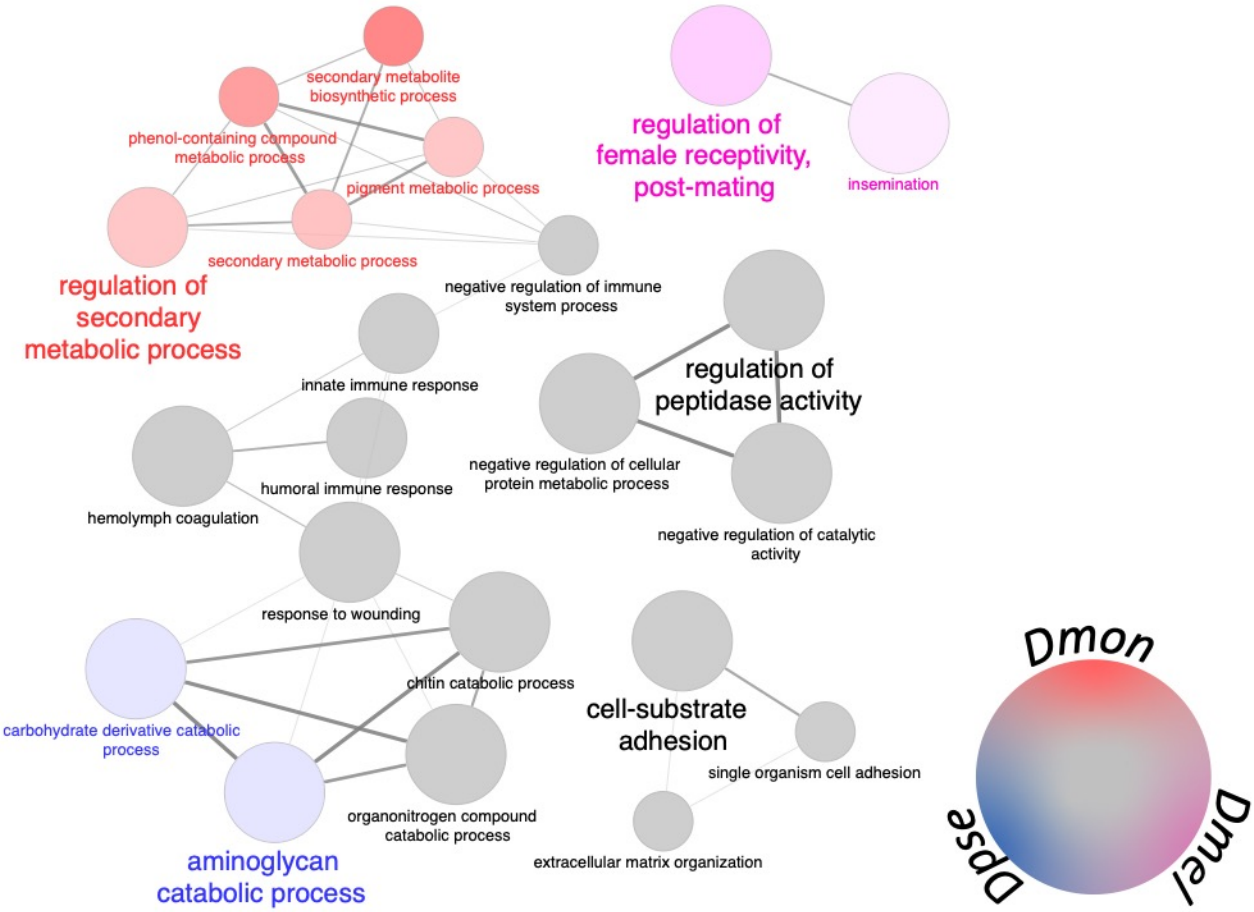
392 Figure 4. Mean non-synonymous (dN) to synonymous (dS) base substitution rate (ω) estimates
393 (± standard error) for *D. montana* genes identified in our LC-MS/MS analysis with equal
394 abundance in the accessory gland proteome and ejaculatory bulb proteome ('background';
395 grey), higher abundance in the accessory gland proteome (turquoise), ejaculatory bulb
396 proteome (orange), or found in the secretome (purple) or Sfps (pink). See Fig. S7 for separate
397 dN and dS plots.

