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Seminal fluid protein divergence among populations exhibiting postmating prezygotic reproductive isolation

Martin D. Garlovsky¹ | Caroline Evans² | Mathew A. Rosenow³ |
Timothy L. Karr⁴ | Rhonda R. Snook⁵

¹Department of Animal and Plant Sciences, The University of Sheffield, Sheffield, UK

²Department of Chemical and Biological Engineering, The University of Sheffield, Sheffield, UK

³Caris Life Sciences, Phoenix, AZ, USA

⁴Centre for Mechanisms of Evolution, The Biodesign Institute, Arizona State University, Tempe, AZ, USA

⁵Department of Zoology, Stockholm University, Stockholm, Sweden

Correspondence

Martin D. Garlovsky, Department of Animal and Plant Sciences, The University of Sheffield, Sheffield, UK.
Email: martingarlovsky@gmail.com

Present address

Martin D. Garlovsky, Department of Biology, Syracuse University, Syracuse, NY, USA

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Abstract

Despite holding a central role in fertilization, 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 the female during and after mating resulting in postmating prezygotic (PMPZ) isolation between populations. Crosses between *Drosophila montana* populations show PMPZ isolation in the form of reduced fertilization success in both non-competitive and competitive contexts. Here we test whether male ejaculate proteins produced in the accessory glands or ejaculatory bulb differ between populations using liquid chromatography tandem mass spectrometry. We find more than 150 differentially abundant proteins between populations that may contribute to PMPZ isolation, including a number of proteases, peptidases and several orthologues of *Drosophila melanogaster* Sfps known to mediate fertilization success. Males from the population that elicit the stronger PMPZ isolation after mating with foreign females typically produced greater quantities of Sfps. The accessory glands and ejaculatory bulb show enrichment for different gene ontology (GO) terms and the ejaculatory bulb contributes more differentially abundant proteins. Proteins with a predicted secretory signal evolve faster than nonsecretory proteins. Finally, we take advantage of quantitative proteomics data for three *Drosophila* species to determine shared and unique GO enrichments of Sfps between taxa and which potentially mediate PMPZ isolation. Our study provides the first high-throughput quantitative proteomic evidence showing divergence of reproductive proteins between populations that exhibit PMPZ isolation.

KEYWORDS

accessory glands, *Drosophila*, ejaculate, postcopulatory sexual selection, postmating prezygotic isolation, proteomics, reproductive isolation, seminal fluid proteins, speciation, tandem mass-spectrometry

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1 | INTRODUCTION

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

PMPZ isolation can result from incompatibilities between the male ejaculate and the female reproductive tract, proteins on the gamete cell surfaces, and/or differences between populations in sperm–female reproductive tract morphology (Howard et al., 2009). The male ejaculate contains a complex mixture including microbes, glycoproteins, sugars, lipids and seminal fluid proteins (Sfps) along with sperm which can impact fertilization success (Avila et al., 2011; Perry et al., 2013; Rowe et al., 2020; South & Lewis, 2011). Of the various molecules found in the ejaculate, Sfps have received the most research attention. Most Sfps are products of male secretory glands (e.g., in mammals, the prostate gland and seminal vesicles; in arthropods, accessory glands and ejaculate ducts/bulb; for a review, see Sirot et al., 2015; Figure S1). Different secretory organs contribute distinct sets of proteins to the ejaculate allowing increased complexity and modulation or tailoring of the ejaculate (Bayram et al., 2019). The majority of work on insect Sfp evolution has been done on *Drosophila melanogaster* with over 200 Sfps identified (Findlay et al., 2008, 2009; Mueller et al., 2005). However, many of the biochemical classes of Sfps are similar across animals; for example, proteases and protease inhibitors, and those with antimicrobial/immune-related functions (Avila et al., 2011; Sirot et al., 2015).

Despite conserved protein classes observed in the seminal fluid of all animals, a large fraction of Sfps show rapid molecular evolution and, therefore, even Sfps of the same classes in different species are not orthologous (Avila et al., 2011; Perry et al., 2013; Sirot et al., 2015). Functional confirmation of Sfps, performed mostly in *D. melanogaster*, indicate they aid in sperm transfer and storage, influence the outcome of sperm competition, and/or alter female physiology, behaviour and reproductive tract morphology after mating (Avila et al., 2011; Avila & Wolfner, 2009; Fedorka et al., 2011; Holman, 2009; Mattei et al., 2015; Ravi Ram & Wolfner, 2007; Wigby et al., 2009; Wolfner, 2009; Wong et al., 2008). When genetically manipulated, some Sfps elicit PMPZ-like phenotypes such as sperm storage abnormalities or reduced oviposition rates (LaFlamme et al., 2012; Ravi Ram & Wolfner, 2007). Moreover, differences in Sfp expression between species, and abnormal gene expression profiles or changes in protein abundance in the female reproductive tract after mating with heterospecific vs. conspecific males are associated with PMPZ isolation (Ahmed-Braimah et al., 2017; Bono et al., 2011; McCullough et al., 2020). Recently, ectopic injection of Sfps between populations of simultaneously hermaphroditic freshwater snails, *Lymnaea stagnalis*, that showed divergent gene expression patterns was shown to alter fecundity and sperm transfer (Nakadera et al., 2020). These shared patterns of divergence between taxa supports a putative role of Sfps as causative agents of PMPZ isolation.

However, while studies showing divergence in gene expression are associated with disrupted ejaculate × female reproductive tract interactions, changes in gene expression may not 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 × female reproductive tract interactions, leading to PMPZ isolation (Goenaga et al., 2015; Nakadera et al., 2020). High-throughput proteomics using liquid chromatography tandem mass spectrometry (LC-MS/MS) has revolutionized identification and quantification of Sfps, revealing that the male ejaculate often contains hundreds of unique proteins (Bayram et al., 2019; Karr, 2019; McDonough et al., 2016; 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” (Ahmed-Braimah et al., 2017; Avila et al., 2011; Bayram et al., 2019; Karr et al., 2019; Sepil et al., 2019). Molecular evolution analyses have shown that proteins with a secretory signal evolve faster than other proteins found in the accessory glands (Ahmed-Braimah et al., 2017; Bono et al., 2015; Karr et al., 2019; Mueller et al., 2005; Ramm et al., 2009; Tsuda et al., 2015; Wagstaff & Begun, 2005).

