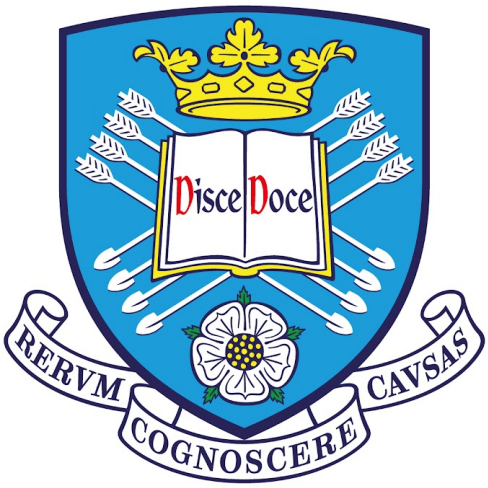

The evolution of postmating prezygotic isolation

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"Without deviation from the norm, progress is not possible."

Frank Zappa

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Statement of intellectual contribution

The work in this thesis owes much to the supervision of Rhonda R. Snook (RRS). Additionally, various aspects of some chapters would not have been possible without the help and support of various collaborators. Acknowledgements and contributions are presented at the beginning of each chapter, all written in the format of scientific publications.

Chapter 1: RRS conceived the idea, MDG and RRS developed the idea and conducted the literature survey. MDG wrote the manuscript with contribution from RRS.

Chapter 2: text is presented in its accepted form for publication in *Ecology and Evolution* (**8**:9062-9073). MDG collected the data with help from Joe Baxter (MBiolSci student). MDG and RRS designed the study and wrote the manuscript. MDG analysed the data.

Chapter 3: written and designed by MDG and RRS and analysed by MDG. Sperm precedence data was collected by MDG, relative reproductive investment and progeny data collected by Tobit Dehnen and Will Leaning, undergraduates at The University of Sheffield, as part of a TA:SURE scheme project awarded to MDG.

Chapter 4: conceived by RRS and data collected by MDG and Caroline Evans (Department of Chemical and Biological Engineering, The University of Sheffield). Data was analysed by MDG with the help of Tim Karr (TLK) and Matthew Rosenow. MDG wrote the manuscript with input from TLK and RRS.

Chapter 5: RRS conceived the study. RRS and Goran Arnqvist (GA) designed the experiments. Long term maintenance of the experimental evolution lines has been undertaken by numerous technicians and past members of the Snook lab. Data on development time, stress resistance, and metabolite composition was collected by MDG, RRS, and Andrew L. Brooks, respectively. Data on metabolic rates was collected by Zorana K. Novicic at Uppsala University, Sweden. MDG and GA analysed the data and MDG and RRS wrote the manuscript.

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Summary

Interactions between the sexes before and after mating can have far reaching implications, from the evolution of traits in populations, to shaping patterns of biodiversity. Here, I investigate the effects of sexual selection and sexual conflict on the evolution of reproductive traits and their consequences for the evolution of interactions within and between populations. The main focus of my thesis is the role of postmating prezygotic isolation during the evolution of nascent reproductive isolation using the malt fly, *Drosophila montana*. In chapter one I review the current literature investigating the mechanisms and evolution of postmating prezygotic isolation across metazoans. In chapter two, I test postmating prezygotic isolation between populations of *D. montana* across genotypes and between different populations within North America. In chapter three, I test whether patterns of reproductive isolation can be explained by episodes of sexual selection acting within populations, and whether different prezygotic isolation barriers cooccur or counter one another. In chapter four, I test whether divergence in male seminal fluid proteomes is responsible for the emergence of postmating prezygotic isolation between populations and provide the first description of the *D. montana* male seminal fluid proteome using high throughput shotgun proteomics. Finally, in chapter five I test how the strength of sexual selection and sexual conflict impacts physiological and life history traits using populations of *D. pseudoobscura* experimentally evolving under elevated polyandry or enforced monogamy. Overall, this thesis offers novel insights in to the evolution of early reproductive isolation by characterising the reproductive barriers and possible mechanisms underlying the evolution of postmating prezygotic isolation. Furthermore, I show how sexual selection can have far reaching consequences beyond reproductive traits, with implications for the evolution of physiological and life history traits.

Introduction

Understanding how new species arise and persist is integral for understanding the generation and maintenance of biological diversity. Yet our understanding of the origin of species remains incomplete (Darwin, 1859; Seehausen et al., 2014; The Marie Curie Speciation Network, 2012). The process of speciation comprises the splitting of lineages from a common ancestor, the evolution of reproductive isolation between them, and the persistence of those lineages through time (Mayr, 1942; Rabosky, 2016). Speciation proceeds across a continuum, ranging from fully interbreeding panmictic populations, to fully reproductively isolated species. Speciation can be considered to be complete when reproductive isolation is complete (Mayr, 1942). This strict definition of the biological species concept provides a practical and definable measure for studying speciation.

Studying pairs of taxa of different ages that fall along the "speciation continuum" allows us to investigate different stages of the speciation process to determine when different barriers to gene flow emerge, the relative role of different evolutionary processes, such as genetic drift and selection, in generating divergence between taxa, and the contribution of different barrier effects to total reproductive isolation during speciation (Coyne and Orr, 2004). To understand the origin of species, the reproductive isolation barriers that emerge early between young sister taxa need to be identified, together with the underlying traits and conditions which enable reproductive isolation to evolve and persist (The Marie Curie Speciation Network, 2012). Understanding how barriers to gene flow evolve that enable the build-up and maintenance of linkage disequilibrium between lineages is the crux of the speciation problem (Felsenstein, 1981).

Barriers to gene flow can act between parental genotypes to prevent the formation of hybrids (prezygotic isolation) or in the hybrids themselves (postzygotic isolation). Postzygotic isolation impedes gene flow when hybrids suffer reduced fitness. Intrinsic postzygotic isolation, where hybrids are sterile or inviable, results from genetic incompatibilities between parental genotypes. Bateson Dobzhansky Muller incompatibilities (BDMIs) provide a simple model for the evolution of genetic incompatibilities between two taxa descended from a common ancestor

(Seehausen et al., 2014). As different alleles fix between populations and/or new alleles arise within populations, hybridisation will bring together combinations of alleles on genetic backgrounds that have not experienced the same evolutionary history, potentially resulting in negative epistatic interactions (Ono et al., 2017). Extrinsic postzygotic isolation can impede gene flow where hybrids do not fit well in either parental habitat, despite being viable and fertile (Cooper et al., 2018). However, hybridisation can also result in sharing of beneficial alleles between populations (adaptive introgression; Oziolor et al., 2019), the ability of hybrids to explore new niches, or the instantaneous generation of new hybrid species (Soltis and Soltis, 2009). Postzygotic isolation may be necessary for the long-term persistence of taxa (Rabosky, 2016). In many cases prezygotic isolation evolves earlier than postzygotic isolation and likely plays a more important role during, as opposed to after, divergence (Coyne and Orr, 1989; Mendelson, 2003; Rabosky and Matute, 2013; Turissini et al., 2018).

Prezygotic isolation can be split further in to barriers that act before or after mating. Premating isolation reduces the frequency of interbreeding via mechanisms of assortative mating (Kopp et al., 2017). Habitat or temporal isolation prevents interbreeding as reproductively mature individuals of different types do not cooccur at breeding sites at the same time (Filchak et al., 2000). The geographic context of speciation, i.e. allopatric vs. sympatric, can be considered a form of assortative mating as individuals in allopatry mate exclusively within their respective populations as they never cooccur (Kirkpatrick and Ravigné, 2002). Behavioural or sexual isolation can prevent interbreeding due to divergent sexual signals and preferences despite individuals occupying the same space at the same time. The build-up of linkage disequilibrium due to assortative mating can then allow further barriers to gene flow to evolve. Prezygotic isolation mechanisms need not stop at mating (Markow, 1997). The contribution of postcopulatory processes to speciation has remained somewhat overlooked due to the cryptic nature of the interactions between the male ejaculate and the female reproductive tract or the gametes themselves (Firman et al., 2017; Howard et al., 2009; Pitnick et al., 2009). However, postmating interactions between the sexes evolve exceptionally rapidly, pointing to an early role during speciation (Ahmed-Braimah et al., 2017; Bono et al., 2015; Kelleher et al., 2007). There is a sparse but growing literature investigating postmating prezygotic isolation in

metazoans and, in **chapter 1**, I review these empirical and theoretical studies in the context of the importance for understanding speciation.

Reproductive isolation can evolve in the absence of selection via the accumulation of incompatibilities by genetic drift or mutation (e.g. leading to BDMIs). However, variation in local conditions and subsequent divergent selection pressures are also likely to play an important role in speciation (Sobel et al., 2010). In areas of sympatry, where species cooccur, natural selection against costly hybridisation can promote the evolution of prezygotic isolation, i.e. reinforcement (Butlin and Smadja, 2017; Servedio and Noor, 2003). Natural or sexual selection may play a more direct role in the evolution of prezygotic isolation by imposing divergent selection that generates barriers to gene flow between populations. As individuals adapt to their local ecological, social, and sexual environment, divergent selection pressures will generate linkage disequilibrium between locally adapted alleles.

Sexual selection theory and the evolution of reproductive isolation

Traits directly involved in reproduction evolve rapidly, potentially accelerated by sexual selection and sexual conflict (Immonen et al., 2014; Simmons and Fitzpatrick, 2019; Swanson and Vacquier, 2002; VanKuren and Long, 2018; Walters and Harrison, 2011). Sexual selection and sexual conflict are therefore expected to generate divergence between taxa more rapidly than natural selection. As reproductive traits evolve independently in different populations, divergence in mating signals and preferences and/or disruption of molecular interactions between male and female reproductive traits will prevent mating or fertilisation between populations.

Stemming from the evolution of anisogamy males typically increase fitness with the number of mates they obtain while females benefit more from increased mate quality (Bateman, 1948; Lessells et al., 2009; Maynard Smith, 1982, 1978). Individuals of one sex (often males) must then compete for the limited resource of mating and fertilisation opportunities (intrasexual selection), and the other sex (often females) may exert a preference over who to mate with (intersexual selection) (Andersson, 1994). The fitness returns with increased mate number may

be more obvious for males, however, females can also benefit from mating with more than one male (Arnqvist and Nilsson, 2000; Jennions and Petrie, 2000; McCullough et al., 2017; Simmons, 2001; Slatyer et al., 2012; Tregenza and Wedell, 2002). Female multiple mating (polyandry) is widespread across the animal kingdom (Taylor et al., 2014). The far reaching ecological and evolutionary implications of polyandry have only recently begun to be appreciated (Pizzari and Wedell, 2013). With polyandry sexual selection continues after mating as the ejaculates of multiple males compete to fertilise a given set of ova (sperm competition) and females can exert preference over which males sperm fertilise their eggs (cryptic female choice) (Birkhead and Pizzari, 2002). Different episodes of sexual selection (pre- vs. post-copulatory) can have important consequences for how sexual selection operates and the response to selection. For instance, strong mating assortment, where the most polyandrous females mate with the most polygynous males can weaken selection on mating success in favour of traits favoured in sperm competition (McDonald and Pizzari, 2018). Much sexual selection research has focused on understanding different episodes of sexual selection such as the evolution of different reproductive tactics or trade-offs between investment in premating ornaments and armaments vs. traits influencing sperm competition (Simmons et al., 2017).

Sexual selection shapes the evolution and elaboration of male traits that influence reproductive success, or aid in female preference, before and after mating (Andersson, 1994; Birkhead and Pizzari, 2002). Sexual selection affects trait evolution within populations, potentially along different evolutionary trajectories. Fisher's sexy-son hypothesis posits that female preference for (perhaps arbitrary) male traits will evolve because their sons risk not finding a mate if other females in the population exert preference for the trait (Fisher, 1930, 1915). Fisher proposed this can result in runaway sexual selection as male trait and female preference coevolve within populations. As trait and preference alleles co-segregate in offspring, linkage disequilibrium will increase between them as they increase in frequency. Thus, runaway sexual selection can result in signal and preference evolving along a line of equilibrium within populations resulting in divergent sexual signals and preferences between populations, and the evolution of sexual isolation (Lande, 1981) (Fig. 1). Two problems arise from the sexy-son hypothesis. First, preference and trait alleles must already be at relatively high frequency for selection to favour their continued expression. Second, if female preference converges on a single trait, then genetic

diversity will be eroded in the population, yet genetic diversity for sexually selected traits is still observed in nature, i.e. the lek paradox (Rowe and Houle, 1996). The lek paradox can be resolved if mutation continually prevents fixation of the trait and preference alleles, or populations are subject to fluctuating conditions (Tomkins et al., 2004). Faria et al. (2018) recently showed that the sexy-son hypothesis can be considered an extension of Hamilton's greenbeard effect (Hamilton, 1963). The greenbeard effect posits individuals carrying a 'greenbeard' allele will discriminate towards individuals expressing the greenbeard, either favourably or agonistically. In the context of reproductive isolation, the greenbeard can be thought of as a species-specific signal. By considering the sexy-son hypothesis as a special case of the greenbeard effect and incorporating population structure in their models, local assortative mating allows the trait and preference allele to reach high enough frequency to be favoured by selection (Faria et al., 2018). Thus, considering the size and structure of populations, including interspecific interactors, will be important for understanding how sexual selection acts both within and between populations (McDonald et al., 2019).

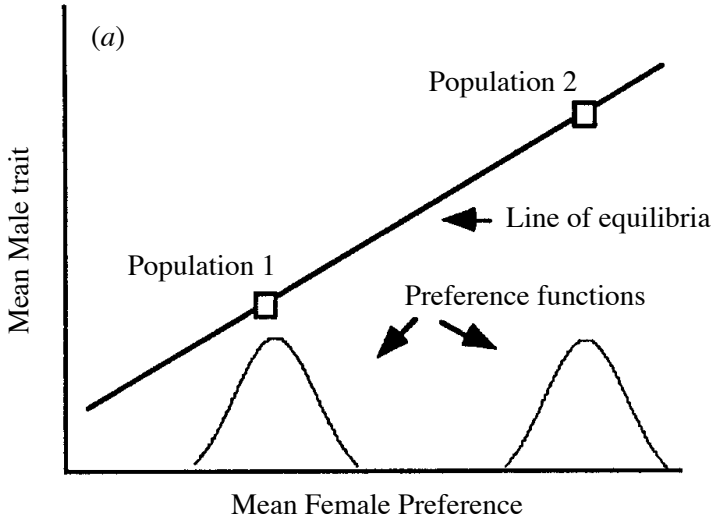

Figure 1. Populations evolve divergent female preferences and male traits along a line of equilibrium via runaway sexual selection (Fisher, 1930, 1915). Given the distribution of female preferences (x-axis), females from population one would not recognise males from population two as potential mates, and vice versa; from Price (1998) after Lande (1981). A similar scenario can be envisioned for the coevolution of a shared trait subject to sexual conflict (e.g. mating rate), where the female preference trait is instead a resistance trait, and the male trait a persistence trait.

Sexual conflict is an inevitable consequence of sexual selection that favours traits in one sex that increase their fitness even at a cost to the other sex, and the different reproductive interests of the sexes (Parker, 1979). Sexual conflict occurs where there is antagonistic selection over traits shared between the sexes (Rowe and Day, 2006). In diploid organisms with two sexes, they share much of the genome. Intralocus sexual conflict arises where alleles shared between the sexes are beneficial in one sex but costly in the other (Bonduriansky and Chenoweth, 2009). For instance, genetic backgrounds increasing female fitness may be negatively correlated with male fitness resulting in a genomic conflict of interests between the sexes (Chippindale et al., 2001). The coevolution of antagonistic alleles may play an important role in the evolution of postzygotic isolation. For instance, the large X-effect observed in many cases of hybrid sterility or inviability may be the result of incompatibilities between driving selfish genetic elements and suppressors in different genetic backgrounds (Masly and Presgraves, 2007; Orr, 1987; Presgraves, 2010; Verspoor et al., 2018).

Interlocus sexual conflict arises where the target of antagonistic selection is on different loci in the two sexes. For instance, sexual conflict over mating rate has resulted in the coevolution of male persistence and female resistance traits both between and within species of pondskaters (Gerridae) (Arnqvist and Rowe, 2002; Perry et al., 2017). Sexual conflict can also continue after mating. Ejaculate traits increasing male fertilisation success or paternity share may be at odds with female interests. Some seminal fluid proteins can delay female remating, or increase female short term fecundity at an expense to later life survival or reproduction (Gioti et al., 2012; Sirot et al., 2015). Furthermore, ejaculates with increased fertilisation potential can increase lethal polyspermy (Snook et al., 2011) necessitating the evolution of counter-adaptations in females to offset costs (Firman et al., 2014). Thus, sexual conflict, both before and after mating, and within and between loci, can generate sexually antagonistic coevolution, resulting in an evolutionary arms race between the sexes (Pizzari and Snook, 2004; Rice, 1996). Sexually antagonistic coevolution is predicted to result in cycles of coevolution within, but not between, populations, promoting the evolution of reproductive isolation (Fig. 1) (Gavrilets, 2000; Panhuis et al., 2001).

Despite theoretical support for a prominent role of sexual selection and sexual conflict in the evolution of reproductive isolation, empirical evidence remains less conclusive (Kraaijeveld et al., 2011; Ritchie, 2007). Comparative studies have aimed to determine if there is a correlation between patterns of diversity and the strength of sexual selection across taxa. In support of a role of sexual selection and sexual conflict increasing diversification, polyandrous insect clades on average comprise four times as many species as monandrous clades (Arnqvist et al., 2000). In birds, some studies have found a positive correlation between the strength of sexual selection and species richness or speciation rate (Barraclough et al., 1995; Cooney et al., 2019), while others do not (Huang and Rabosky, 2014; Morrow et al., 2003). The use of proxies to infer the strength of sexual selection in comparative analyses, such as sexual dichromatism, or the operational sex ratio, may be flawed. Morphological characteristics obvious to us as observers may not be the relevant metric on which sexual selection is acting and such traits may be involved in natural as well as sexual selection. Metrics such as the Bateman gradient, the slope of the least squares regression of relative reproductive success on relative mating success, provide a more direct measure of the strength of sexual selection (Arnold, 1994; Bateman, 1948; Jones, 2009). Using Bateman's metrics, Janicke et al. (2018) did find a positive correlation between the strength of sexual selection and species richness across a wide (Janicke et al., 2018). Thus, at the macroevolutionary scale there appears to be at least a modest contribution of sexual selection increasing diversity.