398

399 *Comparison of male Sfps across species*

400 We identified 61 Sfps (secretome proteins with extracellular annotations) for *D. montana*, 249
401 Sfps for *D. melanogaster*, and 131 Sfps for *D. pseudoobscura* (Fig. S3; Table S2). Comparing
402 functional enrichment of Sfps across species identified a number of shared and unique GO
403 categories. Shared Biological Processes included chitin catabolic process, innate immune
404 response, cell-substrate adhesion, and regulation of peptidase activity (Fig. 5; see Table S8

405 for CC and MF terms). Uniquely enriched BP functions included regulation of secondary
406 metabolic process (*D. montana*); postmating regulation of female receptivity (*D. melanogaster*)
407 and amino glycan catabolic processes (*D. pseudoobscura*) (Fig. 5; see Table S8 for CC and
408 MF terms).
409



410

411 Figure 5. Seminal fluid protein (Sfps) comparisons for GO BP terms across species. Circle size
412 is associated with level of significance with increasing size indicating increasing significance.
413 Node colour indicates proportion of genes from each species associated with a term: *D.*
414 *montana* (red. Dmon), *D. melanogaster* (pink; Dmel), and *D. pseudoobscura* (blue; Dpse),
415 shared terms are shown in grey. Min. GO level = 3, max. GO level = 8. Number of genes/%
416 genes per group: *D. montana* 3/3%, *D. pseudoobscura* 6/6%, *D. melanogaster* 12/12%.
417 Percentage significance = 55%, kappa-score threshold = 0.25.

418 **DISCUSSION**

419 The molecular basis of mechanisms underlying PMPZ isolation are poorly understood. Seminal
420 fluid proteins are likely to contribute to PMPZ isolation due to their effects on sperm use,
421 fertilisation success, and rapid divergent evolution. We used quantitative proteomics to identify
422 proteins produced in the accessory glands and ejaculatory duct and bulb in populations
423 exhibiting PMPZ isolation and found a number of differentially abundant proteins between
424 populations including several orthologs of *D. melanogaster* Sfps. The accessory glands and
425 ejaculatory bulb showed different functions and there were more differentially abundant
426 proteins found in the ejaculatory bulb than the accessory glands. For proteins found in both
427 populations, but in separate tissues, there was strong concordance in abundance between
428 populations. We found secretome proteins evolved at a faster rate than non-secretome
429 proteins, both those differentially abundant between male secretory organs and those showing
430 similar abundance between male tissues. Sfps did not show elevated rates of molecular
431 evolution, likely because identification by extracellular annotation required orthology in *D.*
432 *melanogaster*. Despite shared Sfps, and a core set of shared Sfp biological processes across
433 three *Drosophila* species, there was species-specific enrichment of Sfp function.

434

435 *D. montana* from Colorado and Vancouver show low genome-wide divergence and a history
436 of gene flow (Parker et al. 2018; Garlovsky et al. in review), yet show enrichment of genes with
437 reproductive function that are divergent between populations (Parker et al. 2018). Crosses
438 between Colorado and Vancouver show reduced fertilisation success after a single mating
439 (Jennings et al. 2014; Garlovsky and Snook 2018) and exhibit conpopulation sperm
440 precedence (Garlovsky et al. in review). Between different *Drosophila* species, mechanisms
441 causing PMPZ isolation include abnormal sperm transfer and displacement, or mismatches
442 between sperm length and female tract morphology (Price et al. 2001; Manier et al. 2013).

443 Females receiving a foreign ejaculate comprising an abnormal Sfp complement might also
444 result in mismatched ejaculate x female reproductive tract interactions measured by gene
445 expression differences (Bono et al. 2011; Plakke et al. 2015), although variation between
446 species in ejaculate composition has not been quantified in those studies.
447
448 We identified a number of differentially abundant proteins between populations exhibiting
449 PMPZ isolation, including several orthologs of *D. melanogaster* Sfps. Intriguingly, 11 of 14 of
450 these proteins were more abundant in Vancouver males than Colorado males. PMPZ isolation
451 between *D. montana* populations is asymmetric, with matings between Vancouver males and
452 Colorado females having lower fertilisation success compared to the reciprocal cross
453 (Jennings et al. 2014; Garlovsky and Snook 2018). If Vancouver males transfer more of these
454 Sfps to their mates, then the chemical environment in the reproductive tract of Colorado
455 females may be mismatched, more so than the reciprocal cross.
456
457 Additionally, a number of proteases and peptidases (or inhibitors) were differentially abundant
458 between populations. Proteases and peptidases are central to reproduction across taxa,
459 regulating proteolytic activity and initiating cascades of interactions among downstream
460 proteins (LaFlamme et al. 2012; LaFlamme and Wolfner 2013; Plakke et al. 2015, 2019;
461 Bayram et al. 2017, 2019). Divergence in proteases has been implicated in PMPZ isolation
462 between other insect species in both the male ejaculate and female reproductive tract
463 secretions (Kelleher et al. 2007; Kelleher and Pennington 2009; Marshall et al. 2009, 2011;
464 Meslin et al. 2017; Al-Wathiqui et al. 2018; Plakke et al. 2019).
465
466 In *D. montana* females receive and store motile sperm from incompatible males, but fertilisation
467 success is reduced (Jennings et al. 2014). Failure to either properly orient sperm in storage