These results suggest Sfps are prime candidates for generating PMPZ isolation. However, no study using high-throughput quantitative proteomics has tested the prediction that ejaculate composition will vary between populations exhibiting PMPZ isolation, and that Sfps will evolve more rapidly than other ejaculate proteins (Wagstaff & Begun, 2005). 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. For instance, while many Sfps are secreted by the accessory glands, proteins secreted by the ejaculatory duct and bulb form the *Drosophila* mating plug thought to help retain sperm in storage (Ahmed-Braimah et al., 2017; Lung & Wolfner, 2001). Here we use LC-MS/MS to quantify the proteomes of the accessory glands and the ejaculatory bulb/duct, followed by analysis of molecular evolutionary rates, to test these predictions using the malt fly, *Drosophila montana*. We have focused on two populations (Crested Butte, Colorado, USA; Vancouver, Canada) which show incomplete premating and PMPZ isolation and no evidence of postzygotic isolation (Garlovsky & Snook, 2018; Jennings et al., 2014). Premating isolation is asymmetrical, with Vancouver females accepting mating attempts from Vancouver males around twice as frequently as they will Colorado males, whereas Colorado females will mate with Vancouver males as frequently as with Colorado males (Jennings et al., 2014). PMPZ isolation is also asymmetrical; after a single mating only 50% of eggs oviposited by Vancouver females mated with Colorado males hatch, whereas in the reciprocal cross no more than 30% of eggs hatch after Colorado females mate with Vancouver males (Garlovsky & Snook, 2018; Jennings et al., 2014). Hatching failure results from sperm failure to penetrate the egg, despite successful transfer to, and storage of, sperm in the female reproductive tract. PMPZ isolation is also found where within- and between-population males compete for fertilization (i.e., conspecific sperm precedence), as Colorado male ejaculates outcompete Vancouver male ejaculates in Colorado female reproductive tracts (Garlovsky et al., 2020.d.). Reduced fertilization success and conspecific sperm precedence are both stronger barriers to gene flow in Colorado female reproductive tracts, which suggests a shared mechanism underlying both forms of PMPZ isolation (Garlovsky et al., 2020.d.). Genomic analysis found no fixed single nucleotide polymorphisms (SNPs) between the Colorado and Vancouver populations, probably due to a history of gene flow during divergence (Parker et al., 2018), although genes enriched for biological processes relating to reproductive structure development showed divergence (Parker et al., 2018). Together, these results support focusing on Sfps as potential causative agents of PMPZ isolation. We also use 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 functional types that may contribute to PMPZ isolation.

2 | METHODS

2.1 | 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) (Figure S1). Stocks were established by combining 20 F₃ males and females from 20 isofemale lines (800 flies in total per population) and cultured on Lakovaara malt media (Lakovaara, 1969) in overlapping generations under constant light at 19°C. Flies were collected within 3 days of eclosion and housed in groups of between 10 and 20 single sex individuals in food vials until reproductively mature at 21 days old.

2.2 | Tissue collection and protein extraction

Twenty-one-day-old males were anaesthetized with ether and the accessory glands and ejaculatory duct/bulb separated from non-target tissues, and from each other. We collected three biological replicates, two of which were separated into technical replicates (Figure S1). Following protein extraction and purification, we quantified protein concentration to load 5 µg of protein for each sample into the mass spectrometer (see Figure S2). Samples were reduced with TCEP (tris(2-carboxyethyl)phosphine), alkylated by addition of MMTS (methyl methanethiosulfonate) and digested with trypsin, followed by drying to completion using vacuum centrifugation. Samples were resuspended in 20 µl 3% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid before LC-MS/MS analysis.

2.3 | LC-MS/MS analysis

Detailed description of LC-MS/MS data acquisition and processing can be found in the Supporting Information.

LC-MS/MS was performed by using a nanoflow liquid chromatograph (U3000 RSLCnano, Thermo Fisher) coupled to a hybrid quadrupole-orbitrap mass spectrometer (QExactive HF, Thermo Scientific). Peptides were separated on an Easy-Spray C₁₈ column (75 µm × 50 cm) using a two-step gradient from 97% solvent A (0.1% formic acid in water) to 10% solvent B (0.08% formic acid in 80% acetonitrile) over 5 min then 10% to 50% solvent B over 75 min at 300 nl/min. The full 105-min MS data-dependent acquisition was set up from 375 to 1,500 m/z acquired in the Orbitrap in profile mode at a resolution of 120,000. Subsequent fragmentation was Top 10 in the HCD (Higher-energy collisional dissociation) cell, with detection of ions in the Orbitrap using centroid mode at a resolution of 30,000. MS parameters were as follows: MS1: Automatic Gain Control (AGC) target 1e6 with a maximum injection time (IT) of 60 ms; MS2: AGC target 1e5, IT of 60 ms and isolation window 2 Da. Raw mass spectra have been submitted to the ProteomeXchange via PRIDE with the dataset identifier PXD019634 (Garlovsky et al. 2020a).

We performed label-free quantitative proteomic analysis using MAXQUANT to generate relative peptide and protein intensities (Cox et al., 2014; Tyanova et al., 2016) (see Supporting Information). For protein identification we matched mass spectra to the *D. montana* predicted proteome (Parker et al., 2018), generated using gene predictions from the Maker2 pipeline (Holt & Yandell, 2011) reciprocally blasted against *Drosophila virilis* proteins (Parker et al., 2018).

Data processed in MAXQUANT have been deposited within Dryad (<https://doi.org/10.5061/dryad.pvmcvdnhw>) (Garlovsky et al. 2020b).

2.4 | Gene Ontology (GO) enrichment analysis

We performed network analyses and GO enrichment for Biological Processes (BP), Cellular Components (CC) and Molecular Functions (MF) with the CLUEGO plugin (Bindea et al., 2009) for CYTOSCAPE (Shannon et al., 2003). We used FlyBase gene numbers (FBgns) for *D. virilis* orthologues of *D. montana* genes retrieved from Parker et al. (2018) or *D. melanogaster* orthologues converted via FlyBase.org. Specific settings for network groups are provided in the figure and table legends. For GO enrichment we used right-sided hypergeometric tests with Benjamini–Hochberg multiple test correction.

2.5 | Differential abundance analysis between *D. montana* populations and between tissues

We performed differential abundance analysis of MAXLFQ ion intensities using the “edgeR” (Robinson et al., 2010) and “limma” (Ritchie et al., 2015) packages in R (version 3.5.1) (R Core Team, 2018) (see Supporting Information). We performed TMM (trimmed mean of M-values) normalization using “calcNormFactors” and identified differentially abundant proteins using “voom,” “lmFit,” “eBayes” and “duplicateCorrelation” to account for replicate structure (see Supporting Information). Proteins were considered differentially abundant based on a Benjamini–Hochberg false discovery rate adjusted p -value < 0.05 . To identify differentially abundant proteins between populations, we analysed the accessory gland proteome and the ejaculatory bulb proteome separately. We only considered proteins that were present in all five replicates of each tissue for both populations. To identify differentially abundant proteins between tissues, we analysed each population separately. Again, we only considered proteins that were present in all five replicates of each population for both tissues (Table S1).