Both reproductive isolation and lineage persistence contribute to patterns of biodiversity (Rabosky, 2016). Sexual selection may increase diversification rates, and importantly, might also aid in population persistence. Sexual selection can facilitate species range overlap as signal-preference divergence aid in species recognition and coexistence (Cooney et al., 2017; M'Gonigle et al., 2012; Price, 1998). Sexual selection can also provide genetic benefits to the population, purging the genome of deleterious mutations, increasing both sexual and non-sexual fitness, and potentially protect against extinction (Cally et al., 2019; Dugand et al., 2018; Lumley et al., 2015; Parrett and Knell, 2018; Yun et al., 2018). The benefits of sexual selection for population mean fitness may be particularly stark in harsh or changing environments (Cally et al., 2019). The study of sexual selection may be of wider interest in the context of how populations respond in the face of climate change (Parrett and Knell, 2018).

Experimental studies investigating a link between sexual selection and speciation at the microevolutionary scale have tested whether reproductive isolation is stronger between treatments experiencing stronger sexual selection. For instance, experimental evolution studies manipulated the operational sex ratio (and thus the opportunity for sexual selection and sexual conflict) and then tested for assortative mating or fertilisation between populations after a number of generations. The prediction being that reproductive isolation will be stronger between populations experiencing stronger sexual selection and sexual conflict (i.e. polyandrous lines) than between populations experiencing weakened or absent sexual selection and sexual conflict (i.e. monogamous lines). Stronger sexual isolation evolved between populations experiencing heightened sexual conflict in the dung fly, *Sepsis cynipsea*, but not in *Drosophila melanogaster* (Hosken et al., 2009) or *D. pseudoobscura* (Bacigalupe et al., 2007). This mixed evidence might be explained by the misplaced assumption that sexual isolation will evolve due to sexually selected traits evolving in arbitrary directions in different populations which may not be correct (Snook et al., 2005). Additionally, sexual selection and sexual conflict alone may not be sufficient to generate complete or sufficient assortative mating. Finally, much of this work investigates mechanisms of assortative mating, however, sexual conflict might have greater impacts on postcopulatory traits in these animals (Chapman et al., 1995; Hollis et al., 2019). Therefore, assortative fertilisation mechanisms might evolve more rapidly between populations.

The *Drosophila montana* system

To study the early stages of the speciation process requires studying divergent populations within a species (The Marie Curie Speciation Network, 2012). Given that PMPZ isolation is predicted to emerge early during reproductive isolation, in **chapters 2-4** I test hypotheses about the early emergence of postmating prezygotic isolation using North American populations of the malt fly, *Drosophila montana* (Patterson and Wheeler, 1942). A member of the virilis group, *D. montana* (Fig. 2) is found across the Northern Hemisphere at high altitudes and/or latitudes. *D. montana* last shared a common ancestor with *D. virilis* around 10 million years ago in central Asia. The ancestor subsequently spread throughout Eurasia and in to North America (Morales-Hojas et al., 2011). Scandinavian and North American populations are estimated to

have diverged between 450,000 – 900,000 years ago (Mirol et al., 2007). Due to their cold acclimation, *D. montana* have been the subject of study investigating reproductive diapause and cold adaptation (Parker et al., 2018; Vesala and Hoikkala, 2011). Females can undergo reproductive diapause and overwinter as adults. Where diapausing females overwinter is currently unknown.

Within North America, *D. montana* populations expanded from separate Northern and Southern glacial refugia after the last ice age ca. 20,000 years ago (Hewitt, 2000). Southern populations, such as Colorado, and northern populations, such as Vancouver, fall in to some distinct groups, with no evidence of admixture based on microsatellite markers and mitochondrial DNA sequences (Mirol et al., 2007). Notably, populations differ in their breeding ecology, and current and historic range overlap with close relatives (Fig. 3). *D. montana* in Vancouver are found at low elevations, are adapted to warmer climates, are univoltine, and only recently has *D. flavomontana* become sympatric (Jennings et al., 2011; Poikela et al., 2019). In comparison, *D. montana* in Colorado inhabit higher elevations, uniquely breed on Aspen trees (*Populus spp.*), are bivoltine and share much of their current range with at least two close relatives, *D. borealis* and *D. flavomontana* (Jennings et al., 2011; Routtu et al., 2007). Thus, sexual selection in Vancouver may be dominated by intraspecific interactions, while in Colorado, interspecific discrimination may play a relatively more important role in sexual selection.

Figure 2. Adult *Drosophila montana*. Credit Martin Garlovsky flickr.com.

Populations from North America and Finland show substantial variation in reproductive traits involved in sexual signalling and reproduction, including male song carrier frequency (Klappert et al., 2007), cuticular hydrocarbon profiles (Jennings et al., 2014a; Veltsos et al., 2012), and genital morphology (Routtu et al., 2007). *D. montana* was included in the extensive surveys of *Drosophila spp.* across North America in the first half of the 20th Century (Moorhead, 1954; Patterson, 1941, 1943, 1946, 1952). Moorhead (1954) noted that crosses between 'giant' and 'standard' strains of *D. montana* showed variable fertility, probably the result of postmating prezygotic isolation (Moorhead, 1954). The possibility that postmating prezygotic isolation was acting as a barrier to gene flow between populations of *D. montana* in North America was neglected for more or less the next 50 years (Jennings et al., 2014b, 2011).

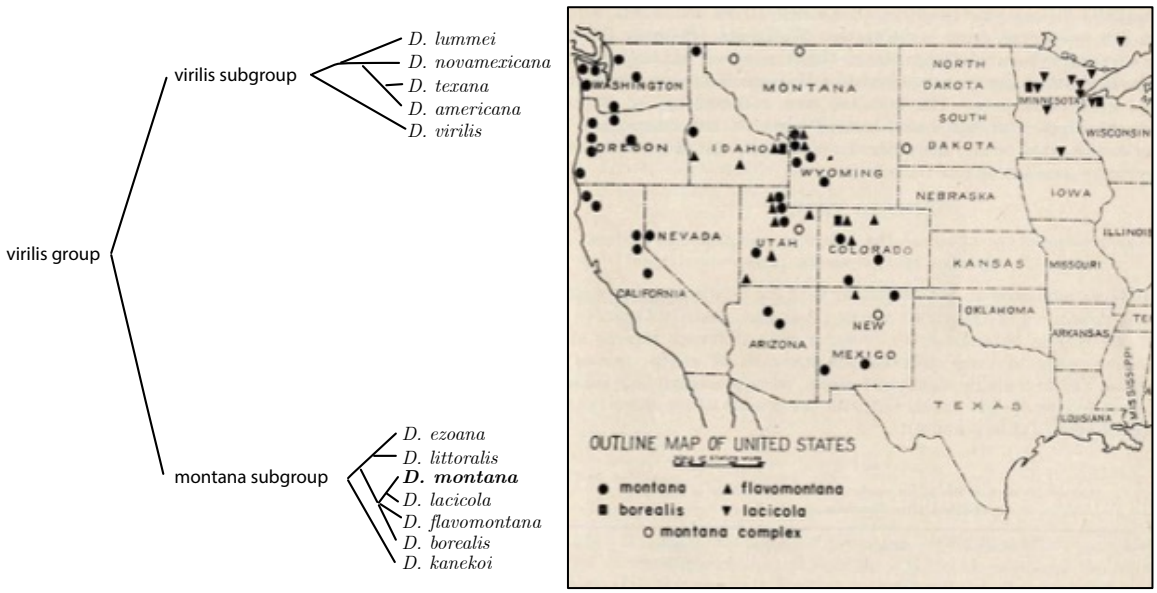

Figure 3. Left: Phylogenetic relationship of the virilis group. Modified from flybase.org. Right: Locations of *D. montana* and relatives from Patterson (1952). Since these collections were made, *D. flavomontana* has expanded its range northward and is now sympatric with *D. montana* in the Northern USA around Washington State and Canada (Poikela et al., 2019).

Previous studies found PMPZ isolation between *D. montana* populations from Finland and North America (Jennings et al., 2014b, 2011). Further sampling in North America was undertaken with the intention of setting up a paired design to test differences between "Colorado-like" populations, i.e. Jackson, Wyoming, USA and Crested Butte, Colorado, USA, and "Vancouver-like" populations, i.e. Ashford, Washington, USA and Vancouver, British

Columbia, Canada (M.G. Ritchie, R.K. Butlin, pers. comms.). In **chapter 2**, published in *Ecology and Evolution* (**8**:9062-9073), I test three main hypotheses. First, to test whether the pattern of PMPZ isolation observed between Colorado and Vancouver was replicated between other populations. Second, due to the recent divergence time between populations, incompatibilities may only be present between particular genotypes, rather than acting at the population level. Therefore, I tested whether different genotypes from similar locations but from different time points showed the same pattern of PMPZ isolation. Finally, PMPZ isolation may be affected by male or female remating rates, as evidenced by other kinds of incompatibilities such as cytoplasmic incompatibility (Karr et al., 1998) or the insemination reaction (Kelleher and Markow, 2007). Therefore, I tested whether remating ameliorated or exacerbated the strength of PMPZ isolation acting between populations.

The role of different modes of prezygotic isolation (premating or PMPZ) during the initial stages of speciation warrants further investigation. In some systems, premating isolation appears to emerge before postmating barriers. However, how episodes of pre- and post-copulatory sexual selection interact and might contribute to speciation is largely unknown. Emerging from chapter 2 – that PMPZ isolation was asymmetrical – I predicted that the strength of PMPZ isolation would reflect the strength of postcopulatory sexual selection acting within populations. To test this prediction, I tested two hypotheses in **chapter 3**. First, I tested whether the strength of PMPZ isolation is correlated with measures of postcopulatory sexual selection acting within populations, and whether conspecific sperm precedence is acting between populations. Second, I tested whether premating isolation and PMPZ isolation cooccur or evolve independently.

For internally fertilising taxa, males transfer seminal fluid proteins in the ejaculate along with sperm (Perry et al., 2013). Postmating prezygotic interactions often necessarily involve protein-protein interactions between the male ejaculate and the female reproductive tract, or between the gametes themselves. In **chapter 4** I test whether populations of *D. montana* exhibiting PMPZ isolation show differences in the composition of the ejaculate using liquid chromatography tandem mass spectrometry (LC-MS/MS). High throughput "shotgun" proteomics is an emerging tool for the study of PMPZ isolation (McDonough et al., 2016). I

identified a number of differentially abundant proteins in each population, including several orthologues of known seminal fluid proteins in *D. melanogaster*. This analysis also provides the first description of the *Drosophila montana* seminal fluid proteome, including the characterisation of both the accessory gland and the ejaculatory duct and bulb proteomes.

Sexual selection and the evolution of life histories

Sexual selection and sexual conflict can have important life histories consequences (Wedell et al., 2006). Variation in reproductive success on which sexual selection can act is affected by many aspects of overall physiological health and condition (Emlen et al., 2012). Therefore, sexual selection will capture many aspects of organismal performance, involving genes across the genome (Rowe and Houle, 1996; Tomkins et al., 2004). Sexual selection will therefore impact not only traits directly involved in reproduction, but the underlying physiological and life history traits that enable the expression and maintenance of sexually selected traits. Sexual conflict can also shape life history traits due to the sexes differing in their optimal development time or resource allocation decisions (Wedell et al., 2006). Differences in life history strategies between the sexes can also evolve to resolve sexual conflict (Blanckenhorn et al., 2007).

Changes in life history strategy such as increased investment in reproduction is predicted to result in trade-offs with other aspects of fitness due to limited time and resources (Roff, 2002). In **chapter 5** I test the prediction that the strength of sexual selection and sexual conflict will result in a coordinated response in the evolution of physiological and life history traits to accommodate differential investment in traits subject to sexual selection and sexual conflict. Using experimental evolution, I test how populations subject to the elevation or relaxation of sexual selection and sexual conflict diverge as they adapt to the local socio-sexual environment. While not directly in the context of speciation, this study illustrates how variation in the strength of sexual selection and sexual conflict can act as a powerful source of divergent selection.

Experimental evolution in *Drosophila pseudoobscura*

Experimental evolution can be a power tool to investigate adaptation and divergent selection (Kawecki et al., 2012). Over the past 15 years, the "Snook lines" have been used to study the impacts of sexual selection and sexual conflict by manipulating the operational sex ratio in replicated populations of *Drosophila pseudoobscura*. The Snook lines were established in 2001 from 50 wild-caught, inseminated, *D. pseudoobscura* females collected near Tucson, Arizona, USA. Four replicate populations were subsequently cultured in discrete generations. After four generations of adaptation to the laboratory, an elevated promiscuity (P) and enforced monogamy (M) treatment was established from each replicate line. In the P lines, the opportunity for sexual selection and sexual conflict was increased by housing one female with six males in each generation. *D. pseudoobscura* is a naturally promiscuous species, with females frequently inseminated by more than one male in the wild (Anderson, 1974; Cobbs, 1977; Partridge et al., 1987). Thus, inter- and intra- sexual selection is intensified in the P treatment, as males must compete for mating and reproductive success, and females may exert preference both before and after mating. In the M lines, one female is housed with one male in each generation, reducing the opportunity for sexual conflict, as the reproductive interests of males and females are tied together, and eliminating the opportunity for sexual selection, as choice and competition are eliminated. A detailed description of the establishment and maintenance of the experimental evolution lines can be found in Crudgington et al. (2005).

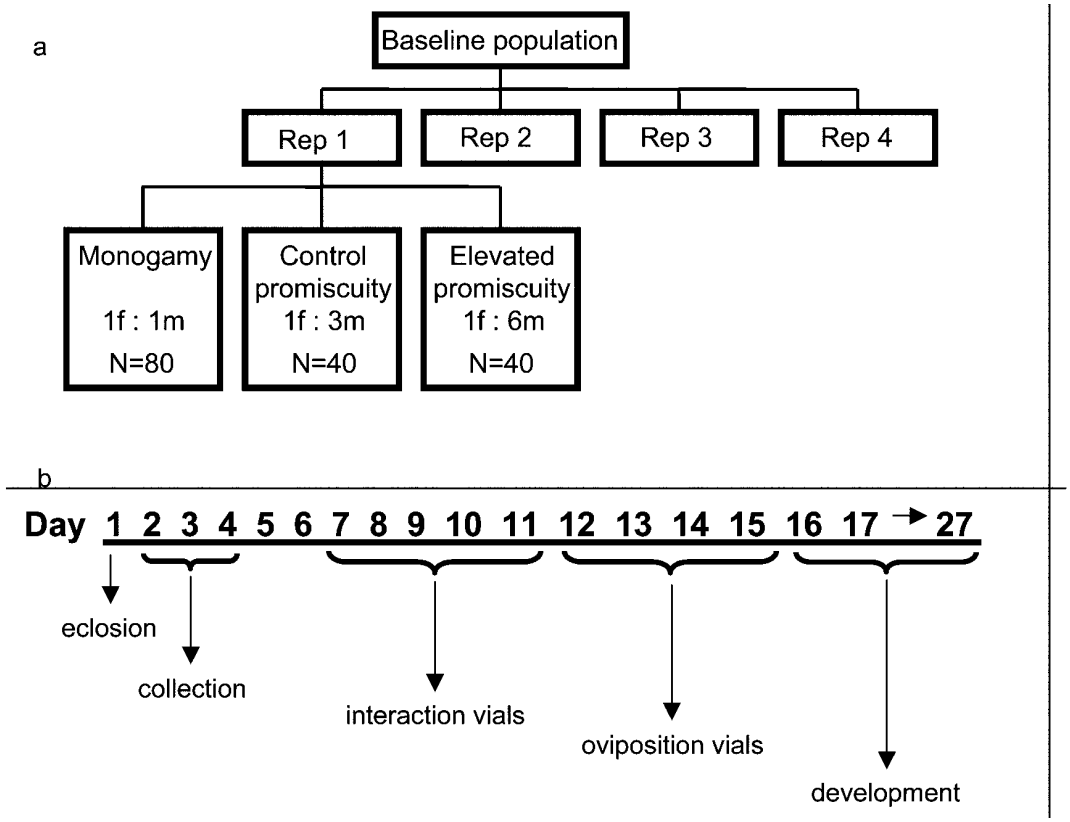

Figure 4. Design of the experimental evolution lines and rearing protocol. N = number of families in each replicate of each treatment, f = female, m = male. Note: The Control promiscuity treatment was subsequently no longer cultured. Modified from Crudgington et al. (2005).

The experiments in **chapter 5** were carried out after more than 175 generations, and up to 199 generations of experimental evolution, the last experiments to be carried out using the Snook lines while still in culture. Previous studies have shown traits favoured by both pre- and post-copulatory sexual selection have diverged between the M and the P treatments. P males sing a faster and more vigorous courtship song, court females more frequently, and have a greater mating capacity and larger accessory glands (Crudgington et al., 2009; Debelle et al., 2017). Female preferences have also diverged, preferring a male courtship song from within their own treatment. However, despite divergent preferences, P males gain more matings with both M and P females, as they outcompete M males (Debelle et al., 2016). Lines also show differential sex-specific gene expression (Immonen et al., 2014; Veltsos et al., 2017). Thus, these changes in traits involved in reproduction are predicted to result in changes in life history traits to accommodate their expression.

1. Cryptic barriers to gene flow: between insemination and fertilisation

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KEYWORDS: Postmating prezygotic isolation, gametic isolation, reproductive isolation, assortative fertilisation, reinforcement, cryptic female choice, sperm competition.

HIGHLIGHTS

- There are a wide range of potential barriers to gene flow that can prevent successful fertilisation that act after mating, or between the gametes themselves.
- Until recently these cryptic barriers to gene flow have received relatively little attention in the speciation literature.
- Internal fertilisation has posed a significant challenge for studying PMPZ isolation due to the difficulties of tracking sperm fate inside the female reproductive tract.
- The study of PMPZ remains in its infancy. However, with modern genomic and proteomic tools it is now possible to begin uncovering these once enigmatic interactions between the sexes and their gametes, to uncover the mechanisms of PMPZ isolation, how they evolve, and their role during the speciation process.