468 (Manier et al. 2013), release sperm from storage, or have sperm release coincide with ovulation
469 (Mattei et al. 2015) could explain PMPZ in this system (Jennings et al. 2014). Some notable
470 differentially abundant Sfps and proteases we identified, and their potential relationship to
471 PMPZ isolation in *D. montana* are seminase, γ-glutamyl transpeptidase, and regucalcin.
472 Seminase (CG10586), is a serine protease and a member of the Sex Peptide (SP) network
473 (Singh et al. 2018). Seminase acts early in the SP network and is required to process other
474 Sfps in the mated female essential for proper sperm storage (Acp36DE) and ovulation (ovulin)
475 (LaFlamme et al. 2012; Singh et al. 2018). RNAi knockdown of seminase in male *D.*
476 *melanogaster* results in failure of mated females to release sperm from the seminal receptacle
477 (LaFlamme et al. 2012). γ-glutamyl transpeptidase (CG6461) functions to maintain a protective
478 redox environment for sperm (Walker et al. 2006). Mismatches between the male ejaculate
479 and the redox environment of the female reproductive tract in which sperm are stored could
480 reduce fertilisation success as sperm subject to increased oxidative stress are less fertilisation
481 competent (Reinhardt and Ribou 2013). Regucalcin (CG1803), a Ca^{+2} binding protein, may
482 also play an anti-oxidative role and, in mammals, is hypothesized to have an anti-capacitation
483 role for sperm (Pillai et al. 2017). One aspect of capacitation, hyperactivation, increases sperm
484 motility which is important for sperm storage in *Drosophila* (Köttgen et al. 2011). Sperm motility
485 behaviour and how this may affect release from storage is unknown. Regucalcin gene
486 expression varies between *D. montana* populations and has been suggested as a cold
487 tolerance gene in diapausing females (Vesala et al. 2012) although it's expression in males
488 has not been studied. These examples provide strong candidates for eliciting PMPZ isolation
489 and will be subject to future studies, for instance using CRISPR/Cas9 gene editing, to further
490 understand the molecular interactions causing PMPZ isolation in *D. montana*.
491

492 Reproductive proteins are predicted to evolve rapidly, driven by postcopulatory sexual
493 selection and sexual conflict (Sirot et al. 2015; Ahmed-Braimah et al. 2017; Firman et al. 2017;
494 Meslin et al. 2017). We found that proteins showing secretory signals (secretome) evolve faster
495 than proteins without this signal. Sfps and secretome proteins were evolving at a similar rate.
496 However, Sfps were not evolving faster than proteins with similar abundance between male
497 tissues, possibly due to showing greater variation than other categories despite having a higher
498 mean rate. The requirement to have extracelluar annotation determined from work in *D.*
499 *melanogaster* limits our ability to identify rapidly evolving Sfps in *D. montana*. Thus, the putative
500 number of Sfps in *D. montana*, 79, is a conservative estimate. Isotopically labelling males to
501 identify proteins transferred to females increased the number of identified *D. melanogaster*
502 Sfps (Findlay et al. 2008, 2009). Future work on *D. montana* can use this technique to identify
503 additional Sfps.
504
505 One goal of this work was to assess whether the different male reproductive secretory organs
506 had different functions, which would not be possible using the heavy labelling technique. Our
507 work provides one of the first proteomic descriptions of both major Sfp secretory organs in
508 *Drosophila*. Previous research in seed beetles has shown that division of labour across
509 secretory organs enables increased complexity to the ejaculate and potential for ejaculate
510 tailoring (Bayram et al. 2019). Most proteins we identified were found in both tissues but
511 showed higher abundance in either the accessory glands or ejaculatory duct and bulb,
512 suggesting these organs provide different functions to the ejaculate. The accessory gland
513 proteome was enriched for translation and biosynthetic processes, whereas the ejaculatory
514 bulb proteome showed enrichment for mainly metabolic processes. We found secretome
515 proteins and Sfps were significantly overrepresented in the set of differentially abundant
516 proteins in the ejaculatory bulb proteome but not the accessory gland proteome, suggesting