2.6 | Characterizing the male seminal fluid proteome across species

We compared differences in GO enrichment of Sfps for three *Drosophila* species for which proteomic data (generated using LC-MS/MS) are available for the male accessory gland and ejaculatory duct and bulb tissues: *D. montana* (this study), *D. melanogaster* (Sepil et al., 2019) and *D. pseudoobscura* (Karr et al., 2019). We retrieved FBgns for *D. melanogaster* genes identified by Sepil et al. (2019) and *D. melanogaster* orthologues for *D. pseudoobscura* genes identified by Karr et al. (2019) and downloaded the corresponding canonical protein sequences from uniprot.org. For proteins we identified in our analysis we retrieved *D. montana* protein sequences from Parker

et al. (2018). We submitted protein sequences for each species to SignalP (Petersen et al., 2011) and Phobius (Käll et al., 2004) and combined the resulting lists of proteins containing a signal peptide to generate a list of secretome proteins for each species. For *D. montana* we converted the corresponding *D. virilis* FBgns for each protein to *D. melanogaster* orthologues via FlyBase.org (for 215/245, 88%). To identify Sfps for each species we submitted secretome lists to FlyBase.org to retrieve genes with GO terms containing “extracellular” (Figure S3; Table S2). To compare GO enrichment between species we adjusted network settings in ClueGO to reflect the different numbers of proteins identified in each species.

2.7 | Evolutionary rates analysis

To obtain sequence divergence estimates for *D. montana* proteins we used a pipeline developed previously (Wright et al., 2015). We obtained protein coding sequences for *D. montana* from Parker et al. (2018) and for *D. pseudoobscura* (r3.04, September 2019) and *D. virilis* (r1.07, August 2019) from FlyBase.org. We identified the longest isoform of each gene for each species and determined orthology with reciprocal BLASTN (Altschul et al., 1990), using a minimum percentage identity of 30% and an E -value cut-off of 1×10^{-10} . We then identified reciprocal one-to-one orthologues across all three species using the highest BLAST score. We identified open reading frames using BLASTX and aligned orthologues using PRANK (Löytynoja & Goldman, 2010). We calculated the ratio of nonsynonymous (dN) to synonymous (dS) nucleotide substitutions, omega (ω), using the CODEML package in PAML (Yang, 2007) (one-ratio estimates, model 0) with an unrooted phylogeny. Results were filtered to exclude orthologues with branch-specific $dS \geq 2$ (due to potential mutational saturation) or where $S \cdot dS \leq 1$.

We then tested for differences in evolutionary rates between sets of proteins we identified in our LC-MS/MS analysis. We relaxed filtering criteria so that a protein need only be identified in a single replicate in a single population or tissue, but still had to be identified by two or more unique peptides. After filtering, we obtained ω values for 757/1,474 (51%) proteins with a reciprocal one-to-one orthologue. We classified genes as belonging to the secretome based on presence of a signal peptide plus orthologues of *D. melanogaster* Sfps. We categorized genes as belonging to the accessory gland proteome or ejaculatory bulb proteome based on whether a protein showed concordant differential abundance between tissues across populations (see Section 3.3). We classified the remainder of proteins that showed equal abundance between tissues (i.e., excluding those with higher abundance in the accessory gland proteome, ejaculatory bulb proteome or putative Sfps) as background proteins. Each class consisted of an exclusive set of proteins, such that the secretome did not include accessory gland proteins, ejaculatory bulb proteins or background proteins; accessory gland proteins did not include ejaculatory bulb proteins or background proteins, etc. We tested for differences in evolutionary rates between groups using a Kruskal–Wallis rank sum test followed by pairwise Wilcoxon rank sum

tests corrected for multiple testing using the Benjamini–Hochberg method.

3 | RESULTS

3.1 | The *D. montana* accessory gland proteome and ejaculatory bulb proteome

We identified 1,711 proteins, of which 1,474 (86%) were identified by two or more unique peptides. The majority of proteins (1,013/1,474; 69%) were shared across male secretory tissues, while 138 (9%) and 323 (22%) proteins were unique to the accessory glands and ejaculatory bulb, respectively (Figure S4a). Proteins identified only in the accessory gland proteome showed a 3.2-fold lower mean abundance compared to the remaining proteins whereas proteins identified only in the ejaculatory bulb proteome showed a 14.9-fold reduction. These proteins probably represent missed rather than truly unique proteins and are not considered further. We identified 79 *Drosophila montana* Sfps, consisting of 38 orthologues of *Drosophila melanogaster* Sfps identified by converting *Drosophila virilis* FBgns on FlyBase.org, plus 55 secretome proteins with extracellular annotations identified by two or more unique peptides (14 of which overlapped) (Figure S4a; Table S3) (Findlay et al., 2008, 2009; Mueller et al., 2005). A multidimensional scaling (MDS) plot of normalized intensities using all proteins ($n = 1,474$) showed a clear separation of samples by tissue type (dimension 1), and separation by population (dimension 2) with clear separation of populations for the accessory gland proteome and marginal overlap between populations in the ejaculatory bulb proteome (Figure 1).

3.2 | Differential abundance of reproductive proteins between populations

The majority of proteins were identified in both populations (1,322/1,474; 90%), while 45 (3%) and 107 (7%) were only identified in Colorado and in Vancouver, respectively (Figure S4b). Proteins only identified in one population showed a 263- and 171-fold lower mean abundance compared to the rest of the proteins in Colorado, and Vancouver, respectively. As above, these low-abundance proteins are not considered further. For shared proteins, we then tested for differential abundance. We identified 154 (out of 725) differentially abundant proteins produced in the accessory glands between populations (Figure 2a), including nine orthologues of *D. melanogaster* Sfps (Table 1). We identified 244 (out of 929) differentially abundant proteins produced in the ejaculatory bulbs (Figure 2b). Again, these included nine orthologues of *D. melanogaster* Sfps, two of which overlapped with those identified in the accessory gland proteome (Table 1). In the accessory gland proteome, Sfps and proteins with a predicted secretory signal were not overrepresented in the cohort of proteins showing differential abundance (Chi-squared test, $\chi^2 = 1.57$, $df = 2$, $p = .456$; Figure 3a) but these were overrepresented