ABSTRACT

Postmating prezygotic isolation has received relatively little attention from the speciation community. However, these cryptic barriers to gene flow are emerging as potentially important early during the speciation process due to the rapid evolution of reproductive traits within populations. Much has been learned over the past 15 years about the emergence and evolution of these enigmatic interactions between the sexes and their gametes, however much more is left unknown. In this review we aim to address current gaps and pitfalls in studies of PMPZ isolation and highlight outstanding questions about the evolution of PMPZ isolation and what role it plays during the evolution of reproductive isolation.

GLOSSARY

- **Conspecific sperm precedence (CSP)**: the non-random use of conspecific sperm over heterospecific sperm to fertilise eggs, regardless of mating order, is a taxonomically diverse form of PMPZ isolation, analogous to conspecific pollen precedence in plants (Howard, 1999).
- **Ejaculate**: the sperm and non-sperm components of male derived secretions transferred during mating. Non-sperm components of the ejaculate may provide secondary functions such as sperm competitive ability and nutrition to the female.
- **Ejaculate x female reproductive tract interactions (EFIs)**: sperm and seminal fluid proteins in the male ejaculate interact with tissues or female reproductive tract secretions to induce behavioural, physiological and morphological changes in the mated female. EFIs can also contribute to male sperm competitive ability and CSP.
- **Fecundity**: the number of eggs laid by a female during a given time period or over a whole lifetime.
- **Fertilisation set**: the population of sperm representing different males that are able to compete to fertilise a given set of ova (Parker et al., 1990).
- **Intracellular sperm-egg interactions (ISEIs)**: interactions between the sperm and egg that take place within the egg cytoplasm after sperm entry inside the egg but before karyogamy.
- **Karyogamy**: the fusion of sperm and egg pronuclei inside the egg resulting in the formation of a zygote.
- **Micropyle**: in insects, fishes, and some cephalopods the physical entry point through which sperm must enter the egg.
- **Polyspermy**: the fertilisation of an ovum by multiple sperm, which in most species' leads to embryo mortality.
- **Positive selection**: the increase in frequency of genetic variants. Signatures of positive selection are often inferred from dN/dS ratios (the number of nonsynonymous (dN) divided by the number of synonymous (dS) base substitutions between lineages) greater than one, a.k.a. omega (ω).
- **Postcopulatory sexual selection**: in polyandrous species where females remate within a reproductive cycle sexual selection can continue after mating. Rival male ejaculates must

then compete for fertilisation of ova (sperm competition) and females may exert choice over which sperm are used to fertilise her eggs (cryptic female choice) (Birkhead and Pizzari, 2002).

- **Postmating prezygotic (PMPZ) isolation:** encompasses all interactions between male and female reproductive tract tissues and their secretions, or the gametes themselves, that reduce the frequency of successfully fertilised eggs in crosses between taxa.
- **Postzygotic isolation**: hybridisation between taxa that results in inviable, sterile, or low fitness offspring.
- **Premating isolation**: barriers to gene flow that reduce the frequency of interspecific matings; due to temporal or spatial isolation reducing the overlap of breeding individuals (ecological isolation), or individuals may discriminate between mates based on sensory stimuli such as visual, olfactory or other cues (sexual isolation).
- **Proteases**: enzymes which catalyse the degradation of complex proteins into polypeptides or single amino acids.
- **Reinforcement**: the strengthening of prezygotic barriers to gene flow resulting from selection against costly hybridisations (as hybrids are sterile, infertile, or less fit) (Servedio and Noor, 2003).
- **Seminal fluid proteins (SFPs)**: proteins in the male ejaculate, which are transferred with sperm, that can influence the outcome of fertilisation.
- **Spermatophore**: a packet of sperm and seminal fluid proteins transferred to females either internally (e.g. Lepidoptera) or deposited and later picked up by females (e.g. Orthoptera). In the Heliconiinae butterflies, the spermatophore delays female remating until it is digested by the female. Spermatophore thickness is greater in polyandrous species, suggesting the co-evolution of spermatophore thickness and its breakdown evolve via sexual selection and sexual conflict (Sánchez and Cordero, 2014).
- **Syngamy**: the fusion of gamete cell surfaces.

Barriers to gene flow: Splitting the dichotomy

To understand the origin of species requires identifying the barriers to gene flow that emerge first or early between divergent taxa and quantifying how different barriers to gene flow contribute to total reproductive isolation (Butlin and Smadja, 2017; Coyne and Orr, 2004; Sobel and Chen, 2014; The Marie Curie Speciation Network, 2012; Turelli et al., 2001). The dichotomy inherent in investigating barriers to gene flow that act before or after mating, or before or after zygote formation, has led to the majority of speciation research focussing on **premating isolation** (see Glossary) and **postzygotic isolation** (Coyne and Orr, 2004). This pedagogy has led to a distinct lack of research on **postmating prezygotic (PMPZ) isolation**, the set of barriers to gene flow that act after mating, but before fertilisation. There has been a modest but growing interest in PMPZ isolation over the past 15 years (Howard et al., 2009). Yet, we identified just 121 studies that measured PMPZ isolation in a literature survey of studies published since 2004 using the Web of Science (wok.mimas.ac.uk) (Fig. 1.1). In comparison we identified 534 studies using the search terms "postzygotic" and "reproductive isolation"; and 816 studies using the terms "premating" and "reproductive isolation" or "sexual isolation". Thus, we still have a fairly poor understanding of the mechanisms and evolution of PMPZ isolation and its role during the evolution of reproductive isolation. This oversight may prove costly as PMPZ isolation barriers appear to arise rapidly and early during the evolution of reproductive isolation.

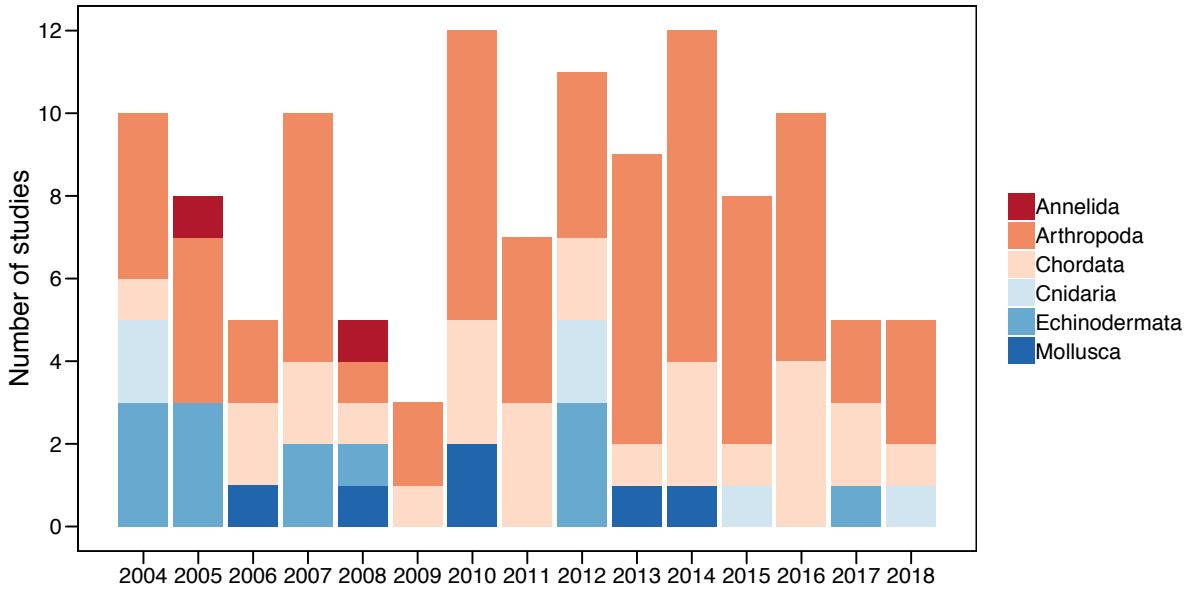

Figure 1.1. Number of studies that measured postmating prezygotic isolation published since 2004 identified using the Web of Science (wok.mimas.ac.uk). Of the total 121 studies we identified, the majority were studies of arthropods, notably insects (66/121; 55%).

PMPZ isolation often involves microscopic or molecular interactions between gametes or protein-protein interactions within an opaque female reproductive tract. Consequently, as a detailed understanding of postmating female responses has remained elusive in studies of **postcopulatory sexual selection** (Firman et al., 2017), the cryptic nature of postmating interactions has posed a major challenge to the study of PMPZ isolation. Theoretical advances, the emergence of new tools, and reductions in costs have allowed deeper insights in to these cryptic barriers to gene flow (McDonough et al., 2016). The fundamental role of postmating interactions to fitness also highlights the study of PMPZ barriers as an important avenue of research which could benefit our understanding of animal fertility more generally. Here, we aim to identify current gaps in our understanding of PMPZ isolation in metazoans and highlight what we consider to be important avenues of research that warrant further investigation. We start by describing what we know about the evolution of PMPZ isolation and then explore the mechanisms responsible.

The evolution of postmating prezygotic isolation

Where does postmating prezygotic isolation fit in the speciation timeline?

When does PMPZ isolation emerge between taxa during the evolution of reproductive isolation? What is the strength of PMPZ isolation compared to premating or postzygotic isolation at different timepoints during the course of speciation? What evolutionary processes (selection or drift) contribute to the evolution of PMPZ isolation? These questions are fundamental to understanding how important PMPZ isolation is during the speciation process, as opposed to playing a role in maintaining species integrity after divergence (Coyne and Orr, 2004).

The early emergence of PMPZ isolation is predicted given the rapid evolution of reproductive traits within populations. As postmating interactions rapidly coevolve between the sexes within populations relating to interactions between the sexes or the gametes fundamental to successful fertilisation, PMPZ isolation phenotypes are expected to emerge between populations early during divergence. Reproductive traits fundamental to reproduction are expected to maintain a conserved role, yet reproductive genes are among the fastest evolving (Swanson and Vacquier, 2002) and show exceptionally fast rates of diversification compared to non-reproductive traits (Ahmed-Braimah et al., 2017; Ramm et al., 2009; Rowe et al., 2015). Likewise, genes with male biased expression show elevated expression and sequence divergence (Zhang et al., 2007), and rapid gene turnover between species (Harrison et al., 2015). Sexual selection and sexual conflict are often invoked as catalysts of the rapid evolution of reproductive traits, as male and female traits coevolve within, but not between populations (Gavrilets, 2000; Lande, 1981). In support of a role of sexual selection and sexual conflict in the evolution of PMPZ isolation, the same processes involved in postcopulatory sexual selection and sexual conflict often generate PMPZ isolation. For instance, the same genes conferring advantages in sperm competition within species are found to influence PMPZ isolation (Britch et al., 2007; Castillo and Moyle, 2014; Civetta and Finn, 2014). Experiments in muroid rodents and marine invertebrates suggest a role of sexual conflict and sexually antagonistic co-evolution in the evolution of postmating prezygotic incompatibilities (Firman, 2018). Sperm competition favouring male **ejaculate** traits increasing fertilisation success may lead to increased incidence of lethal **polyspermy** (Snook et al., 2011). As females (or their ova) evolve counter-adaptations to defend against polyspermy, this may generate asymmetries in the strength of sperm-egg incompatibilities between taxa

[BOX 1]. Studies in house mouse (*Mus domesticus*) have also shown that populations differ in their 'ova defensiveness' to fertilisation, and manipulating females perceived risk of sperm competition can result in plastic responses in ova defensiveness (Firman and Simmons, 2013). Further, experimental evolution altering the intensity of sperm competition (and thus risk of polyspermy) can result in the evolution of increased ova defensiveness (Firman et al., 2014). These experiments provide evidence of a crucial first step towards the evolution of PMPZ isolation arising between populations resulting from sexual selection and sexual conflict acting within populations. However, direct evidence for sexual selection and sexual conflict generating PMPZ isolation has proved more elusive.

BOX 1 – Asymmetrical postmating prezygotic isolation

In reciprocal crosses between populations, reproductive isolation may be greater in one direction of the cross. Asymmetry is often, if not always, observed in the strength of PMPZ isolation between pairs of taxa and provides possible insights in to the evolutionary forces generating PMPZ isolation.

Asymmetry might reflect the consequences of different demographic histories, historical bottlenecks, or interspecific interactions. As populations lose genetic diversity going through bottlenecks, genetically diverse populations may maintain compatibility with a wide range of (heterospecific) genotypes, whereas populations that have undergone strong reductions in genetic diversity will only maintain compatibility with a restricted range of (conspecific) genotypes (Ahmed-Braimah and McAllister, 2012). The "rarer-female" hypothesis posits that in areas of sympatry, females from the rarer species will encounter heterospecific males more frequently and therefore more often incur costs of hybridisation, strengthening selection on prezygotic isolation (i.e., **reinforcement**) (Yukilevich, 2012).

Dubbed the weak inbreeder strong outbreeder, or "WISO" hypothesis (see Ting et al., 2014), asymmetric PMPZ isolation may reflect the strength of sexual selection and sexual conflict acting within populations. In the house mouse (*Mus*) species complex, species experiencing stronger intraspecific sperm competition have greater fertilisation success with heterospecifics (strong outbreeders) and are more resistant to heterospecific fertilisation by species that

experience weaker intraspecific sperm competition (weak inbreeders) (Martín-Coello et al., 2009). Experimental evolution also provides insights in to differential sperm usage dynamics and the role of sexual selection in generating asymmetrical PMPZ isolation. For instance, populations that have evolved longer sperm in response to heightened postcopulatory sexual selection might always outcompete shorter sperm (Godwin et al., 2017), or sperm that more precisely match the female reproductive tract may be favoured (Miller and Pitnick, 2003).

Heterospecific mating need not always result in lower fitness asymmetries. Heterospecific mating can elevate female fecundity above that of a conspecific (Fricke et al., 2006), heterospecific sperm may outcompete conspecific sperm (Dean and Nachman, 2009; Hosken et al., 2002), or mating with a conspecific can lead to the greatest reduction in lifespan (Fricke and Arnqvist, 2004).

END BOX

An early role of postmating prezygotic isolation during the speciation process is supported by numerous empirical studies showing PMPZ isolation is the primary or only barrier to gene flow between some closely related taxa (Ahmed-Braimah and McAllister, 2012; Devigili et al., 2018; Fricke and Arnqvist, 2004; Friesen et al., 2013; Garlovsky and Snook, 2018; Larson et al., 2012; Riginos et al., 2006; Soudi et al., 2016; Styan et al., 2008). In *Drosophila* and birds premating isolation evolves faster than postzygotic isolation (Rabosky and Matute, 2013). Where PMPZ isolation fits in the "speciation timeline" is less clear. In polychaete worms (*Galeolaria caepitosa*) PMPZ isolation scaled with genetic distance (Styan et al., 2008). Similarly, across the *Drosophila melanogaster* species group PMPZ isolation increased with genetic distance, evolving faster than postzygotic isolation but not as fast as premating isolation (Turissini et al., 2018). In other groups the relationship between the strength of PMPZ isolation and genetic distance is less clear. PMPZ isolation was stronger than premating isolation between populations of *Drosophila montana* (Jennings et al., 2014) and in toads (*Bufo spp.*) postzygotic isolation increased with genetic distance, whereas PMPZ isolation did not (Malone and Fontenot, 2008). PMPZ isolation is apparently the only form of reproductive isolation acting between crickets in the *Allonemobius socius* species complex (Marshall et al., 2011) and between laboratory strains of bean weevils (*Callosobruchus maculatus*) (Fricke and Arnqvist, 2004).

These studies suggest PMPZ isolation can emerge before other prezygotic barriers and so might be particularly important early during reproductive isolation.

Between ejaculation and fertilisation: mechanisms of PMPZ isolation

Once released, either into the external environment, or directly into the female reproductive tract, sperm must move towards the site of fertilisation and enter the egg. The path of sperm towards the egg is no mean feat. There are a multitude of potential barriers to fertilisation that can act during or after mating or the release of gametes, and eventual **karyogamy**.

Extrinsic PMPZ isolation

Extrinsic postzygotic isolation, where hybrids do not fit well to either parental habitat, are relatively well studied (Coyne and Orr, 2004). In a similar manner, PMPZ isolation could reflect differences in locally optimal conditions (van Doorn et al., 2009) or evolved responses to changes in life history strategy or shifts in reproductive mode, i.e. pleiotropy (e.g. transition from external to internal fertilisation) (Weber et al., 2017). There is growing interest in environmental effects on fertility (Porcelli et al., 2017, 2016; Sales et al., 2018), particularly in the context of how populations may respond to climate change (Walsh et al., 2019). Extrinsic PMPZ isolation barriers, such as thermally induced infertility, might only be apparent under certain conditions. For instance, sperm viability may be compromised at high temperatures for some, but not all populations or species (Matute et al., 2009; Porcelli et al., 2017). Common garden experiments might therefore over- or under- estimate PMPZ isolation present in nature. This fact highlights the importance of taking in to account each population's local biology and ecology when quantifying PMPZ isolation and reproductive isolation more generally.

Sperm-egg (gametic) isolation

True gametic isolation involves interactions between the gametes at, or close to, the cell surface that subsequently prevent **syngamy**. The diversity of mechanisms and difficulties associated with sperm-egg interactions are discussed in detail elsewhere (Karr et al., 2009). With respect to PMPZ isolation, an overarching theme is that gametes possess protein binding regions on the cell surfaces (ligands and receptors) that require species-specific signalling molecules (Palumbi, 2009). Much of what we know about such gametic incompatibilities comes from

externally fertilising marine invertebrates where the lack of direct interactions between individuals (mating) means prezygotic isolation is limited to these gametic incompatibilities.