517 the two male secretory organs may contribute differently to PMPZ isolation. Most past work on
518 Sfp evolution has focused on the accessory glands, which could skew understanding of not
519 only molecules involved in reproduction but those reproductive molecules that may elicit PMPZ
520 isolation.
521
522 We took advantage of recent accessory gland proteomes for three *Drosophila* species
523 generated using high throughput LC-MS/MS to characterise shared and enriched functions of
524 Sfps between species. Using the same identification criteria for all species (secretory sequence
525 and extracellular annotation), we identified a set of shared GO categories between species
526 that last shared a common ancestor 40 million years ago. This core set included immune-
527 related genes, which are associated with sexual conflict in *D. melanogaster* (Innocenti and
528 Morrow 2009). We also found species-specific GO-enrichment of Sfps, suggesting divergence
529 in how they contribute to male ejaculate function between species. Differences may reflect how
530 selection has targeted particular ejaculate traits in different mating systems (Markow 2002).
531 Differences will also reflect the use of *D. melanogaster* as the reference for GO annotation. For
532 instance, *D. melanogaster* showed enrichment for reproductive genes but Sfps in the other
533 species clearly have reproductive functions. It is likely that reproductive genes which
534 experience strong divergent selection may no longer resemble *D. melanogaster* genes. Our
535 work offers a first insight into the proteomic composition of male ejaculate characteristics
536 across species. As understanding of the molecular interactions between the sexes matures, it
537 will be important to determine whether shared or divergent functions between species are more
538 likely to contribute to PMPZ isolation and when during speciation such divergence occurs. Are
539 shared functions more likely to diverge within populations early during speciation or are Sfps
540 that already show some species specificity more likely to contribute to early PMPZ isolation?
541

542 Here we have tested whether reproductive proteins show differential abundance between
543 populations that exhibit PMPZ isolation. Our study has focussed on *Drosophila*, a model
544 system for studying the evolution of reproductive processes and the evolution of reproductive
545 isolation in metazoans. However, reproductive processes, classes of reproductive proteins,
546 and the action of PMPZ isolation across animals show similarities. For example, differentially
547 abundant proteins between *D. montana* populations we found included a number of proteases
548 or peptidases which are common and important mediators of reproductive processes in all
549 animals. Differentially abundant proteins also included several orthologs of *D. melanogaster*
550 Sfps with functions that may be similar to altered reproductive processes generating PMPZ
551 isolation in *D. montana*, such as noncompetitive gametic isolation and conspecific sperm
552 precedence (Jennings et al., 2014; Garlovsky et al. in review). These reproductive isolating
553 mechanisms are found in many other metazoan taxa (for brief review, see Turissini et al. 2018).
554 We also showed secretome proteins are evolving faster than other proteins found in the
555 accessory glands or ejaculatory duct and bulb, and at the same rate as Sfps. Such rapid
556 evolution is frequently attributed to sexual selection and sexual conflict, and these dynamic
557 processes may contribute to speciation (Gavrilets 2000; Panhuis et al. 2001). Male
558 reproductive secretory tissues had divergent functions with the ejaculatory bulb contributing
559 more differentially abundant proteins than the accessory glands, and the direction and severity
560 of asymmetrical PMPZ isolation mirrors differential abundance. We also identified shared and
561 species-specific GO enrichment of male reproductive proteins that influence reproductive
562 processes, although whether PMPZ isolation more likely arises due to divergence in one or the
563 other of these categories requires additional data. Democratisation of high throughput
564 proteomics will facilitate understanding the evolution of male reproductive proteins, their
565 influence on reproductive processes per se, and their contribution to reproductive isolation.
566

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AUTHOR CONTRIBUTIONS

RRS, TLK and CE conceived the study. RRS and MDG received funds for the work. MDG and CE collected the data. MDG and TLK analysed the data. MAR provided additional analysis tools. MDG and RRS wrote the manuscript with contributions from all authors. All authors agreed on the final version of the manuscript.

DATA ACCESSIBILTY

Supplementary material, data and R code used to perform analyses can be found at: https://github.com/MartinGarlovsky/Dmon_ejaculate_proteomics. Additional data has been submitted to Dryad (https://doi.org/10.5061/dryad.pvmcvdnhw). The mass spectrometry

592 proteomics data have been deposited to the ProteomeXchange Consortium
593 (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno et
594 al. 2016) with the dataset identifier PXD019634.
595

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