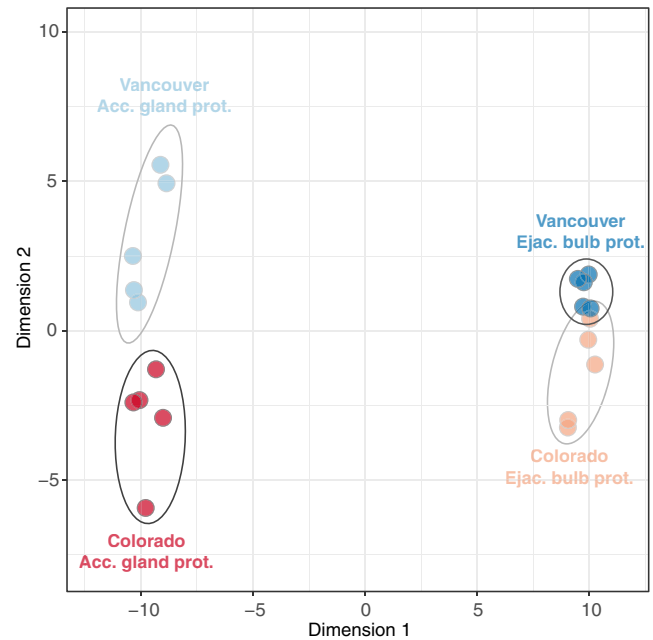


FIGURE 1 Multidimensional scaling (MDS) plot of normalized intensities for proteins identified by two or more unique peptides ($n = 1,474$) in each replicate (points). Dimension 1 separates the two tissue types (accessory glands and ejaculatory bulbs). Dimension 2 separates the two populations (Colorado and Vancouver)

in the cohort of differentially abundant proteins in the ejaculatory bulb proteome ($\chi^2 = 44.56$, $df = 2$, $p < .001$; Figure 3b). Two genes showing differential abundance between populations in either the accessory gland proteome or the ejaculatory bulb proteome overlapped with genes showing divergence between populations identified by Parker et al. (2018): FBgn0208933 and FBgn0203373.

Of 45 proteins that were significantly differentially abundant between populations in both male reproductive tissues, 36 showed a concordant pattern; that is, showed higher abundance in one population or the other in both tissues (Figure S5). The nine genes that showed a discordant pattern of differential abundance between populations included two orthologues of *D. melanogaster* Sfps (Cystatin-like and Calreticulin), two other putative Sfps we identified in *D. montana* with orthologues in *D. virilis* (FBgn0209753 and FBgn0198572), and five proteins without orthologues. Significantly enriched GO categories for proteins showing differential abundance between populations are given in Tables S4–S6.

3.3 | The accessory gland and ejaculatory bulb proteomes differ in protein functional types

To test whether the accessory glands and ejaculatory bulb provide different protein functional types we performed differential abundance analysis between tissues for Colorado and Vancouver separately. We found 524 (out of 652) differentially abundant proteins between tissues in Colorado. Similarly, in Vancouver we found 557 (out of 676) differentially abundant proteins. The majority of these

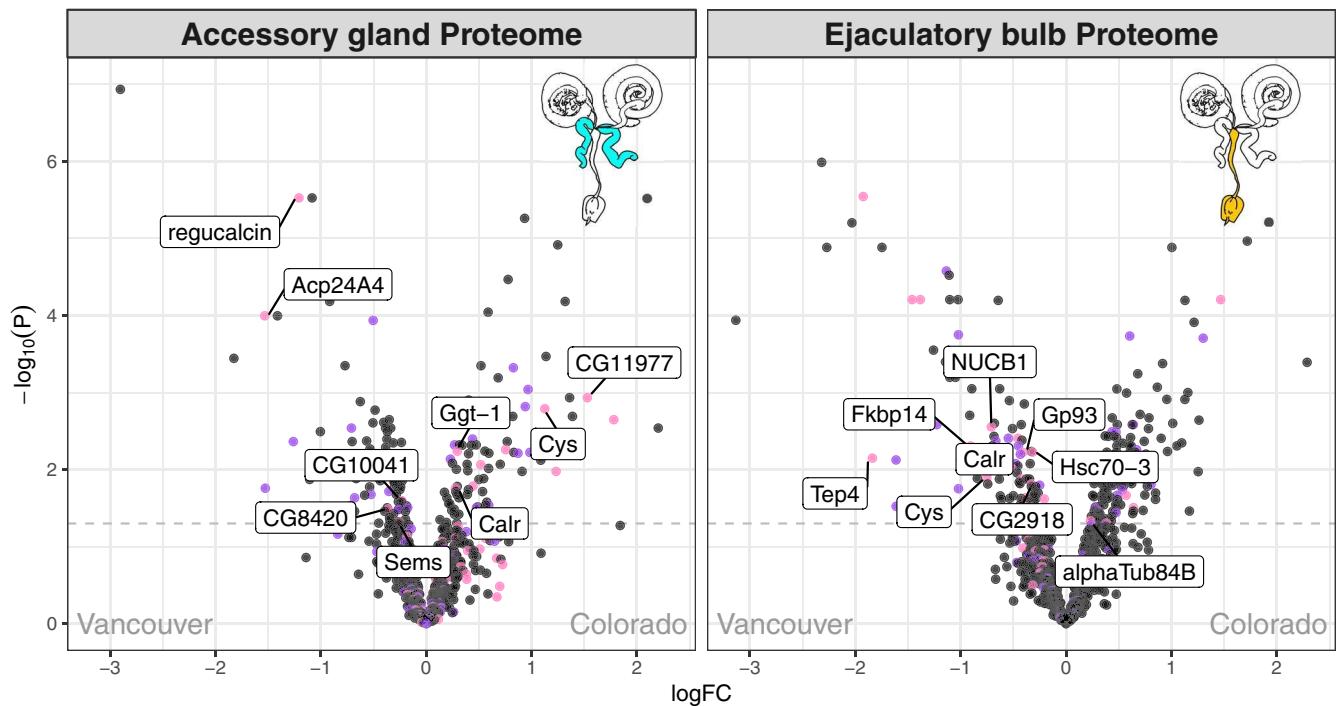


FIGURE 2 Differentially abundant proteins between Colorado and Vancouver in (a) the accessory glands ($n = 725$) and (b) the ejaculatory bulbs ($n = 929$). Secretome proteins are shown in purple and seminal fluid proteins (Sfps) in pink. Significantly differentially abundant proteins with a known Sfp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

proteins were found in both populations (609 proteins). To identify consistently differentially abundant proteins between tissues, we compared the \log_2 -fold change in abundance in each population of these 609 proteins. Proteins with higher abundance in the accessory gland proteome or ejaculatory bulb proteome in Colorado generally also showed higher abundance in Vancouver (Spearman's rank correlation, $\rho = .945$, $p < .001$, $n = 609$) (Figure 4a). Five proteins showed a discordant pattern of differential abundance between tissues, two with orthologues of *D. melanogaster* Sfps; Cystatin-like and Imaginal Disc Growth Factor 3. GO analyses identified both tissues as having enrichment for GO terms expected for highly metabolically active secretory organs (Table S7). Different GO terms were enriched in each tissue, highlighting that the two secretory organs provide distinct roles in reproduction and the ejaculate (Figure 4b; Table S7).