Chemoattractants may be important in helping sperm navigate towards eggs, effectively increasing the target area for guiding sperm towards the egg, particularly useful in dense multispecies gamete mixes in the sea (Riffell et al., 2004). In heterospecific gamete mixes, sperm chemotaxis towards the egg may be reduced. For instance, brittle star (*Ophioderma spp.*) sperm possess divergent receptors for egg chemoattractants that result in reduced sperm attraction towards heterospecific eggs (Weber et al., 2017). If heterospecific sperm do contact the egg, sperm attachment and entry may be reduced. In sea urchins (e.g. *Echinometra spp.*), to break down and pass through the egg envelope sperm require species-specific bindin proteins to bind with proteins (EBR1) on the egg membrane. Similarly, abalone (*Haliotis spp.*) sperm require species-specific lysin proteins to properly interact with the vitelline envelope receptor for lysin (VERL) found on the egg surface. Lysin evolves to complement VERL in a species-specific manner via **positive selection**. In mammals, the zona pellucida proteins, ZP2 and ZP3, found on the egg membrane, show similar patterns of positive selection to the VERL-lysin system in abalone (reviewed in Wilburn and Swanson, 2016). Whether egg surface proteins such as ZP2 and ZP3 (and the corresponding sperm ligands) contribute to PMPZ isolation in mammals is currently unknown. Major histocompatibility complex (MHC) genotype at gamete surfaces influences fertilisation success in birds and mice acting as a mechanism for inbreeding avoidance (Firman and Simmons, 2015; Løvlie et al., 2013; Rülicke et al., 1998). Similar mechanisms could contribute to outbreeding avoidance (i.e. against heterospecifics). Indeed, selection for immune compatibility at CMAH/Neu5Gc egg cell-surface antigens may have contributed to PMPZ isolation early in our own evolutionary history (Ghaderi et al., 2011).

In marine invertebrates, birds, and mammals, the acrosome reaction is required for sperm entry in to the egg (Karr et al., 2009). In insects, fishes and cephalopods, there is no acrosome reaction. Instead sperm enter the egg through the **micropyle** (Yanagimachi et al., 2013). Precise molecular or morphological interactions may be essential for sperm entry through the micropyle. However, very little is known about sperm-micropyle interactions and any potential barriers to gene flow they might pose.

Intracellular sperm-egg interactions

Intracellular sperm-egg interactions (ISEIs), taking place inside the egg after syngamy but before karyogamy, present a relatively underexplored realm in which PMPZ isolation might act (Bugrov et al., 2004; Karr et al., 2009; Snook et al., 2009, 2011). Between Zimbabwe (Z) and Metropolitan (M) strains of *Drosophila melanogaster*, fertilisation success is reduced in one direction, as M sperm do not take up the correct 3D structure inside the egg and/or are unable to properly penetrate and fertilise Z eggs (Alipaz et al., 2001). Further examples of ISEIs are rare in part due to the difficulties of studying these microscopic interactions. While ISEIs warrant further investigation, the fundamental role ISEIs hold to fertility suggest they could be highly conserved across taxa presenting only a modest, if any, contribution to PMPZ isolation (Southern et al., 2018).

Ejaculate x female reproductive tract interactions

For internally fertilising taxa, the passage of sperm through the female reproductive tract involves a series of **ejaculate x female reproductive tract interactions (EFIs)** (Pitnick et al., 2009). Mismatches between the male ejaculate and the female reproductive tract, or an ejaculate deficient of particular **seminal fluid proteins (Sfps)**, may not elicit the proper behavioural, physiological or morphological **postmating female response** necessary for efficient fertilisation (LaFlamme et al., 2012; Mattei et al., 2015; Ravi Ram and Wolfner, 2009; Singh et al., 2018; Yapici et al., 2008). EFIs can also contribute to other PMPZ isolation barriers, such as problems with sperm transfer, transport, and storage, or **conspecific sperm precedence (CSP; [BOX 2])**.

BOX 2 – Conspecific sperm precedence

A single heterospecific mating may not result in infertility such that PMPZ isolation is only evident where the ejaculates of both con- and hetero-specific males compete for fertilisation. Conspecific sperm precedence (CSP) can be broken down in to three components. First, males from species experiencing heightened postcopulatory sexual selection may fare better in interspecific sperm competition (Martín-Coello et al., 2009). Conspecific males may also increase representation in the fertilisation set by displacing sperm from a previous (heterospecific) mating more efficiently (Manier et al., 2013; Rivera et al., 2004), or mating for

longer (Price et al., 2001). However, success in intraspecific sperm competition may trade-off with CSP where interspecific interactions shape the postcopulatory selective environment (Castillo and Moyle, 2019). Second, cryptic female choice may favour conspecific sperm, conferring a 'home turf advantage' in a native female reproductive tract. Females may disproportionately favour conspecific sperm or discriminate against heterospecific sperm due to the same mechanisms generating PMPZ isolation after a single heterospecific mating. Heterospecific sperm may be lost from storage at a faster rate, be preferentially ejected by females, or females may bias use towards sperm storage organs containing conspecific sperm (Manier et al., 2013). Female derived secretions may hamper heterospecific sperm motility (Cramer et al., 2016) or aid conspecific sperm motility (Devigili et al., 2018; Yeates et al., 2013). Finally, the interaction between sperm competition and cryptic female choice might influence the outcome of sperm competition where con- and hetero-specific ejaculates compete. Seminal fluids can protect sperm from spermicidal secretions of the female reproductive tract (including sperm from other males) (Holman, 2009; Holman and Snook, 2008; Liberti et al., 2018). Non-self sperm can also reduce the fertility of own sperm (den Boer et al., 2010). Thus, mixing of con- and hetero-specific ejaculates might increase the fertilisation success of heterospecific sperm, or, heterospecific ejaculates might sabotage the fertilisation efficiency of conspecific sperm. Whether female mediated spermicide can target heterospecific sperm, or whether mixing of con- and hetero-specific ejaculates might save, or sabotage fertility warrants further investigation.

Differentiating between biased patterns of paternity arising under equal sperm concentrations from other mechanisms which stack the deck to bias representation of conspecific sperm in the fertilisation set may be challenging. For instance, heterospecific sperm might have reduced fertilisation success despite reaching the site of fertilisation first. Paternity share inferred from genotyping or other phenotypic markers will show a bias towards conspecific males, indicative of conspecific sperm precedence. However, this in fact will be the result of sperm-egg (gametic) incompatibilities rather than biased use of conspecific sperm by females or conspecific ejaculates faring better in sperm competition.

END BOX

Postmating female responses

Mating itself, and the transfer of the ejaculate, can have far reaching consequences in the mated female long after copulation has ended and beyond the female reproductive tract wall (Delbare et al., 2017; Yapici et al., 2008). Abnormal postmating female responses may generate a number of PMPZ isolation phenotypes. Short term or lifetime **fecundity** may be reduced after a heterospecific mating, although the mechanism is rarely reported. Heterospecific sperm may be lost (or die), not stimulating females to oviposit (Matute, 2010a). Further, heterospecific sperm might physically prevent ovulation or incorrectly interact with eggs (Ting et al., 2014). Reduced female survival after a heterospecific mating has also been reported as a PMPZ isolation mechanism; presumably due to reductions in lifetime fecundity (Kao et al., 2015; Ting et al., 2014).

Sperm transfer and transport towards the site of fertilisation

A number of barriers can prevent sperm ever reaching the vicinity of the egg. Heterospecific sperm numbers may be disproportionately diluted by the external environment (Levitan, 2017) or in transit through the female reproductive tract (Cramer et al., 2016). Sperm approach towards the egg may be helped by conspecific female secretions such as ovarian fluid or egg coat proteins (Yeates et al., 2013). The transition from external fertilisation to internal fertilisation presents a host of additional PMPZ isolation barriers as sperm traverse the female reproductive tract. Similar to the effects of female derived secretions of external fertilisers, female reproductive tract fluid (Cramer et al., 2016) or ovarian fluid (Devigili et al., 2018) can favour conspecific sperm transport within the female reproductive tract towards the site of fertilisation.

Once copulation begins, abnormally short copulations between heterospecifics may result in insufficient numbers of sperm being transferred during mating (Coyne, 1993). While prolonged copulations may generate a conflict between the sexes, females may benefit from longer copulations with compatible (i.e. conspecific) males (Price et al., 2001). External fertilisers are amenable to experimental manipulation of sperm concentrations to determine heterospecific sperm fertilisation efficiency (e.g. Styan et al., 2008). Artificial insemination experiments to control for sperm numbers in internally fertilising taxa are more challenging or intractable.

Research investigating PMPZ isolation in internal fertilisers should nevertheless aim to standardise or control for sperm number to avoid confounding effects (i.e. standardise or control for copulation duration or statistically including copulation duration or sperm numbers as covariates in analyses, e.g. (Hosken et al., 2001)).

Sperm storage dynamics

If sufficient numbers of sperm are transferred during a heterospecific mating, sperm may be lost or deteriorate at an accelerated rate before reaching the sight of fertilisation. In species where females store sperm after mating, potentially for extended periods (Pitnick et al., 1999), sperm entry into, and exit from, storage may be compromised. Heterospecific sperm may be lost from storage at an accelerated rate (Ahmed-Braimah, 2016; Sagga and Civetta, 2011), suffer reduced viability in storage (Rose et al., 2014), be a 'bad fit' to the female storage organ(s) (Miller and Pitnick, 2003), never exit storage (Avila et al., 2015), or females may preferentially dump heterospecific sperm (Manier et al., 2013). The majority of research on sperm storage dynamics and speciation comes from insects, particularly Diptera and Coleoptera. Extending the study of long term sperm storage dynamics, sperm competition, and EFIs in eusocial insects (e.g. Baer et al., 2006; den Boer et al., 2010; Liberti et al., 2018) to include heterospecific crosses could prove informative in the context of PMPZ isolation. In birds, females store sperm in specialised sperm storage tubules (SSTs), more focussed study of which could provide a more general understanding of interspecific sperm storage dynamics (Birkhead and Brillard, 2007). In mammals, females may store sperm intermittently in the cervix providing the potential for reduced sperm survival (Richardson et al., 2019). Studies of mid- to long-term sperm storage in vertebrates are rare but could provide information to improve artificial insemination protocols for livestock purposes or those aimed at improving fertilisation success for endangered species.

Seminal fluid proteins

Transferred along with sperm in the ejaculate are a cocktail of seminal fluid proteins (Sfps) often providing essential reproductive functions to ensure optimal fertility (Perry et al., 2013). Even in species where males do not possess dedicated tissues for the production of seminal fluids, other secretions may be co-opted to provide a reproductive function (Borziak et al.,

2016). Genes encoding seminal fluid proteins are among the fastest evolving ever recorded, showing signatures of positive selection and rapid divergence between closely related taxa (Ahmed-Braimah et al., 2017; Bono et al., 2015; Civetta and Finn, 2014; Findlay et al., 2014; Marshall et al., 2011; Ramm et al., 2009; Vacquier and Swanson, 2011; Walters and Harrison, 2011). The advent of high-throughput "shotgun" proteomics is providing novel insights in to reproductive trait evolution and the contribution of Sfps to PMPZ isolation (McDonough et al., 2016). Proteomics studies characterising the male seminal fluid proteome and differences between populations often identify many novel peptides (Bayram et al., 2019; Civetta and Finn, 2014; Marshall et al., 2009; Rowe et al., 2018). Novel function and rapid gene turnover of genes fundamental to reproduction suggest Sfps as prime targets for future investigations of PMPZ isolation.

Although focus has shifted from the male dominated perspective of EFIs, much less research has investigated the role of the female reproductive tract secretions in PMPZ isolation. The cabbage white butterfly (*Pieris rapae*) shows rapid divergence in both proteins found in the male **spermatophore**, and female reproductive tract **proteases** which break down the spermatophore envelope, relative to non-reproductive tissue (Meslin et al., 2017). In many *Drosophila* species the insemination reaction, a swelling of the female bursa copulatrix, physically prevents oviposition after mating (Patterson, 1946). The reaction mass takes longer to subside after heterospecific matings (Kelleher and Markow, 2007). Heterospecifically mated *D. mojavensis* females show major disruption of postmating molecular interactions in the female reproductive tract (Bono et al., 2011). In *D. arizonae* there is evidence of gene duplication and positive selection of female reproductive tract digestive proteases, indicating the evolution of species-specific postmating female responses contributing to EFIs that aid in degrading the insemination reaction mass (Kelleher et al., 2007). Using heavy isotope labelling techniques to identify male and female proteins separately can provide a powerful approach to dissect postmating female responses at the molecular level (Bayram et al., 2019; Degner et al., 2019). Similarly, comparing the proteomes of virgin vs. mated male and female reproductive tracts can improve our understanding of male and female realised contributions to mating and may prove fruitful for understanding the molecular basis of PMPZ isolation (Meslin et al., 2017; Sepil et al., 2019).

Fuzzy borders

The fuzzy borders between premating and postmating interactions may in part explain why PMPZ isolation has been somewhat overlooked. While PMPZ isolation is by definition a prezygotic barrier to gene flow, the distinction between pre- or post- mating barrier effects may not always be clear. Female remating rate can bias representation of 'preferred' (i.e. conspecific) male sperm in the fertilisation set (Hook, 2017) and thus could impact CSP. Females may remate earlier if their first mate was a heterospecific or be unwilling to remate after first mating with a conspecific (Chang, 2004). Remating rate can also be influenced by copulation duration, Sfps or mate guarding (including the use of copulatory plugs) (Stockley, 1997). Copulation duration – affecting sperm transfer – may depend on premating cues (Li et al., 2012), and thus span premating and postmating prezygotic interactions. Likewise, males may invest more in sperm production in the presence of conspecific females ("sperm priming"; Aspbury and Gabor, 2004), and genitalic mismatch, a form of mechanical isolation, may reduce sperm transfer efficiency (Dopman et al., 2010; Sánchez-Guillén et al., 2012; Wojcieszek and Simmons, 2013). How premating stimuli influence later acting reproductive barriers is a largely unexplored area at the intersection between animal behaviour and reproductive physiology. The fuzzy borders surrounding PMPZ isolation during the reproductive cycle may also have implications for the role of PMPZ isolation in reinforcement **(BOX 3)**.

BOX 3 - PMPZ isolation and reinforcement

PMPZ isolation could emerge as the result of the co-evolution of reproductive traits within populations or from natural selection against costly hybridisation (reinforcement) (Butlin and Smadja, 2017; Servedio and Noor, 2003). The role of PMPZ isolation in reinforcement is not entirely clear or necessarily predictable. Reinforcement might target premating isolation and weaken selection on the evolution of PMPZ isolation if heterospecific encounter rates are high and/or in areas of historical sympatry (Poikela et al., 2019). Conversely, reinforcement might target PMPZ isolation if gametes from multiple species frequently intermix (Riffell et al., 2004). Conspecific sperm precedence can evolve via an assortative fertilisation mechanism to offset the costs of heterospecific matings. For instance, if conspecific mating opportunities are rare, due to low population densities or frequent interspecific interactions, females may employ a bet hedging strategy and mate with poor quality males (i.e. heterospecifics) and later 'trade-up'

(Kokko and Mappes, 2005) – relying on PMPZ mechanisms (i.e. CSP) to restore fertility. Conspecific sperm precedence can also slow reinforcement. If females remate frequently the costs of heterospecific mating are reduced, thus selection for assortative mating will be reduced (Lorch and Servedio, 2007; Marshall et al., 2002).

The role of PMPZ isolation in reinforcement is also possibly paradoxical, as PMPZ isolation holds a unique role; straddling the traditional pre- vs. post- zygotic border, depending on when PMPZ isolation acts during the reproductive cycle (e.g. before or after energy is expended on gametes). PMPZ isolation as the selective force strengthening (premating) prezygotic isolation has received relatively little theoretical consideration (but see Servedio, 2001). Reduced fertilisation success, where females expend energy towards unfertilised eggs, essentially producing inviable hybrid embryos, will have similar costs to postzygotic isolation and so could be the selective force driving the evolution of prezygotic (including other PMPZ) isolation. Therefore, PMPZ isolation has the potential to act as both the agent, and target, of reinforcement; an idea that needs to be explored in more detail to see if it is theoretically plausible and occurs in nature.

Empirical evidence for a link between PMPZ isolation and reinforcement comes mostly from *Drosophila*. PMPZ isolation is greater within the hybrid zone between *D. yakuba* and *D. santomea* on the island of São Tomé (Chang, 2004; Matute, 2010a). Matute (2010b) also showed low levels of hybridisation and migration and strong selection against hybrids resulted in stronger (i.e. reinforcement of) premating- and PMPZ- isolation within only a few generations of experimental evolution. Conspecific sperm precedence is also stronger in areas of sympatry between *D. pseudoobscura* and *D. persimillis* (Castillo and Moyle, 2019), however, remating rate did not show evidence of reinforcement in *D. pseudoobscura* (Davis et al., 2017) and bindin evolution was not driven by reinforcement in *Arabacia* sea urchins (Lessios et al., 2012).

END BOX

(A)biotic factors influencing the occurrence of PMPZ isolation

The types of isolation mechanisms that evolve between populations will depend on the biology and ecology of the taxa involved, including the number and types of interspecific interactions

(McDonald et al., 2019; Rivera et al., 2004), and fundamental aspects of a species reproductive biology. For species in which gametes from multiple species overlap, notably broadcast spawners and internally fertilising species that frequently cooccur at mating sites, selection against heterospecific fertilisation might be particularly strong. EFIs influencing the outcome of postcopulatory sexual selection in favour of conspecifics will be increased for polyandrous species with internal fertilisation. The opportunity for PMPZ isolation to evolve relating to sperm viability/storage will be increased where females store sperm for prolonged periods. PMPZ isolation will also be important for external fertilisers where other barriers (i.e. sexual isolation) aside from temporal or spatial isolation are absent (Fogarty et al., 2012; Klibansky and McCartney, 2014).

An emerging pattern from the sexual selection literature is that the strength of pre- and post-copulatory sexual selection can interact (Evans and Garcia-Gonzalez, 2016; McDonald and Pizzari, 2018; Simmons et al., 2017). At present, theory is sparse regarding how the interaction between pre- and post-copulatory sexual selection might impact the evolution of premating and PMPZ isolation and their interaction. In *Heliconius* butterflies there is little evidence of PMPZ isolation (Mérot et al., 2017), despite sexual selection and sexual conflict acting on postmating phenotypes within species (Sánchez and Cordero, 2014; Walters and Harrison, 2011). Strong selection against hybrids (Darragh et al., 2017; Merrill et al., 2019, 2012), suggests that strong premating isolation may preclude the evolution of postmating prezygotic isolation. A better understanding of the interplay between different episodes of sexual selection acting within populations, taking in to account local ecological conditions and (a)biotic and interspecific interactions will help inform our understanding of the evolution of PMPZ isolation.