3.4 | Evolutionary rates analysis

We tested whether genes with higher protein abundance in either the accessory gland proteome, the ejaculatory bulb proteome, the secretome (proteins with a secretory signal [i.e., putative Sfps]), or background proteins (i.e., those proteins that do not differ in protein abundance between the accessory glands and ejaculatory bulb and excluding the secretome) were evolving at different rates. There was a significant difference between protein groups in evolutionary rates (Kruskal-Wallis test, $\chi^2 = 40.3$, $df = 3$, $p < .001$) (Figure 5; Figure S6). The secretome (including putative Sfps) was evolving faster than proteins with higher abundance in the accessory gland proteome,

ejaculatory bulb proteome or background (pairwise Wilcoxon rank sum test with Benjamini-Hochberg adjustment, all $p < .003$). Proteins with higher abundance in the accessory gland proteome and ejaculatory bulb proteome were evolving at similar rates ($p = .072$), and slower than the remaining background proteome (accessory gland proteome versus background, $p < .001$; ejaculatory bulb proteome versus background, $p = .010$).

3.5 | Comparison of male Sfps across species

We identified 61 Sfps (secretome proteins with extracellular annotations) for *D. montana*, 249 Sfps for *D. melanogaster* and 131 Sfps for *D. pseudoobscura* (Figure S3; Table S2). Comparing GO enrichment of Sfps across species identified a number of shared and unique GO categories. Shared Biological Processes included chitin catabolic process, innate immune response, cell-substrate adhesion and regulation of peptidase activity (Figure 6; see Table S8 for CC and MF terms). Uniquely enriched BP functions included regulation of secondary metabolic process (*D. montana*); postmating regulation of female receptivity (*D. melanogaster*) and aminoglycan catabolic processes (*D. pseudoobscura*) (Figure 6; see Table S8 for CC and MF terms).

4 | DISCUSSION

The molecular basis of mechanisms underlying PMPZ isolation are poorly understood. Seminal fluid proteins are likely to contribute to

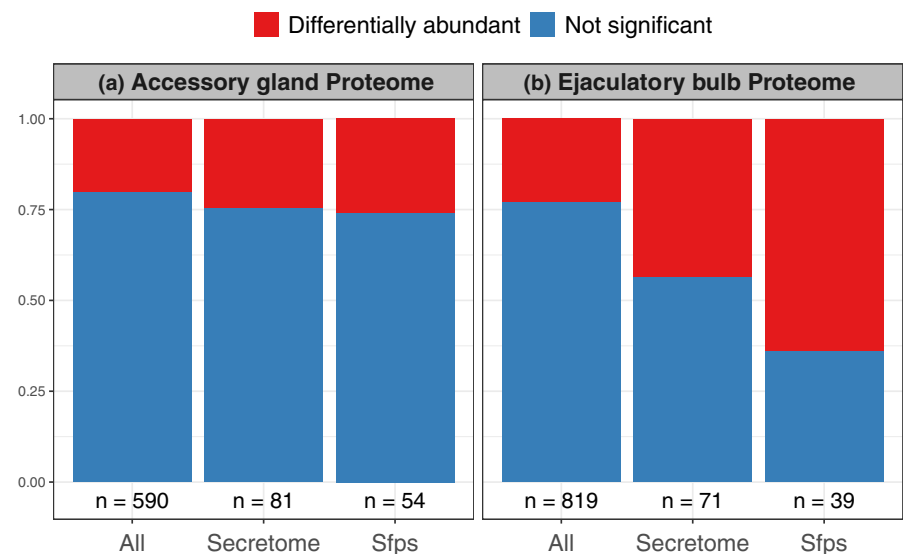
TABLE 1 Differentially abundant proteins between *Drosophila montana* populations with a known seminal fluid protein (Sfp) orthologue in *D. melanogaster*

Tissue comparison	Gene name	Higher abundance
Accessory glands	CG11977	Colorado
	γ -Glutamyl transpeptidase	Colorado
	Acp24A4	Vancouver
	CG10041	Vancouver
	CG8420	Vancouver
	Regucalcin	Vancouver
	Seminase	Vancouver
Ejaculatory bulbs	α -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}

Note: Gene names were retrieved from FlyBase.org using the corresponding *D. virilis* FBgns for *D. montana* proteins we identified via LC-MS/MS. The population for which each protein showed higher abundance is given. Proteins found in both tissue comparisons indicate in which population there was higher abundance.

Abbreviations: Acgs, accessory glands; Ebs, ejaculatory bulbs.

FIGURE 3 Representation of secretome and seminal fluid proteins (Sfps) in the set of differentially abundant proteins between populations. Secretome proteins and Sfps were equally represented in the set of differentially abundant proteins between Colorado and Vancouver in the accessory gland proteome (a) but were overrepresented in the differentially abundant proteins in the ejaculatory bulb proteome (b). *n* = numbers of proteins in each group



PMPZ isolation due to their effects on sperm use, fertilization success and rapid divergent evolution. Previously, divergent gene expression profiles of Sfps and female reproductive tracts have been found between species that show PMPZ isolation. We used quantitative proteomics to identify proteins produced in the accessory glands and ejaculatory duct and bulb in populations exhibiting PMPZ isolation and found a number of differentially abundant proteins between populations including several orthologues of *D. melanogaster* Sfps. The accessory glands and ejaculatory bulb showed enrichment

of different GO terms and there were more differentially abundant proteins found in the ejaculatory bulb than in the accessory glands. For proteins found in both populations, but in separate tissues, there was strong concordance in abundance between populations. We found that secretome proteins, including putative Sfps, evolved at a faster rate than nonsecretome proteins, both those differentially abundant between male secretory organs and those showing similar abundance between male tissues. Despite shared Sfps, and a core set of shared Sfp biological processes across three *Drosophila*

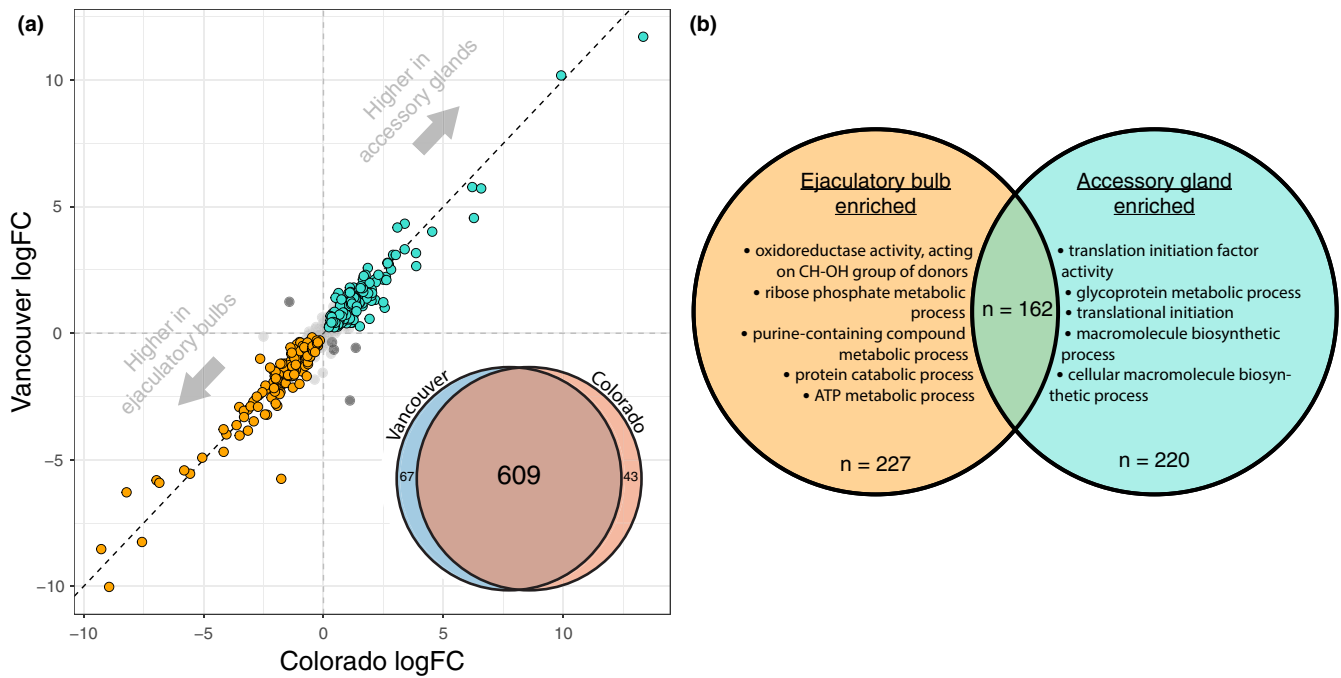


FIGURE 4 Populations show consistent differential abundance between secretory organs. (a) Concordance between populations in \log_2 -fold change (FC) in abundance of proteins found in either the accessory gland proteome or the ejaculatory bulb proteome. Positive values indicate proteins with higher abundance in the accessory glands in both populations (top right), and negative values indicate proteins with higher abundance in the ejaculatory bulb (bottom left). Proteins are coloured based on whether they showed a concordant pattern of significantly higher abundance in the accessory gland proteome (turquoise, $n = 220$), the ejaculatory bulb proteome (orange, $n = 227$), were discordant (black, $n = 5$) or were not significantly different between tissues (grey, $n = 157$). Dashed black line shows 1:1. Inset: Venn diagram showing numbers of proteins included in separate differential abundance analysis between tissues in each population and overlap. (b) Top five significantly enriched GO Biological Process terms ranked by percentage identity of proteins to each tissue (see Table S7 for full list)

species, there was species-specific enrichment of Sfp protein functional types.

Drosophila montana from Colorado and Vancouver show low genome-wide divergence and a history of gene flow (Garlovsky et al., 2020.d.; Parker et al., 2018), yet show enrichment of reproductive genes that are divergent between populations (Parker et al., 2018). Crosses between Colorado and Vancouver show reduced fertilization success after a single mating (Garlovsky & Snook, 2018; Jennings et al., 2014) and exhibit conpopulation sperm precedence (Garlovsky et al., 2020.d.). Mechanisms causing PMPZ isolation include sperm-egg incompatibilities, abnormal sperm transfer and displacement, or mismatches between sperm length and female reproductive tract morphology (Howard et al., 2009; Manier et al., 2013; Price et al., 2001). Females receiving a foreign ejaculate comprising an abnormal Sfp complement might also result in mismatched ejaculate \times female reproductive tract interactions (Bono et al., 2011; Plakke et al., 2015).

We identified a number of differentially abundant proteins between populations exhibiting PMPZ isolation, including several orthologues of *D. melanogaster* Sfps. Intriguingly, 11 of 14 of these proteins were more abundant in Vancouver males than in Colorado males. PMPZ isolation between *D. montana* populations is asymmetric, with matings between Vancouver males and Colorado females having lower fertilization success compared to the reciprocal cross

(Garlovsky & Snook, 2018; Jennings et al., 2014). If Vancouver males transfer more of these Sfps to their mates, then the chemical environment in the reproductive tract of Colorado females may be mismatched, more so than for the reciprocal cross. While our results show divergence in Sfp abundance is a potential mechanism underlying the asymmetry and occurrence of PMPZ isolation between *D. montana* populations, further studies are required to discern any causal relationship. A recent study showed ectopic injection of divergent Sfps between freshwater snail populations was associated with differences in the effects of these Sfps on fecundity and sperm transfer (Nakadera et al., 2020). PMPZ isolation in *D. montana* may also result from differences between populations in sperm length and female sperm storage organ morphology, direct interactions between the sperm and egg cell surfaces, or a combination of these factors (Howard et al., 2009). We are currently exploring these other possibilities.

We identified a number of proteases and peptidases (or inhibitors) that were differentially abundant between populations. Proteases and peptidases are central to reproduction across taxa, regulating proteolytic activity and initiating cascades of interactions among downstream proteins (Bayram et al., 2017, 2019; LaFlamme et al., 2012; LaFlamme & Wolfner, 2013; Plakke et al., 2015, 2019). Divergence in proteases has been implicated in PMPZ isolation between other insect species in both the male ejaculate and the female

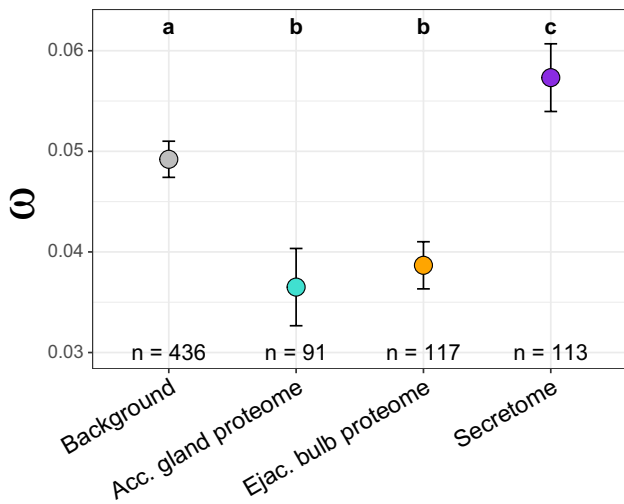


FIGURE 5 Mean nonsynonymous (dN) to synonymous (dS) base substitution rate (ω) estimates (\pm SE) for *Drosophila montana* genes identified in our LC-MS/MS analysis with equal abundance in the accessory gland proteome and ejaculatory bulb proteome ("background"; grey), higher abundance in the accessory gland proteome (turquoise), ejaculatory bulb proteome (orange), or found in the secretome (including putative seminal fluid proteins [Sfps]; purple). Different letters above points indicate groups that show significant differences from pairwise Wilcoxon rank sum tests corrected for multiple testing. See Figure S6 for plots showing dN and dS separately.

reproductive tract secretions (Al-Wathiqui et al., 2018; Kelleher & Pennington, 2009; Kelleher et al., 2007; Marshall et al., 2009, 2011; McCullough et al., 2020; Meslin et al., 2017; Plakke et al., 2019).