Concluding remarks

The PMPZ barriers described above need not work independently. Several barriers may interact or act concurrently, producing an overall stronger barrier to gene flow (Butlin and Smadja, 2017). Few studies have investigated multiple barriers to gene flow simultaneously (Jennings et al., 2014; Poikela et al., 2019; Rose et al., 2014). This is necessary to understand the individual contribution of different barrier effects to total reproductive isolation (Butlin and Smadja, 2017; Sobel and Chen, 2014). Still fewer studies have tried to tackle the daunting

prospect of assessing the contribution of premating, PMPZ, and postzygotic reproductive barriers across a complete phylogeny. The single example of Turissini et al. (2018) provides a unique insight in to the evolution of PMPZ isolation, where they found that barriers to gene flow that act earlier during reproduction (i.e. premating) evolve faster than later acting barriers (i.e. postmating prezygotic, or postzygotic) across the *D. melanogaster* clade, demonstrating the power of a phylogenetically informed approach.

The study of taxa amenable to laboratory experiments (notably insects and marine invertebrates) has provided insights into the types of PMPZ isolation barriers that can emerge between taxa, indeed in some cases providing the only examples of PMPZ isolation. However, to understand the role of PMPZ isolation in nature it may be necessary to move the laboratory in to the field (Cramer et al., 2016), collecting field estimates of interspecific fertilisation success (Demont et al., 2011), and exploring the interaction between natural and sexual selection in different populations under varying (a)biotic conditions (Perry et al., 2017). A better understanding of the eco-evolutionary feedback between individuals and their environments (Svensson, 2018) contributing to the evolution of postmating prezygotic isolation will improve our understanding of the forces contributing to the evolution of reproductive isolation in nature.

The study of PMPZ isolation remains in its infancy. Available empirical and theoretical evidence support a role of PMPZ isolation early during the evolution of reproductive isolation. Thus, greater consideration of PMPZ isolation across taxa, especially where it has potentially been misclassified, overlooked or underestimated, will improve our knowledge of the early stages of the speciation process. Studying birds and mammals poses a significant challenge in studying PMPZ isolation due to difficulties in determining fertilisation success. Looking beyond speciation research, a better understanding of the traits and genes or loci underlying PMPZ isolation will benefit our understanding of animal fertility more generally. The studying of PMPZ isolation will shed light on the mechanisms and evolutionary processes affecting animal fertility which could help in providing targets to help couples with fertility issues and conservation efforts of endangered species or provide targets for insect biocontrol.

2. Persistent postmating, prezygotic reproductive isolation between populations

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Authorship contributions

MDG collected the data and performed analyses. MDG and RRS designed the experiments and wrote the manuscript.

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Data Accessibility Statement

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ABSTRACT

Studying reproductive barriers between populations of the same species is critical to understand how speciation may proceed. Growing evidence suggests postmating, prezygotic (PMPZ) reproductive barriers play an important role in the evolution of early taxonomic divergence. However, the contribution of PMPZ isolation to speciation is typically studied between species in which barriers that maintain isolation may not be those that contributed to reduced gene flow between populations. Moreover, in internally fertilizing animals, PMPZ isolation is related to male ejaculate – female reproductive tract incompatibilities but few studies have examined how mating history of the sexes can affect the strength of PMPZ isolation and the extent to which PMPZ isolation is repeatable or restricted to particular interacting genotypes. We addressed these outstanding questions using multiple populations of *Drosophila montana*. We show a recurrent pattern of PMPZ isolation, with flies from one population exhibiting reproductive incompatibility in crosses with all three other populations, while those three populations were fully fertile with each other. Reproductive incompatibility is due to lack of fertilization and is asymmetrical, affecting female fitness more than males. There was no effect of male or female mating history on reproductive incompatibility, indicating that PMPZ isolation persists between populations. We found no evidence of variability in fertilization outcomes attributable to different female x male genotype interactions, and in combination with our other results, suggests that PMPZ isolation is not driven by idiosyncratic genotype x genotype interactions. Our results show PMPZ isolation as a strong, consistent barrier to gene flow early during speciation and suggest several targets of selection known to affect ejaculate-female reproductive tract interactions within species that may cause this PMPZ isolation.

KEYWORDS: Speciation, postmating prezygotic isolation, gametic isolation, sexual selection, sexual conflict, *Drosophila montana*.

INTRODUCTION

Speciation requires the accumulation of barriers to gene flow between populations and subsequent taxa. Identifying the barriers that act early during the evolution of reproductive isolation is critical to determine how speciation proceeds (Turelli et al. 2001; Coyne and Orr 2004; Butlin et al. 2012). Reproductive barriers to gene flow can broadly be classified in to three categories. Premating reproductive barriers reduce the incidence of hybridization events between taxa (Murray and Clarke 1980; Hoskin et al. 2005; Dopman et al. 2010; Lackey and Boughman 2017), while postzygotic reproductive barriers are those that result in reduced fitness of hybrid offspring, either due to intrinsic genetic defects (i.e. sterility or inviability), or low fitness in either of the parental habitats (Wu and Ting 2004; Presgraves 2010; Cooper et al. 2017). The third class of reproductive barriers are postmating, prezygotic (PMPZ) reproductive barriers – incompatibilities relating to interactions between the sexes that act after copulation, but before karyogamy – preventing successful fertilization between populations or taxa. Both premating and postzygotic reproductive barriers to gene flow have been studied extensively, however only relatively recently have PMPZ reproductive barriers begun to be considered in more detail as potentially important reproductive barriers.

The fast-paced molecular evolution of reproductive tract tissues within populations, accelerated by sexual selection and sexual conflict, is predicted to result in rapid divergence between populations in allopatry and the emergence of PMPZ reproductive incompatibilities between populations early during reproductive isolation (Eady 2001; Panhuis et al. 2001). In polyandrous mating systems with internal fertilization, where females mate multiply within a single reproductive cycle, the ejaculates of multiple males may overlap within the female reproductive tract. Different males' ejaculates must then compete to fertilize ova (sperm competition), and females retaining sperm from multiple males may bias paternity (cryptic female choice). Such postcopulatory sexual selection, and its attendant sexual conflict within populations (Andersson 1994; Gavrilets 2000; Arnqvist and Rowe 2002; Andersson and Simmons 2006), can shape the evolution of inter-sexual interactions during copulation and fertilization (Birkhead and Pizzari 2002; Bernasconi et al. 2004; Firman et al. 2017). Rapid evolution of such phenotypes is supported by evidence that genes encoding reproductive tract

proteins are among the fastest evolving, showing rapid protein sequence and gene expression evolution (Swanson and Vacquier 2002; Hollis et al. 2014; Perry et al. 2016; Veltsos et al. 2017).

PMPZ isolation in external fertilizers is mostly limited to incompatibilities relating to chemo-attraction between gametes (Weber et al. 2017) and/or gamete interactions at the cell surface (Vacquier and Swanson 2011). For internal fertilizers, an additional array of potential PMPZ reproductive barriers can act as a result of the complex series of events that take place within the female reproductive tract after mating (Bloch Qazi et al. 2003; Schnakenberg et al. 2012; Orr and Brennan 2015). In single hetero-specific matings, successful fertilization can be decreased or prevented by reduced sperm transfer by males, and/or reduced transport, storage, and viability of hetero-specific sperm in females (Reinhardt 2006; Kelleher and Markow 2007; Larson et al. 2012; Manier et al. 2013; Rose et al. 2014; Ahmed-Braimah 2016; Kohyama et al. 2016). PMPZ isolation has also been suggested to occur when hetero-specific matings result in reduced egg production compared to con-specific matings, even though fertilization is successful (e.g. Matute and Coyne 2010; Turissini et al. 2018). PMPZ isolation in internally fertilizing animals may also be manifested only when con- and hetero-specific ejaculates are in competition. For instance, conspecific sperm precedence occurs when paternity is biased to sperm from the conspecific male even though hetero-specific male sperm may fertilize ova in single matings (Price 1997; Yeates et al. 2013; Castillo and Moyle 2014; Cramer et al. 2016).

A growing body of literature now shows PMPZ isolation is the primary or only barrier to gene flow in some closely related taxa, suggesting an important role in the early evolution of reproductive isolation (Dean and Nachman 2009; Bono et al. 2015; Cramer et al. 2016; Soudi et al. 2016; Ahmed-Braimah et al. 2017; Turissini et al. 2018). However, the majority of research has focussed on incompatibilities arising between species even though barriers that maintain reproductive isolation after divergence may not be the same barriers that were important in reducing gene flow during the initial stages of the speciation process (Turelli et al. 2001; Coyne and Orr 2004; Butlin et al. 2012). Therefore, to understand the factors important during the initial stages of divergence more focus is needed on the reproductive barriers acting between recently diverged populations of the same species.

Drosophila montana provides an opportunity to study the role of PMPZ isolation and the early stages of the speciation process. This species is distributed across the northern hemisphere at high altitudes and latitudes with a well-documented ecology and phylogeographic history (Aspi et al. 1993; Mirol et al. 2007). Investigating the contribution of both pre- and post- mating reproductive barriers between three *D. montana* populations, two from North America and one from Europe, found hybrid crosses between populations exhibited PMPZ isolation (Jennings et al. 2014). PMPZ isolation was a consequence of sperm failing to penetrate eggs, even though motile sperm were transferred by males and stored by females (Jennings et al. 2014). These populations also exhibited premating isolation which increased with genetic distance, suggesting isolation by distance, however, there was no clear relationship between genetic distance and the strength of PMPZ isolation. While premating reproductive barriers to gene flow are undoubtedly important in these populations, strong PMPZ isolation that is not associated with isolation by distance suggests PMPZ isolation may be especially important early during the evolution of reproductive isolation.

Yet, there remains several open questions about the evolution of PMPZ isolation, both specifically for this system and generally. Are patterns of PMPZ isolation unique to these populations or more widespread? Are PMPZ isolation patterns repeatable with individuals tested from the same location but collected at different times? Do all individuals show similar strengths of PMPZ isolation or is PMPZ isolation idiosyncratic between some individuals? Additionally, male and female mating history may also influence the expression and strength of PMPZ isolation with consequences for the importance of PMPZ isolation in limiting gene flow between populations. Mating history is known to have both ameliorating and exacerbating effects on other types of reproductive incompatibility, such as cytoplasmic incompatibility between *Wolbachia*-infected *D. simulans* males and uninfected females which is ameliorated if males have remated frequently (Karr et al. 1998; Awrahman et al. 2014). In contrast, receipt of multiple foreign ejaculates by females may amplify infertility due to receipt of toxic foreign ejaculates (Knowles and Markow 2001; Kelleher and Markow 2007). Do male and female mating history influence the strength of PMPZ isolation?

We addressed these outstanding questions about the evolution of PMPZ isolation by testing both recent collections from the same North American locations as previously described and additional new populations. We also assessed whether PMPZ isolation is acting at the population level or only between specific genotype x genotype interactions from different populations. Furthermore, we determined whether the presence and strength PMPZ isolation is affected by intrinsic infertility or male and female mating history.

METHODS

Fly stocks

Adult *D. montana* were collected from riparian habitats using malt bait buckets and mouth aspirators from Ashford, Washington, USA, in 2013 (referred to as Ashford, and abbreviated as A); Crested Butte, Colorado, USA in 2009 and 2013 (referred to as Colorado, abbreviated as C); Jackson, Wyoming, USA in 2013 (referred to as Jackson; abbreviated as J); and Vancouver, British Columbia, Canada (referred to as Vancouver; abbreviated as V) in 2008 and 2014 (Fig. 2.1, Table S2.1). Both iso-female lines and population cages were tested for PMPZ isolation. Population cages for Colorado (2013) and Vancouver (2008) were established by combining 20 F3 progeny of each sex from each of 20 iso-female lines. The population cage for Vancouver (2014) was established in the same way except F4 progeny from 21 iso-female lines were merged. All populations and iso-female lines were cultured in the laboratory on Lakovaara malt medium (Lakovaara 1969) in overlapping generations at 19^{o}C in constant light (Jennings et al. 2014). Flies used for experimentation were collected within three days of eclosion, as male reproductive maturity does not occur until at least 8 days post-eclosion (Pitnick et al. 1995). All experiments were carried out using flies aged between 21-28 days from eclosion. In each experiment, we carried out all four possible crosses between the two focal populations being tested, where the female population is always indicated first (e.g. AA is a cross between Ashford females and Ashford males and AC is a cross between Ashford females and Colorado males).

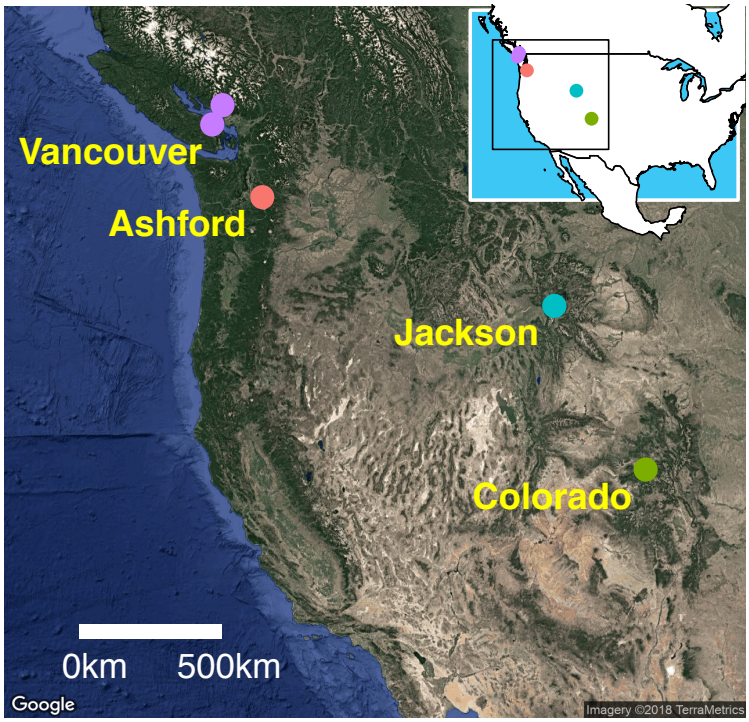

Figure 2.1. Collection locations of *Drosophila montana* populations. Maps created using the 'ggmap' package in R (Kahle and Wickham 2013).

Statistical analysis

We outline specific statistical tests for each experiment and trait we analyse at the end of each section (described below). All statistical analyses were performed in R (version 3.3.0) (R Core Team 2016). Generalised linear mixed effects models (GLMMs) and parametric bootstrap simulations to obtain model predicted values (± 95% confidence intervals) were fitted using the 'lme4' package (Bates et al. 2015). We tested for significance of fixed effects and interactions via likelihood ratio tests (LRT), or parametric bootstrapped simulations using the PBmodcomp function from the 'pbkrtest' package (Halekoh and Højsgaard 2014). When necessary, we performed post-hoc Tukey's honest significant difference (HSD) tests using the glht function from the 'multcomp' package (Hothorn et al., 2008).

Postmating, prezygotic isolation between North American populations of D. montana.

To test the pattern of PMPZ isolation previously reported (Jennings et al. 2014) with a new Colorado population and to identify other populations showing evidence of PMPZ isolation, we performed a series of crosses between Colorado and Vancouver, and two previously untested populations - Ashford and Jackson. PMPZ isolation is measured by egg hatch success (number

of eggs oviposited that hatched) and/or number of progeny produced. For each pair of focal populations, we performed fully factorial experiments, generating data from both parental crosses and the two reciprocal between-population crosses. We refer to the four crosses within each pair-wise comparison as the cross-type. Final sample sizes and details of the specific strain x strain cross-types performed in each of the pair-wise combinations between the four populations, and a summary of PMPZ outcomes are presented in Fig. S2.1 and Fig. S2.3.

For each cross, we assessed PMPZ isolation by mating single virgin males and females (n = 30 per cross-type per block). Note all crosses were observed for mating over a four-hour period to exclude confounding sources of reproductive isolation. If mating did not occur within this timeframe, then we discarded that pair. If mating occurred, then the pair was mouth-aspirated into a chamber of an oviposition "manifold" after mating (Jennings et al. 2014). Manifolds were connected to oviposition plates containing a molasses-agar egg laying medium with a drop of dried yeast paste added and incubated at 19^{o}C. Females were left to oviposit for two days, before changing the oviposition plate, and allowing a further two days of oviposition. Following the second two-day oviposition period, flies were discarded, the numbers of eggs laid were counted (fecundity), and the oviposition plate returned to the incubator. Two days later, the numbers of unhatched eggs on the second oviposition plate were counted again. Females that did not oviposit were excluded from analyses.

To assess differences between cross-types in fecundity, we fitted GLMMs with Poisson errors and a log link, using the total number of eggs laid as the response variable. To assess differences between cross-types in hatching success rates, we fitted GLMMs with binomial errors and a logit link, using the numbers of hatched eggs ("successes") and unhatched eggs ("failures") as the response variable. All models included cross-type as the only fixed effect, and we performed analyses on each of the 6 crosses separately. We included random effects for the specific strains tested from within each population, and for experimental block, to account for variation between strains tested in each cross-type, and variation between blocks testing each cross between populations, respectively. All models also included an observation level random effect (OLRE) to account for overdispersion (Harrison 2014, 2015). To test whether there was a significant effect of cross-type on each measure of PMPZ isolation, we compared each model to

a null model including the global intercept (~1) as the only fixed effect (but with the same random effects structure), with 10,000 parametric bootstrapped simulations.

Postmating, prezygotic isolation mechanism

Previous work showed that PMPZ isolation was manifested by the lack of fertilization, despite males transferring and females storing motile sperm (Jennings et al. 2014). To confirm that egg hatching failure was due to lack of fertilization in additional crosses and populations, eggs from a subset of crosses were scored for development following the same protocol as previously used (Jennings et al. 2014). Briefly, for each of the four cross-types in the Colorado 2013-Jackson 2013 and Colorado 2013-Vancouver 2008 cross (Table S2.1), we mouth aspirated flies (30-40 of each sex) in to half-pint bottles covered with an oviposition plate, containing molasses-agar egg laying medium with a drop of dried yeast paste added. Oviposition plates were replaced every 24 hours, and eggs were collected en masse, fixed and stained using DAPI. Eggs were inspected using fluorescence microscopy to score for development. Non-developing eggs were further inspected using differential interference contrast (DIC) microscopy to score eggs for presence or absence of sperm in the egg, indicating whether fertilization had been successful. We tested for differences in the numbers of fertilized eggs in each cross-type using Pearson's Chi-squared test. Note that fertilization failure cannot be due to cytoplasmic incompatibility as a consequence of Wolbachia infection in our stocks because we found no visual evidence of Wolbachia (Stouthamer et al. 1999) and previous analyses investigating Wolbachia prevalence across the Drosophila phylogeny found no molecular evidence of Wolbachia in the virilis group (Bourtzis et al. 1996; Mateos et al. 2006).