In *D. montana*, females receive and store motile sperm from incompatible males, but fertilization success is reduced (Jennings et al., 2014). Failure to either properly orient sperm in storage (Manier et al., 2013), release sperm from storage or have sperm release coincide with ovulation (Mattei et al., 2015) could explain PMPZ isolation in this system (Jennings et al., 2014). Some notable differentially abundant Sfps and proteases we identified, and their potential relationship to PMPZ isolation in *D. montana* are seminare, γ -glutamyl transpeptidase and regucalcin. Seminare (CG10586) is a serine protease and a member of the Sex Peptide (SP) network (Singh et al., 2018). Seminare acts early in the SP network and is required to process other Sfps in the mated female essential for proper sperm storage (Acp36DE) and ovulation (ovulin) (LaFlamme et al., 2012; Singh et al., 2018). RNA interference knockdown of seminare in male *D. melanogaster* results in failure of mated females to release sperm from the seminal receptacle (LaFlamme et al., 2012). γ -Glutamyl transpeptidase (CG6461) functions to maintain a protective redox environment for sperm (Walker et al., 2006). Mismatches between the male ejaculate and the redox environment of the female reproductive tract in which sperm are stored could reduce fertilization success as sperm subject to increased oxidative stress are less fertilization-competent (Reinhardt & Ribou, 2013). Regucalcin (CG1803), a Ca^{2+} binding protein, may also play an anti-oxidative

role and, in mammals, is hypothesized to have an anti-capacitation role for sperm (Pillai et al., 2017). One aspect of capacitation, hyperactivation, increases sperm motility which is important for sperm storage in *Drosophila* (Köttgen et al., 2011). Sperm motility behaviour and how this may affect release from storage is unknown. Regucalcin gene expression varies between *D. montana* populations and has been suggested as a cold tolerance gene in diapausing females (Vesala et al., 2012) although its expression in males has not been studied. These examples provide strong candidates for eliciting PMPZ isolation and will be subject to future studies, for instance using CRISPR/Cas9 gene editing, to further understand the molecular interactions causing PMPZ isolation in *D. montana*.

Reproductive proteins evolve rapidly (Ahmed-Braimah et al., 2017; Dapper & Wade, 2020; Firman et al., 2017; Meslin et al., 2017; Sirot et al., 2015). We also found that proteins showing secretory signals (i.e., the secretome and putative Sfps) evolve faster than proteins without this signal. When considered separately, Sfps and secretome proteins were evolving at a similar rate. However, despite having a higher mean rate, Sfps were not evolving faster than proteins with similar abundance between male tissues. This may be due to Sfps showing greater variation in evolutionary rates than other categories (data not shown). In addition, the requirement to have extracellular annotation determined from work in *D. melanogaster* limits our ability to identify rapidly evolving Sfps in *D. montana*. Thus, the 79 putative Sfps in *D. montana* we identified is surely a conservative estimate. Isotopically labelling males to identify proteins transferred to females increased the number of identified *D. melanogaster* Sfps (Findlay et al., 2008, 2009). Future work on *D. montana* can use this technique to identify additional Sfps.

One goal of the present study was to assess whether the different male reproductive secretory organs contribute differently to reproduction and the ejaculate, which would not be possible using the heavy labelling technique. Our work provides one of the first proteomic descriptions of both major Sfp secretory organs in *Drosophila* (Takemori & Yamamoto, 2009). In seed beetles, division of labour between secretory organs enables increased complexity and potential for ejaculate tailoring (Bayram et al., 2019). Most proteins we identified were found in both tissues but showed higher abundance in either the accessory glands or the ejaculatory duct and bulb, suggesting these organs provide different roles to reproduction. The accessory gland proteome was enriched for terms indicating a more direct contribution to the ejaculate, such as translation and biosynthetic processes. The ejaculatory bulb proteome showed enrichment for mainly metabolic processes which suggests this organ may instead provide a more "housekeeping" role. We found secretome proteins and Sfps were significantly overrepresented in the set of differentially abundant proteins in the ejaculatory bulb proteome but not the accessory gland proteome, suggesting the two male secretory organs may contribute differently to PMPZ isolation. Our analysis shows that studies of Sfp evolution focused solely on the accessory glands may have biased our understanding of not only molecules involved in reproduction but also those reproductive molecules that may elicit PMPZ isolation.

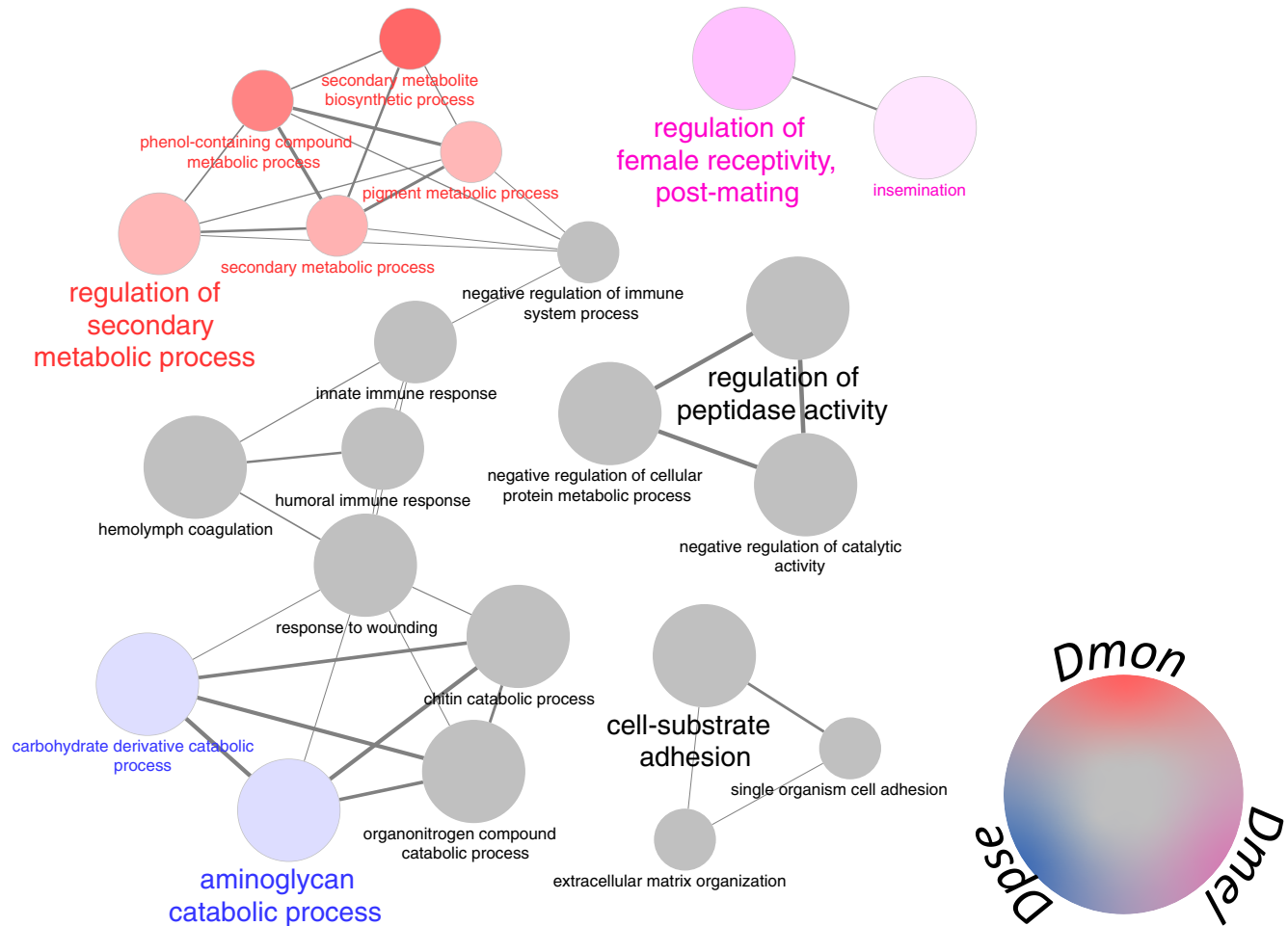


FIGURE 6 Seminal fluid protein comparisons for GO Biological Process terms across species. Circle size is associated with level of significance with increasing size indicating increasing significance. Node colour indicates proportion of genes from each species associated with a term: *Drosophila montana*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

We took advantage of recent accessory gland proteomes for three *Drosophila* species generated using high-throughput LC-MS/MS to characterize shared and enriched protein functional types of Sfps between species. Using the same identification criteria for all species (secretory signal sequence and extracellular annotation), we identified a set of shared GO categories between species that last shared a common ancestor 40 million years ago. This core set included immune-related genes, which are associated with sexual conflict in *D. melanogaster* (Innocenti & Morrow, 2009). We also found species-specific GO enrichment of Sfps, suggesting divergence in how they contribute to the male ejaculate between species. Differences may reflect how selection has targeted particular ejaculate traits in different mating systems (Markow, 2002). Differences will also reflect the use of *D. melanogaster* as the reference for GO annotation. For instance, *D. melanogaster* showed enrichment for reproductive genes but Sfps in the other species clearly have a role during reproduction. It is likely that reproductive genes that have undergone rapid evolutionary change may no longer resemble *D. melanogaster* genes. Our work offers a first insight into the proteomic composition

of male ejaculate characteristics across species. As understanding of the molecular interactions between the sexes matures, it will be important to determine whether shared or divergent protein classes between species are more likely to contribute to PMPZ isolation and when during speciation such divergence occurs. Are shared protein functional types more likely to diverge within populations early during speciation or are Sfps that already show some species specificity more likely to contribute to early PMPZ isolation?

Here we have tested whether reproductive proteins show differential abundance between populations that exhibit PMPZ isolation. Our study has focused on *Drosophila*, a model system for studying the evolution of reproductive processes and the evolution of reproductive isolation in metazoans. However, reproductive processes, classes of reproductive proteins and the action of PMPZ isolation across animals show similarities. For example, differentially abundant proteins between *D. montana* populations we found included a number of proteases or peptidases which are common and important mediators of reproductive processes in all animals. Differentially abundant proteins also included several orthologues

of *D. melanogaster* Sfps with functions that may be similar to altered reproductive processes generating PMPZ isolation in *D. montana*, such as noncompetitive gametic isolation and conspecific sperm precedence (Garlovsky et al., 2020.d.; Jennings et al., 2014). These reproductive isolating mechanisms are found in many other metazoan taxa (for a brief review, see Turissini et al., 2018). We also showed that the secretome and putative Sfps are evolving faster than other proteins found in the accessory glands or ejaculatory duct and bulb. Such rapid evolution is frequently attributed to sexual selection and sexual conflict, and these dynamic processes may contribute to speciation (Dapper & Wade, 2020; Gavrillets, 2000; Panhuis et al., 2001). Male reproductive secretory tissues showed enrichment of different protein types with the ejaculatory bulb contributing more differentially abundant proteins than the accessory glands, and the direction and severity of asymmetrical PMPZ isolation mirrors differential abundance. We also identified shared and species-specific GO enrichment of male reproductive proteins that influence reproductive processes, although whether PMPZ isolation more probably arises due to divergence in one or the other of these categories requires additional data. Democratization of high-throughput proteomics will facilitate understanding the evolution of male reproductive proteins, their influence on reproductive processes per se, and their contribution to reproductive isolation.

5 | DATA ACCESSIBILITY STATEMENT

Supplementary material, data and R code used to perform analyses can be found at: https://github.com/MartinGarlovsky/Dmon_ejaculate_proteomics. Additional data have been submitted to Dryad (<https://doi.org/10.5061/dryad.pvmcvdnhw>). The MS proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (Vizcaino et al., 2016) with the data set identifier PXD019634.

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AUTHOR CONTRIBUTIONS

R.R.S., T.L.K. and C.E. conceived the study. R.R.S. and M.D.G. received funds for the work. M.D.G. and C.E. collected the data. M.D.G. and T.L.K. analysed the data. M.A.R. provided additional analysis tools. M.D.G. and R.R.S. wrote the manuscript with contributions from all authors. All authors agreed on the final version of the manuscript.

ORCID

Martin D. Garlovsky  <https://orcid.org/0000-0002-3426-4341>

Caroline Evans  <https://orcid.org/0000-0003-4356-9216>

Mathew A. Rosenow  <https://orcid.org/0000-0001-5522-5715>

Timothy L. Karr  <https://orcid.org/0000-0002-4180-6583>

Rhonda R. Snook  <https://orcid.org/0000-0003-1852-1448>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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