Testing intrinsic male infertility

To assess whether low fertilization success in between-population crosses could be confounded by poor male fertility irrespective of the identity of his mate, we mated focal males to both a between- and within-population female. For this experiment we used flies from the Colorado 2013 and Vancouver 2008 population cages (Table S2.1). Focal males were paired individually with two virgin females on consecutive days, one within- and one between- population female. To account for any mating order effects, we randomly assigned half of males (n = 20 per cross-type) to have a between-population female as the first mate, and the other half of males, a

within-population female as the first mate. All matings were observed; if mating did not occur, pairs were discarded. Mated females were mouth aspirated singly to a manifold chamber after mating and data collected for hatching success, as described above. Males were transferred to new vials containing malt medium and mated the next day with the other female. Second females were mouth aspirated singly to a manifold chamber after mating and data collected for hatching success, as described above. To test if males with low fertilization success in between-population crosses also had low within-population fertilization success we calculated Spearman's rank correlation coefficient for the proportion of eggs hatching between males' first and second mating, for each set of males separately (i.e. Colorado males having a between-population partner first or second, Vancouver males having a between-population partner first or second).

Consistency of postmating, prezygotic isolation across different genotypes

Reproductive incompatibility could be the result of idiosyncratic genotype x genotype interactions between males and females from different populations, rather than a population level effect. To assess whether individual specific female genotype x male genotype interactions yielded variable fertilization outcomes, we used matings within and between –individuals from the Colorado 2013 and Vancouver 2008 population cages (Table S2.1). Focal males (n = 10 per cross-type) were paired individually with a virgin female and monitored for mating. Mated females were mouth aspirated singly to a manifold chamber after mating, while males were transferred to new vials containing malt medium. The next day, focal males were presented with another virgin female from the same population as on the previous day. Mated females were mouth aspirated singly to a manifold chamber after mating. We repeated this for 5 consecutive days. Mated females were processed for egg hatch success as previously described. To assess the between individual variance in hatching success, for those males that mated three or more times, we fitted a GLMM with binomial errors and a logit link, using egg hatch success (i.e. counts of hatched and unhatched eggs) as the response variable and mating day as the only fixed effect. We fitted a model for each cross-type separately, as combining groups across the different cross-types would artificially inflate the between-group variance. Models included a random effect for male identify and an OLRE.

Effects of male multiple mating on postmating, prezygotic isolation

To assess the effect of multiple mating on male fertilization success, we used the data collected from "Consistency of postmating, prezygotic isolation across different genotypes" above, and fitted a GLMM with binomial errors and a logit link, using egg hatch success (i.e. counts of hatched and unhatched eggs) as the response variable, with cross-type, mating number, and the cross-type x mating number interaction as fixed effects and male identity as a random effect, and an OLRE.

Effects of female multiple mating on postmating, prezygotic isolation

To test whether multiple insemination affected the strength of PMPZ isolation we mated focal females to multiple males. For each of the four cross-type combinations between the Colorado 2013 and Vancouver 2008 population cages (Table S2.1), focal females (n = 15 per cross-type) were paired individually with a virgin male. Males were discarded immediately after mating. The next day, focal females were mouth aspirated into a new vial housing a virgin male from the same population as on the previous day. We repeated this for 5 consecutive days. Only females who mated on three or more consecutive days were kept for analysis. All progeny eclosing from each oviposition vial were subsequently counted and sexed. To test the effect of multiple insemination on the total strength of PMPZ isolation, we fitted a GLMM with Poisson errors, using the total number of progeny eclosed as the response variable, with cross-type, mating number, and the cross-type x mating number interaction as fixed effects, and a random effect for female identity, and an OLRE.

RESULTS

Postmating, prezygotic isolation between North American populations of D. montana.

We performed a series of pair-wise fully factorial crosses between four *D. montana* populations from across North America to identify populations showing evidence of PMPZ isolation. Previous studies have included reduced female fecundity following mating with a foreign male as a PMPZ reproductive barrier (Matute 2010; Matute and Coyne 2010; Turissini et al. 2018). Here we found a significant effect of cross-type on female fecundity in three (Ashford-Jackson, Ashford-Vancouver and Jackson-Vancouver crosses) of the six pair-wise population crosses

(Table 1). However, these responses were asymmetric and not in the predicted direction if PMPZ isolation was acting (Fig. S2.1 & S2.2). In Ashford-Jackson crosses, one between-population cross had greater fecundity than both the reciprocal cross and one of the parental crosses; Jackson males elevated Ashford female fecundity above that of the reciprocal cross (AJ vs. JA; Tukey's HSD; $p = 0.013$; Table S2.4) and the within-population Ashford cross (AJ vs. AA; Tukey's HSD, $p = 0.019$; Table S2.4). The same pattern was found for the Ashford-Vancouver cross; Ashford males elevated Vancouver female fecundity above the reciprocal cross (VA vs. AV; Tukey's HSD, $p = 0.026$; Table S2.5) and the within-population Vancouver cross (VA vs. VV; Tukey's HSD, $p = 0.003$; Table S2.5). In the Jackson-Vancouver cross, between-population crosses differed from each other, but not from either within-population cross (JV had lower fecundity than VJ (Tukey's HSD; $p = 0.007$; Table S2.8) but these did not differ from parental crosses). Moreover, these pair-wise population comparisons showed no effect of cross-type on hatching success (all $P > 0.06$, Table 2.1; Fig. S2.3 & S2.4). Thus, there is no evidence of PMPZ isolation between these three populations.

The three pair-wise population comparisons involving the Colorado population showed no difference in fecundity between cross-types (Table 2.1). However, there was a significant effect of cross-type on hatching success (Fig. 2.2; Table 2.1). Hatching success was high and similar ($\geq$75%) for within-population crosses (Tukey's HSD; all $P > 0.5$; Table S2.4, S2.6 & S2.7), whereas the reciprocal between-population crosses were all significantly different from both within-population crosses, and from each other (Fig. 2.2; Tukey's HSD; all $P < 0.003$; Table S2.4, S2.6 & S2.7). Colorado females mated to a foreign male had less than 20% hatching success and, in the reciprocal crosses, foreign females mated to Colorado males had ~50% hatching success (Fig. 2.2). In summary, crosses that involved flies from Colorado exhibited asymmetrical PMPZ isolation with all three other populations tested. In contrast, the crosses between pairs of those three populations showed no evidence of PMPZ isolation (Fig. S2.3 & S2.4).

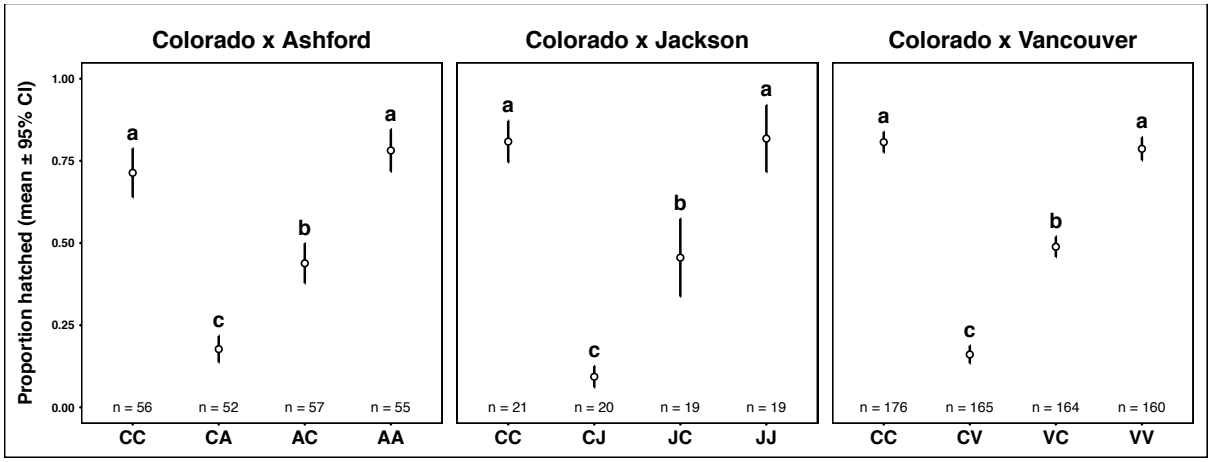

Figure 2.2. Proportion of eggs hatching (mean ± 95% confidence intervals) in crosses involving Colorado. Within each panel different letters indicate significant differences from post-hoc Tukey's HSD. Letters are recycled in each panel, however, supplementary analyses showed that letters shared across panels also represent statistically equivalent groups (see results). Cross-types are abbreviated with the female population given first. A, Ashford; C, Colorado; J, Jackson; V, Vancouver. N = number of mating pairs over all experimental blocks. N.B. Crosses not showing PMPZ isolation are shown in Fig. S2.4.

Table 2.1. Measures of postmating, prezygotic isolation (fecundity and hatching success) between North American populations of *D. montana*. P-values obtained from 10,000 parametric bootstrap simulations, comparing the model including cross-type as the only fixed effect against the null (intercept only) model. Cross lists the two populations being fully reciprocally crossed (e.g. A x C = AA, AC, CA, CC where A, Ashford; C, Colorado; J, Jackson; V, Vancouver; the population of the female is listed first). Because each cross contained all four cross types for each measure of PMPZ, df = 3 for all models. Number in parentheses after the cross is the number of replicate blocks. Total sample sizes for each cross provided in Figure 2.2.

	Cross											
	A x C (3)		A x J (1)		A x V (3)		C x J (1)		C x V (7)		J x V (1)	
Measure of PMPZ	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P	X^2	P
Fecundity	2.96	0.590	**11.56**	**0.015**	**10.14**	**0.050**	3.26	0.366	7.31	0.144	**10.20**	**0.023**
Hatch success	**21.74**	**0.005**	1.06	0.802	2.52	0.690	**107.08**	**< 0.001**	**39.571**	**< 0.001**	7.76	0.062

To determine whether the strength of PMPZ isolation with the Colorado population depended on the non-Colorado population, we tested whether there was a significant difference in hatching success by pooling data across all experimental blocks for all between-population crosses involving only Colorado females or Colorado males (i.e. incompatible crosses). Colorado females showed equally low hatching success, regardless of the origin of the between-population male (LRT = 0.99, df = 2, p = 0.610). Likewise, Colorado males showed equivalently low hatching success, regardless of the origin of the between-population female (LRT = 1.99, df = 2, p = 0.372). To determine whether egg hatch success varied between compatible crosses, we pooled data across all experimental blocks but excluded all between-population crosses involving both Colorado males and Colorado females. Compatible crosses showed high hatching success that did not differ between crosses (LRT = 7.95, df = 9, p = 0.539). In summary, the strength of PMPZ isolation involving Colorado was equal across all populations, regardless of the population origin of the foreign mating partner (see legend in Fig. 2.2) whereas all other between-population crosses had hatching success equivalent to within-population success (Fig. S2.4).

Postmating, prezygotic isolation mechanism

After surveying all populations for evidence of PMPZ isolation, we scored oviposited eggs for development and fertilization status in the Colorado 2013-Vancouver 2008 cross to confirm low hatching rates were due to the same pattern of fertilization failure previously reported (Jennings et al. 2014). We also scored eggs from the Colorado 2013-Jackson 2013 cross to confirm whether this was a consistent PMPZ isolating mechanism. We found a significant effect of cross-type on the number of eggs fertilized in the Colorado-Vancouver ($\mathbf{X^2}$ = 766.55, df = 3, $p < 0.001$) and in the Colorado-Jackson ($\mathbf{X^2}$ = 160.56, df = 3, $p < 0.001$) crosses. While most eggs were developing in all within-population crosses, eggs oviposited by Colorado females mated to foreign males had less than 25% of eggs fertilized, and foreign females mated to Colorado males had less than 50% of eggs fertilized (Fig. 2.3).

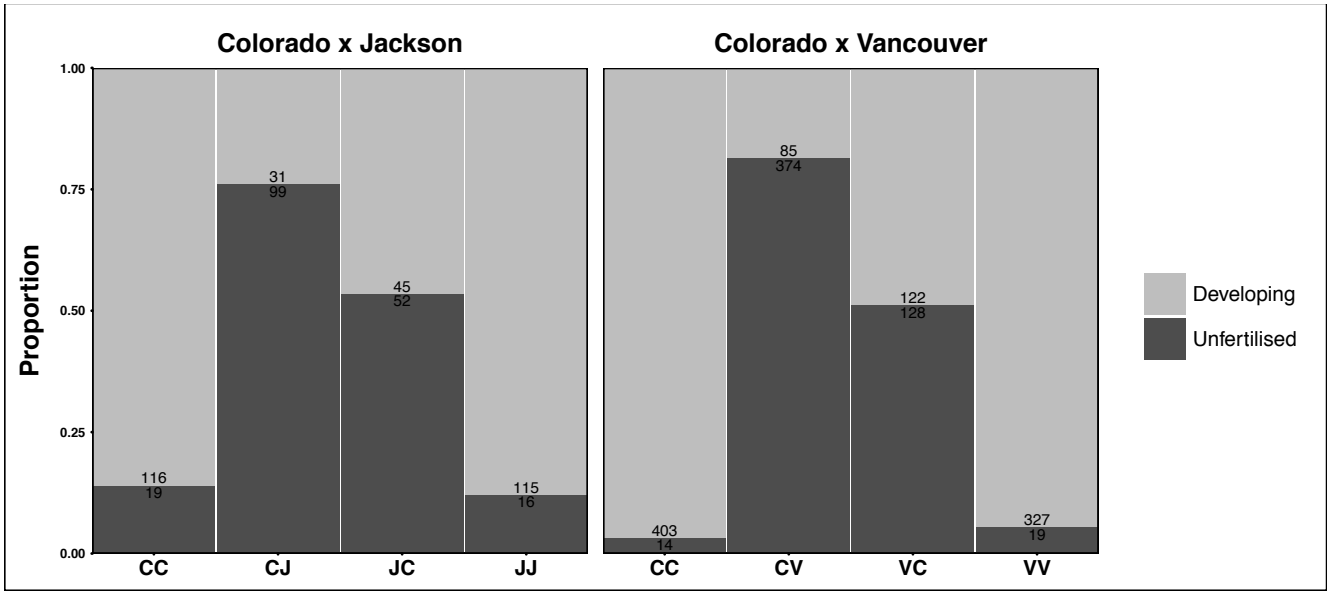

Figure 2.3. Proportion of developing (light grey) and unfertilized (dark grey) eggs in each cross-type. Cross-types are abbreviated with the female population given first. C, Colorado; J, Jackson; V, Vancouver. Numbers in bars indicate the total number of eggs counted.

Testing intrinsic male infertility

To test whether reduced fertilization success could be due to intrinsic male infertility, we calculated Spearman's rank correlation coefficient for the proportion of eggs that hatched for males mated to both a within- and between-population female (Table 2.2, Fig. S2.5). There was no correlation in the level of fertility between the first and second mating, regardless of mating order (Spearman's rank correlation, all $P > 0.48$, Table 2.2). Therefore, hatching success rates can be attributed to the cross-type alone, and are not confounded by male infertility.

Table 2.2. Spearman's rank correlation coefficients calculated for the proportion of eggs hatching for males mated to virgin within- and between-population females.

Male population	Female population		N	Spearman's rho	P
	First mating	Second mating			
Colorado	Colorado	Vancouver	20	-0.032	0.896
	Vancouver	Colorado	18	0.176	0.482
Vancouver	Vancouver	Colorado	18	0.003	0.990
	Colorado	Vancouver	19	0.093	0.713

Consistency of postmating, prezygotic isolation across different genotypes

To test whether PMPZ isolation was due to either specific female genotype x male genotype interactions or a population-level phenomenon, in the Colorado 2013 – Vancouver 2008 population cage cross (Table S2.1), we assessed the between individual variance in hatching success for males that mated at least three times over consecutive days (most males mated the maximum of 5 times; median number of mates = 5). Half of males were mated to virgin females from their own population, and the other half of males were mated to foreign females, and we modelled each cross-type separately to properly partition between-group variance. In all cases, estimates of between individual variance (male identity random effect variance) were 0 signifying inclusion of the male identity random effect was not warranted in the models, and models including male identity as a random effect had higher AICc scores than those without (Table S2.9). Thus, between male variance in hatching success was negligible, indicating a consistent pattern of PMPZ isolation acting across a range of female x male genotype interactions between populations.

Effects of male and female multiple mating on postmating, prezygotic isolation

To test whether male multiple mating affected the strength of PMPZ isolation, in the Colorado 2013 – Vancouver 2008 population cage cross (Table S2.1), we assessed the effect of multiple mating of males on fertilization success (Fig. 2.4). There was a significant effect of cross-type (LRT = 106.08, df = 3, $p < 0.001$; incompatible crosses had low egg hatch success) and a

marginally significant effect of mating number on egg hatch success (LRT = 9.323, df = 4, p = 0.054) suggesting that males improve fertilization success as they mate more. The cross-type x mating number interaction was not significant (LRT = 2.29, df = 3, p = 0.514). Thus, there was no effect of male mating history on the strength of PMPZ isolation.

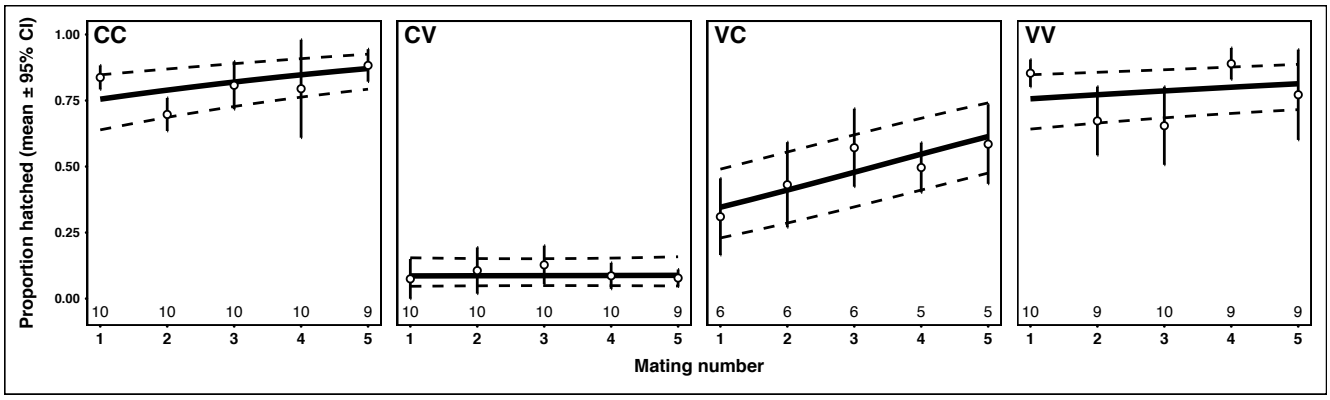

Figure 2.4. Proportion of eggs hatching (mean and model predicted values ± 95% CI) per day for males mated to between three and five within- or between- population females over consecutive days. Cross-types are abbreviated with the female population given first. A, Ashford; C, Colorado; J, Jackson; V, Vancouver. Numbers below points indicate sample sizes (number of mating pairs each day).

We also tested the effect of females receiving multiple ejaculates on the strength of PMPZ isolation, by counting the total number of adult progeny produced each day by females inseminated by up to 5 males. As with males, almost every female mated every day (median number of mates = 5). Like males, we found a significant effect of cross-type (LRT = 58.57, df = 6, $p < 0.001$; incompatible crosses had low fertility). Unlike males, we saw a strong effect of mating number of progeny production per day (LRT = 24.36, df = 4, $p < 0.001$); the rate of progeny production increased with mating number similarly in all four crosses (Fig. 2.5). However, we still found no effect of the cross-type x mating number interaction (LRT = 2.71, df = 3, p = 0.438), thus there was no effect of female mating history on the strength of PMPZ isolation.

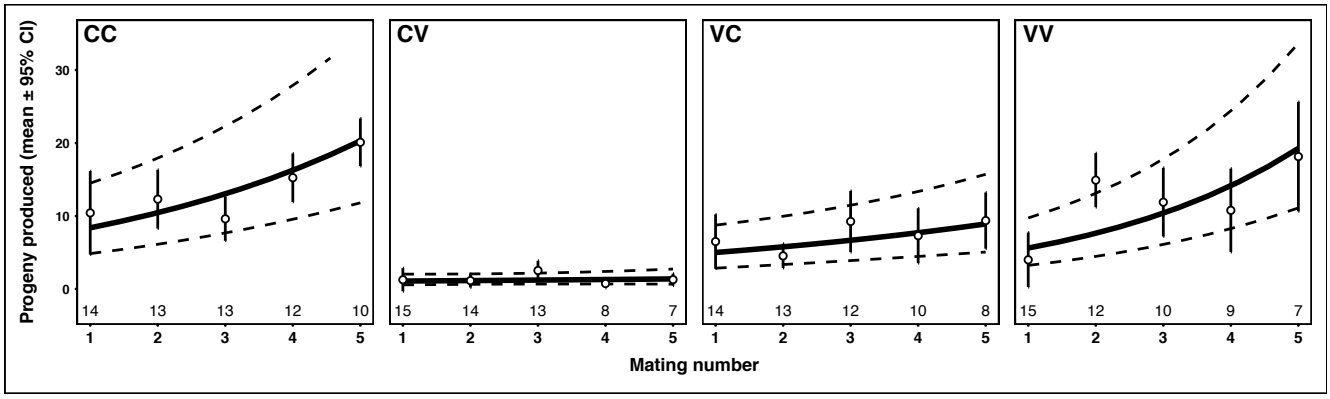

Figure 2.5. Per-day progeny production (mean and model predicted values ± 95% CI) for females mated to multiple within- or between- population males over consecutive days. Cross-types are abbreviated with the female population given first. A, Ashford; C, Colorado; J, Jackson; V, Vancouver. Numbers below points indicate sample sizes (number of mating pairs each day).

DISCUSSION

Identifying early acting reproductive barriers is central to understanding the factors that contribute to the initial stages of the speciation process. While recent efforts have increasingly identified PMPZ isolation as critical in these early stages (Soudi et al. 2016; Devigili et al. 2018; Turissini et al. 2018), outstanding questions remain about factors that could influence the extent of gene flow between populations exhibiting PMPZ isolation. We addressed the repeatability and consistency of PMPZ isolation acting between different populations, the mechanism of PMPZ isolation, and how male and female mating history influences the strength of PMPZ incompatibility. We found a recurrent and robust pattern of PMPZ isolation between *D. montana* populations. Crosses involving either males or (particularly) females from Colorado exhibited PMPZ isolation with three other populations, while crosses between those three populations remained fertile with each other. Incompatibility was due to fertilization failure but was not a consequence of intrinsic male infertility. As reproductive isolation is not complete between these populations, incompatibilities may only be present between specific female x male genotype interactions, but we found no variation in hatching success attributable to male identity. Thus, we show that PMPZ isolation, at least between Colorado and Vancouver, is acting at the population level. Multiple mating by males did not influence fertilization competency; compatible crosses remained compatible and incompatible crosses remained incompatible. Likewise, while multiple insemination of females increased the number of progeny produced per day in all crosses, incompatible crosses still produced significantly fewer progeny compared to within-population crosses. Thus, male or female multiple mating neither exacerbated nor ameliorated incompatibility. These patterns suggest that gene flow will be limited between Colorado individuals and the other populations, at least under these conditions.

Other studies of PMPZ isolation between species have suggested that reduced fecundity is a PMPZ reproductive barrier, even if fertilization occurs normally (Matute 2010; Turissini et al. 2018). Here we show that in some between-population crosses, fecundity is the same as at least one of the parental crosses so PMPZ isolation is not due to a reduction in fecundity. Instead, PMPZ isolation is manifested as a consequence of reduced fertilization rates. For normal and efficient fertilization, a coordinated series of ejaculate-female reproductive tract interactions are

required (Bloch Qazi et al. 2003; Pitnick et al. 2009; Wolfner 2009; Avila et al. 2010b; Mattei et al. 2015). The emergence of PMPZ reproductive barriers may be due to mismatched ejaculate-female reproductive tract interactions, deriving from population differentiation arising from either selection and/or genetic drift. However, Jennings et al. (2014) found no relationship between genetic distance and the strength of PMPZ isolation, suggesting divergence is not simply a result of isolation by distance. Instead, PMPZ isolation likely emerges as a by-product of both sexual selection and sexual conflict which are important in shaping the rapid co-evolution of ejaculate-female reproductive tract interactions (Pitnick et al. 2009; Mendelson et al. 2014; Bono et al. 2015; Ahmed-Braimah et al. 2017). Given that *D. montana* males transfer and females store motile sperm for fertilization but (most of) these sperm do not penetrate eggs, incompatibility is likely because of mismatches between sperm and egg release. These incompatibilities may arise due to variation between populations in seminal fluid proteins (Sfps) in the male ejaculate that cause profound behavioural, morphological, and physiological changes in the mated female (Ravi Ram and Wolfner 2007; Pitnick et al. 2009; Wolfner 2009; Avila et al. 2010b; Perry et al. 2013). Candidate Sfps include sex peptide (SP) which binds to the female sex peptide receptor (SPR) in the mated female and is essential for proper release of sperm from storage to ensure efficient fertilization in *D. melanogaster* (Avila et al. 2010a, 2015) and/or Acp36DE and ovulin which are required for efficient sperm storage and oocyte release in *D. melanogaster* (Avila and Wolfner 2009; Mattei et al. 2015). Future work should examine population variation in *D. montana* Sfp composition to test their potential role in mediating PMPZ isolation and to identify underlying "speciation genes" (Presgraves 2010; Nosil and Schluter 2011; Butlin et al. 2012).

Reproductive incompatibility in this system is asymmetrical, which may also help to understand the evolution of ejaculate-female reproductive tract interactions and the emergence of PMPZ reproductive barriers. Fertilization was reduced more in crosses involving Colorado females (<20% of eggs hatched) than in crosses involving Colorado males (ca. 50% of eggs hatched). Asymmetries in reproductive barriers could result from differences between populations in the strength of sexual selection (Boughman et al. 2005) and the action of sexual conflict (Arnqvist et al. 2000). For example, considering the male ejaculate as a polygenic trait, in populations where females have evolved preferences for high trait values of males, females will impose

stronger selection on males, thus reproductive isolation will be stronger in crosses involving those females. However, females from a population where trait values are lower on average, may still accept males (from another population) having higher trait values (Boughman et al. 2005), generating asymmetries in reproductive isolation. Such asymmetries generate predictions to test in future research: if postmating sexual selection is stronger in the Colorado population, then Colorado males should have more competitive and/or otherwise preferred ejaculates than Vancouver males. While a previous study did not find PMPZ isolation between these two populations under a sperm competitive scenario (Ala-Honkola et al. 2016), the Colorado population they used had very low within-population fertilization success and subsequently went extinct in the laboratory, suggesting some kind of inbreeding depression. Our current research shows recurrent, strong PMPZ isolation between these populations that is not dependent on a particular collection from a particular time and we conclude that PMPZ isolation occurs consistently between these populations (see also Moorhead 1954).

Reproductive isolation is not complete between the Colorado population and any of the others we tested it against, so it was important to establish whether PMPZ isolation was an interaction between specific female x male genotypes or a more widespread pattern acting across a range of genotypes. We tested focal males against multiple incompatible females and found small between-male variance in fertilization success, which did not warrant including male identity in the model. Low between-male variance in fertilization success indicates PMPZ isolation was acting consistently across the range of genotype x genotype interactions tested and was present at the population level. It may be that genotypes were limited after being in culture for a period of time, however, we observe high fertilization success in within-population crosses suggesting no inbreeding depression. Moreover, our results between Colorado and Vancouver populations were similar regardless of which Colorado and Vancouver populations/isofemale lines were being tested (this study and Jennings et al. 2014). Even if genetic variability has been eroded during the course of laboratory culture, then this means that alleles of large effect are likely fixed within populations, making future studies identifying speciation genes/loci causing PMPZ isolation easier to detect.

Male and female mating history is known to influence the extent of reproductive incompatibility, which could then influence the strength of PMPZ isolation. For example, Sfps are harmful to females (Chapman et al. 1995; Wolfner 2009; Sirot et al. 2015) and foreign seminal fluids may be even more so (Knowles and Markow 2001; Kelleher and Markow 2007), thus multiple mating by females may increase reproductive incompatibility. Males can also modify ejaculate composition depending on whether a female is virgin or mated (Sirot et al. 2011), which may elicit different effects on female postmating physiology including fertilization efficiency. However, we found no interaction between cross-type and mating number for either sex, indicating consistent intrinsic incompatibilities between populations. Both male and, to a greater extent, female reproductive success was increased by multiple mating, but this increase was the same relative amount for all crosses. This could be due to several different mechanisms such as females becoming more efficient fertilizers as they age, increased sperm viability and/or sperm number, and ejaculate composition modification.

In summary, we focussed on recently diverged populations of the same species to better understand PMPZ reproductive barriers that could act at the very earliest stages of the speciation process (Shaw and Mullen 2011; Butlin et al. 2012; Servedio and Boughman 2017; Tinghitella et al. 2017), the extent to which these barriers are consistent between populations collected at different times and between different genotypes, and how mating histories of the sexes influenced the strength of PMPZ isolation. While there is no guarantee that these populations will continue along the speciation process, we showed consistent, persistent and reproducible isolation between *D. montana* populations that is manifested at the population level and not influenced by either male or female mating history. PMPZ isolation was asymmetrical and occurred between Colorado individuals crossed with all other tested populations and was a consequence of fertilization failure, likely due to mismatches between ejaculate-female reproductive tract interactions. Future work will determine the nature of these mismatches and aim to identify the loci contributing to PMPZ isolation.

3. Postmating prezygotic isolation in the absence of strong sexual isolation

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CONTRIBUTIONS

MDG collected and analysed the data. MDG and RRS designed the study and wrote the manuscript.

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ABSTRACT

Sexual selection can drive the evolution of reproductive isolation as male signals and female preferences diverge between populations. Postmating prezygotic isolation (PMPZ) is thought to result from divergence of postmating reproductive traits, possibly as the result of postcopulatory sexual selection (PCSS) and sexual conflict acting within populations. However, how different episodes of sexual selection acting in different populations might contribute to the emergence of sexual isolation or PMPZ isolation is poorly understood. Here, we test whether the strength of PMPZ isolation reflects the strength of PCSS acting within populations and whether sexual isolation and PMPZ isolation cooccur between populations of the malt fly, *Drosophila montana*, from North America. We used the irradiated male technique to determine whether conspecific sperm precedence (CSP) was acting between populations, and whether the strength of CSP differed between populations. We also tested whether the strength of PMPZ

isolation reflected the risk of sperm competition experienced within populations, measuring male investment in traits which are selected for under an increased risk of sperm competition. Here we show sexual isolation was stronger in Vancouver in accordance with previous studies, whereas CSP was acting in Colorado but not Vancouver. However, males did not differ in investment in traits favoured by PCSS. Thus, while the strength of sexual isolation reflects the strength of sexual selection acting within populations, our results suggest PMPZ isolation can evolve independently of the strength of sexual selection via an as yet unknown mechanism.

KEYWORDS: Sperm competition, cryptic female choice, postcopulatory sexual selection, gametic isolation, postmating prezygotic isolation, reproductive isolation.

INTRODUCTION

Understanding how reproductive isolation evolves and the role that different barrier effects have during the speciation process is key to understanding the origin of species (Butlin and Smadja, 2017). Barriers to gene flow can act at the prezygotic (between parents) or postzygotic (in the hybrid offspring) stage of the reproductive cycle. Prezygotic isolation is thought to be particularly important during the initial stages of speciation given that postzygotic isolation, where hybrids suffer reduced fitness, evolves later (Coyne and Orr, 1989; Turissini et al., 2018). Prezygotic isolation can further be divided in to barriers to gene flow that act before (premating isolation) or after mating (postmating prezygotic isolation). Premating isolation, including sexual isolation, prevents interbreeding due to assortative mating within populations. An important role of sexual isolation during the speciation process has long been recognised (Wu et al., 1995). Postmating prezygotic (PMPZ) isolation involves incompatibilities between the male ejaculate and the female reproductive tract and/or between the gametes themselves that prevent fertilisation. Only recently has PMPZ isolation gained attention as a potentially important mechanism acting to restrict gene flow early during speciation (Howard et al., 2009).

Traits involved in reproduction evolve rapidly, possibly accelerated by sexual selection and sexual conflict (Swanson and Vacquier, 2002). Sexual selection can contribute to reproductive isolation between populations as male signals and female preferences coevolve within

populations such that individuals from different populations no longer recognise each other as potential mates (Lande, 1981). For example, divergent signals and preferences within populations has facilitated reproductive isolation in threespine stickleback (*Gasterosteus spp.*). Populations adapted to different light environments show divergence in male nuptial colouration that matches female preferences, which correlates with the strength of reproductive isolation (Boughman, 2001). Similarly, sexual conflict can drive cycles of sexually antagonistic co-evolution within populations, resulting in the divergent reproductive phenotypes between populations (Gavrilets, 2000). Populations of the dung fly (*Sepsis cynipsea*) subject to heightened sexual conflict showed stronger sexual isolation than populations experiencing weak or absent sexual conflict (e.g. Hosken et al., 2009; but see Bacigalupe et al., 2007).

In polyandrous species the ejaculates of multiple males compete to fertilise a given set of ova (sperm competition), and females can bias paternity towards particular male genotypes (cryptic female choice). Postcopulatory sexual selection (PCSS) may be responsible for the particularly rapid divergence of postmating reproductive traits (Birkhead and Pizzari, 2002). Sperm and ejaculate traits show exceptionally rapid diversification between species and populations within species (Ahmed-Braimah et al., 2017; Bono et al., 2015). Female reproductive tract tissues also show evidence of rapid evolution and divergence between taxa (Bono et al., 2011; Kelleher et al., 2007). As the male ejaculate and female reproductive tract coevolve within populations mismatches between the male ejaculate and the female reproductive tract may result in disruption of the necessary ejaculate x female reproductive tract interactions leading to successful fertilisation (Pitnick et al., 2009).

If sexual selection contributes to the evolution of prezygotic reproductive isolation, then how different episodes of sexual selection (pre- vs. post-copulatory) operate in different populations, and hence contribute to the evolution of barriers to gene flow, will depend on aspects of the local mating system. For instance, sexual selection acting on mating success could weaken postcopulatory sexual selection and slow divergence of postcopulatory traits that contribute to the evolution of PMPZ isolation. Furthermore, barriers to gene flow acting earlier in the reproductive cycle (e.g. sexual isolation) can impede the evolution of later acting barriers (e.g. postmating isolation) (Coyne and Orr, 2004). On the other hand, selection on mating success

is weakened in populations with high mating rates and positive mating assortment, where the most polyandrous females mate with the most polygynous males (McDonald and Pizzari, 2018). In such mating systems, sexual selection may instead act more strongly on ejaculate traits influencing the outcome of sperm competition. Thus, stronger postcopulatory sexual selection could drive divergence between populations in traits relating to postmating interactions and the emergence of postmating prezygotic isolation (Birkhead and Pizzari, 2002). At present, theory is sparse regarding how the interaction between pre- and post-copulatory sexual selection might impact the evolution of sexual isolation, PMPZ isolation, and their interaction.

The malt fly, *Drosophila montana*, provides the opportunity to test some predictions regarding the interaction between different episodes of sexual selection and speciation. Populations from North America and Europe differ in a number of male traits used in sexual signalling, including wing morphology, courtship song and cuticular hydrocarbon profiles (Jennings et al., 2014a; Klappert et al., 2007; Routtu et al., 2007). Females from Vancouver, British Columbia, Canada, and Oulanka, Finland prefer a higher male courtship song carrier frequency, while females from Crested Butte, Colorado, USA, have a preference for a lower carrier frequency which is overall weaker (Klappert et al., 2007). Similarly, females from Vancouver are also more discriminatory based on cuticular hydrocarbons than females from Colorado or Oulanka (Jennings et al., 2014a). The strength of sexual isolation between populations appears to correspond with the strength of sexual selection acting on mating signals within populations. Flies from Vancouver mate more assortatively, preferring partners from within their own population, whereas flies from Colorado and Oulanka are less choosey with whom they mate based on these signal and preference traits (Jennings et al., 2014b, 2014a; Klappert et al., 2007; Routtu et al., 2007). Sexual isolation is therefore likely to play an important role in reducing gene flow between populations, particularly in Vancouver.

Crosses between populations also yield PMPZ isolation. The strongest PMPZ isolation is found in crosses involving the Colorado population. Colorado females have reduced fertilisation success ($<25\%$) when mating with foreign males, while in the reciprocal crosses, Colorado males fertilise only around 50% of foreign female ova (Garlovsky and Snook, 2018; Jennings et al., 2014b). Stronger sexual isolation coupled with stronger precopulatory sexual selection in

Vancouver, as opposed to stronger PMPZ isolation in Colorado, suggests that postcopulatory sexual selection may be stronger in Colorado. Here we test two predictions arising from this pattern. First, if precopulatory sexual selection is stronger in Vancouver, then we predicted that Vancouver males will invest less into traits favoured by PCSS than Colorado males. Therefore, we tested whether males from Colorado and Vancouver differed in relative investment in the ejaculate and/or mating capacity. In populations experiencing heighten PCSS, where males face an increased risk of sperm competition, they should invest relatively more into their ejaculate. Additionally, in populations where males faced an increased risk of sperm competition, males should have an increased mating capacity (Pizzari and Parker, 2009; Tomkins and Simmons, 2002; Wedell et al., 2002). Both traits increase a males success in sperm competition and have shown to be under selection in mating systems where males face an increased risk of sperm competition (Crudgington et al., 2009; Hosken et al., 2001). Thus, we predicted that Colorado males will have a greater relative investment in reproductive mass and greater mating capacity. Second, if the asymmetry in PMPZ isolation reflects the strength of PCSS acting within populations, then we predicted that another form of PMPZ isolation, conspecific (in this context and hereafter con-*population*) sperm precedence (CSP) will also be stronger in Colorado. For many insects, including *Drosophila spp.*, the second (or last) male to mate normally sires the majority of offspring (Parker, 1970). However, when females mate multiply with both a con- and hetero-specific male, the conspecific male often sires the majority of offspring, regardless of mating order (Howard et al., 2009; Pitnick et al., 2009; Price, 1997). We tested whether CSP was acting in either population and whether the strength of CSP was stronger in Colorado or Vancouver by measuring male offensive paternity share (P_2) in both inter- and intra-population crosses.

METHODS

Fly stocks

A detailed description of the fly stocks used can be found in (Garlovsky and Snook, 2018). Briefly, *Drosophila montana* were collected from riparian habitats in Crested Butte, Colorado, USA (38°49'N, 107°04'W) in 2013 (referred to as Colorado, C), and Vancouver, British Columbia, Canada (48°55'N, 123°48'W) in 2008 (referred to as Vancouver, V). Stocks were subsequently maintained on Lakovaara malt media (Lakovaara, 1969) in overlapping generations in constant light at 19^{o}C to prevent females entering reproductive diapause. All flies used in experiments were collected within three days of eclosion and maintained in single sex vials of 10-20 individuals until reproductive maturity (21-28 days old).

Statistical analysis

All statistical analyses were performed in R (v. 3.5.1) (R Core Team, 2018). We performed post-hoc Tukey's honest significant difference (HSD) tests using the *glht* function from the 'multcomp' package (Hothorn et al., 2008).

Male relative reproductive investment

Approximately 100 flies of mixed sex were collected from population cages and allowed to mate and oviposit for two days in small plastic bottles covered with a molasses-agar oviposition plate with a drop of dried yeast paste added (n = 3 per population). Oviposition plates were changed after 2 days and females allowed another 2 days of oviposition, after which flies were discarded. Oviposition plates were returned to the incubator to allow eggs to develop. Controlled density vials (CDVs) were seeded with 50 first instar larvae from the oviposition plates in to food vials (n = 6 per population) to control for density dependent effects. Adults were collected from CDVs on the day of eclosion and kept in single sex vials of between 10-20 individuals until 21 days old and then frozen at -20^{o}C. Population identity was replaced with a unique identifier to blind experimenters prior to taking measurements. Males were thawed and the entire reproductive tract (testes, accessory glands, ejaculatory duct and bulb) dissected on a pre-weighed piece of foil in a drop of dH_20 which was then transferred to a second piece of pre-weighed foil. Carcasses and reproductive tracts were dried overnight at 60^{o}C before weighing

(METTLER TOLEDO® UMX2 ultra-microbalance). Male reproductive investment was analysed using an ANCOVA, with log transformed dry reproductive tract mass as the response variable, and population ID (Colorado or Vancouver), log transformed dry soma mass, and their interaction as predictors. After removing five outliers (2 Colorado, 3 Vancouver) we tested for significance using type II sum of squares using the 'Anova' function from the *car* package (Fox and Weisberg, 2011).

Female dry mass

We measured dry mass of females emerging from CDVs (N = 60 per population). Frozen females were thawed and dried overnight at 60^{o}C and weighed individually on a weighing boat. We tested for differences in female dry mass between populations with a t-test.

Male mating capacity

We recorded the total number of sequential matings performed by males (n = 20 per population) with females from their own population within a 4-hour period. Virgin males were mouth aspirated without anaesthesia into a vial containing two virgin females. Once copulation was initiated the unmated female was removed from the vial. After mating, the male was transferred to a new vial housing another two virgin females. This procedure of allowing males to start mating with one female and then immediately transferring him to a new vial containing two more virgin females was repeated ad libitum. Mated females were returned to the incubator in their oviposition vial for 4 days, and then transferred to second food vial for a further 4 days of oviposition, after which females were discarded. The total number of adult flies emerging was counted for each female (combined across both oviposition vials). To assess differences between populations in the total number of matings males attempted during the observation period we used a GLM with Poisson errors with male population identity as the only predictor. To assess male per-mating investment we tested for differences between populations in the numbers of offspring sired using a GLMM with Poisson errors, including population, mating number, and the population x mating number interaction as fixed effects, and a random effect for male identity and an observation level random effect to account for overdispersion (Harrison, 2014). We also assessed differences in reproductive success between populations using a t-test of the differences between populations in the total number of progeny sired by males across all

their mates. Two males were lost during the experiment (one Colorado, one Vancouver) and subsequently excluded from analysis.

Con-population sperm precedence

We assessed male sperm offensive paternity share (P_2) using the irradiated male technique (Boorman and Parker, 1976). Delivering a non-lethal dose of gamma irradiation causes dominant lethal mutations in sperm, rendering males sterile, yet fertilisation competent. Eggs fertilised by irradiated sperm will not develop beyond early stages of development, such that eggs will not hatch enabling paternity assignment of embryos. Males were irradiated with a 100Gy dose of gamma radiation with a ^{137}Cs gamma source (dose rate 189.2 rads min^{-1}), which was sufficient to achieve 100% hatching failure in eggs fertilised by irradiated sperm. Virgin males were irradiated less than 24 hours before mating trials started and housed singly overnight in food vials. Control males were handled in exactly the same way, placed in a canister identical to the one placed in the irradiator.

We performed experiments in 3 blocks for each focal female population separately, in which females (n = 10) were randomly assigned to one of 14 treatments. To assess the natural level of fertility after a double mating (used to calculate p in equation (1) below), focal females were mated to two nonirradiated 'control' males, in all possible crossing combinations between females and two males from Colorado and/or Vancouver. During pilot studies we collected additional data on these control crosses which we included in our estimate of p. To assess the efficacy of the irradiation technique (used to calculate z in equation (1)), we mated females to two irradiated males, both from either her own or the foreign population. Irradiated treatments (x in equation (1)) consisted of all possible crossing combinations between females and males from Colorado and/or Vancouver, with either the first or second male irradiated.

Virgin females were presented with a virgin male from the appropriate treatment group and allowed a 4-hour mating opportunity. After mating, females were transferred to one of twenty chambers in an oviposition manifold and males discarded (Garlovsky and Snook, 2018; Jennings et al., 2014b). Manifolds were returned to the incubator for oviposition. Two days later, females were presented with another virgin male from the appropriate treatment and allowed a 4-hour

remating opportunity. Females that remated were returned to the incubator and allowed to oviposit for a further 2 days on a new oviposition plate and then discarded. We ensured no females remated during either 4-hour observation period. The numbers of eggs laid on each oviposition plate was counted immediately after removing the female (for remating or discard). The oviposition plate was then returned to the incubator for a further two days after which the numbers of unhatched eggs was counted again to measure hatching success. The proportion of eggs fertilised by the irradiated male after the second mating, P_R, can be calculated using equation (1) from Boorman and Parker (1976):

$$P_R = \left(1 - \frac{x}{p}\right) + \frac{z}{p} * \left(\frac{1-(x/p)}{1-(z/p)}\right),$$

where x is the observed proportion of developing eggs, p is the level of fertility observed in a double mating for a given 'control' cross-type, and z is the level of sterility achieved by the irradiation. We calculated P_R using a value of p equal to the maximum observed hatching success of a given control cross-type (rather than the average observed hatching success) to capture as much variation in fertilisation success as possible. As $z = 0$ in this case, the equation can be simplified to $P_R = 1 - x/p$. Therefore, if the irradiated male mated first, then the proportion of eggs fertilised by the second male, $P_2 = x/p$. If the irradiated male mates second then $P_2 = P_R$ (Boorman and Parker, 1976). After applying this formula, one P_2 value was greater than 1.0 (a CVC cross) and was excluded from our analysis.

We assessed differences in P_2 between cross-types using generalised linear models (GLMs) with binomial errors and a logit link. The total number of eggs laid by each female after the second mating was multiplied by the calculated P_2 value and rounded to a whole number to give the estimated number of offspring sired by the second male and the remaining offspring inferred to be sired by the first male. The binary response was then the numbers of offspring sired by the second male ("successes") and numbers of offspring sired by the first male ("failures"). Models included cross-type, irradiation order and their interaction as fixed effects and we analysed responses in Colorado and Vancouver females separately as female populations were never tested together. Preliminary analysis indicated that the data was overdispersed, so we used quasibinomial errors. Due to the very low fertility in crosses between Colorado females and Vancouver males (CVV hatching success = 0.115 ± 0.03 [mean ± standard error]), we did not

have sufficient power to assess P_2 in the CVV cross, which was subsequently excluded from our analysis.

Hatching success rates

For our nonirradiated controls we assessed differences between crosses in hatching success rates (a proxy for fertilisation success (Garlovsky and Snook, 2018; Jennings et al., 2014b)) after the first and second mating. We fitted GLMs with quasibinomial errors and a logit link, using the numbers of hatched ("successes") and unhatched ("failures") eggs as the response variable. For the first mating the only predictor was the male population ID (Colorado or Vancouver). For the second mating the only predictor was the cross-type. As for con-population sperm precedence analyses we analysed responses in Colorado and Vancouver females separately.

Premating isolation

We assessed the probability of females to (re)mate using a logistic regression with binomial errors and a logit link. The binary response was whether the female (re)mated (1) or not (0). We assessed female mating latency for the first mating only using a GLM with quasipoisson errors and a log link. We also assessed copulation duration in both the first and second mating using a GLM with quasipoisson errors and a log link. All models included cross-type and whether males were irradiated as predictors.

RESULTS

Male relative reproductive investment

Males from Vancouver were larger than males from Colorado (t-test, t = 11.163, df = 95.31, p < 0.001; Colorado = 0.49 ± 0.01 mg [mean ± standard error], n = 58, Vancouver = 0.67 ± 0.01 mg, n = 57). Reproductive tract mass increased with body size ($F_{1,111}$ = 34.93, p < 0.001) but there was no effect of either population ($F_{1,111}$ = 0.791, p = 0.376) or the population x body mass interaction ($F_{1,111}$ = 0.044, p = 0.835). Thus, male relative reproductive investment did not differ between populations (Fig. 3.1).

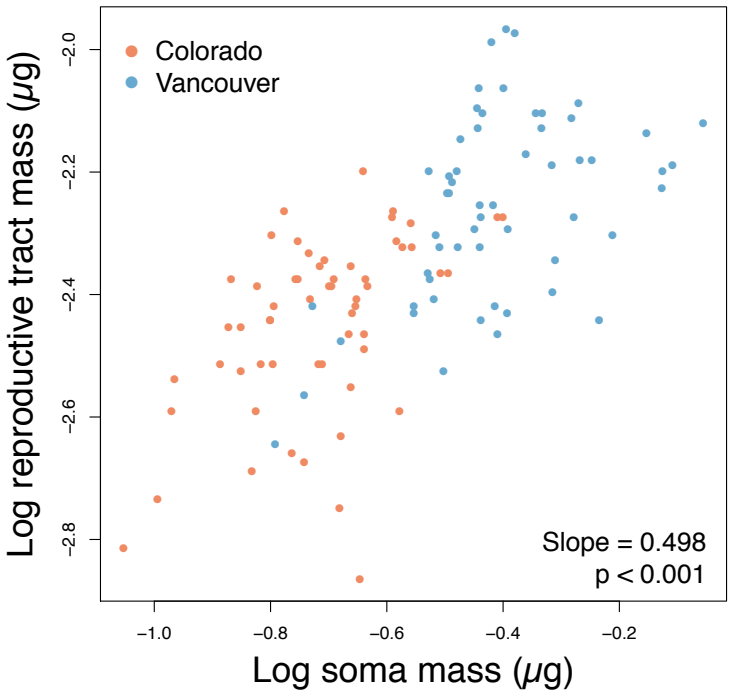

Figure 3.1. Log reproductive tract mass increased with log soma mass. Colorado (red) and Vancouver (blue). 5 outliers were removed (2 Colorado, 3 Vancouver).

Male mating capacity

Males did not differ in the mean number of sequential matings initiated during the 4-hour observation period (Poisson GLM, X^2 = 0.01, df = 1, p = 0.939; Colorado = 4.25 ± 0.44 [mean ± standard error], n = 20; Vancouver = 4.30 ± 0.59, n = 20). However, in total, Vancouver males sired more offspring than Colorado males (t-test, t = -2.50, df = 30.11, p = 0.020; Colorado = 93 ± 16, n = 19; Vancouver = 166 ± 25, n = 19). The greater number of offspring in Vancouver crosses may be explained by Vancouver females being larger than Colorado

females on average (t-test, t = 5.81, df = 89.49, p < 0.001; Colorado = 0.77 ± 0.01 µg, Vancouver = 0.922 ± 0.02 µg). Note, we did not measure body size of females used in the mating capacity experiments. The number of progeny sired per-mating declined with mating number (Poisson GLMM: $X^2 = 5.35$, df = 1, p = 0.021) but there was no significant difference in the rate of decline between populations (population x mating number interaction; Poisson GLMM: $X^2 = 0.44$, df = 1, p = 0.504), suggesting that males invested similarly on a per-mating basis (Fig. 3.2).

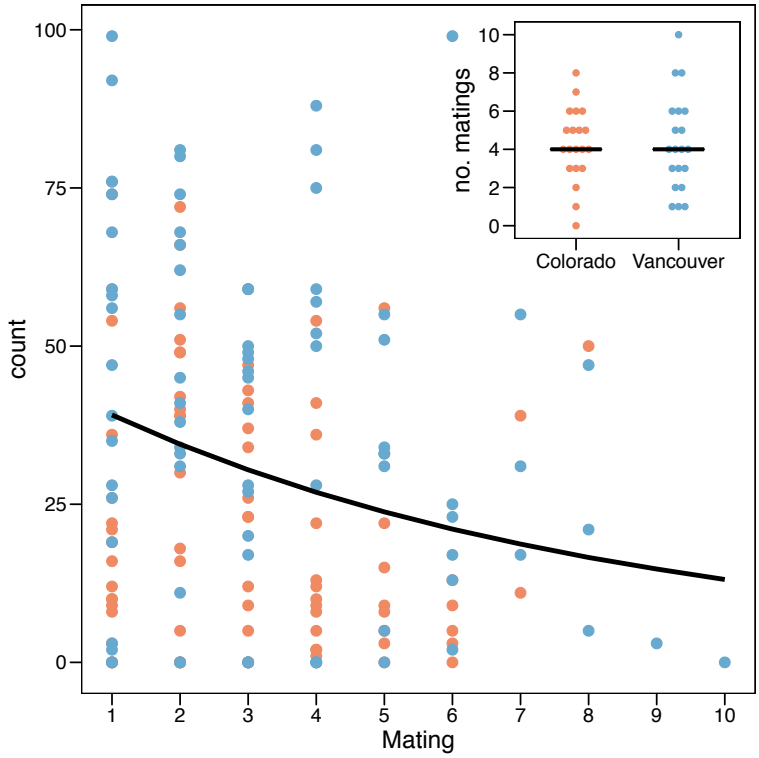

Figure 3.2. The numbers of offspring sired decreased with increasing mating number but did not differ between populations (common slope: β = -0.131 ± 0.06, z = -2.29, p = 0.022). Inset: Total number of matings that males performed during the 4-hour observation period did not differ between populations. Colorado, red; Vancouver, blue.

Premating isolation of the first mating

Colorado females were equally likely to mate with Colorado and Vancouver males (binomial GLM: $X^2 = 0.53$, df = 1, p = 0.468) and showed no difference in mating latency based on male population identity (quasipoisson GLM: $F_{1,438} = 0.90$, p = 0.342), irradiation treatment (quasipoisson GLM: $F_{1,437} = 1.30$, p = 0.254), or their interaction (quasipoisson GLM: $F_{1,436}$ =

0.24, p = 0.627). However, Colorado females mated for longer with Colorado males (quasipoisson GLM: $F_{1,434} = 26.27$, $p < 0.001$; $\beta = -0.20 \pm 0.05$). Vancouver females were more likely to mate with Vancouver males (binomial GLM: $X^2 = 14.98$, df = 1, $p < 0.001$). Vancouver females mating with Colorado males did not differ in mating latency when compared to mating with Vancouver males (quasipoisson GLM: $F_{1,403} = 0.90$, p = 0.344), but did prefer to mate with nonirradiated males (quasipoisson GLM: $F_{1,402} = 5.93$, p = 0.015; $\beta = -0.33 \pm 0.15$). Vancouver females also mated for longer with Colorado males (quasipoisson GLM: $F_{1,399} = 9.76$, p = 0.002; $\beta = 0.13 \pm 0.05$). Therefore, our results are in accordance with previous studies showing that Vancouver females are more choosey with whom they mate than Colorado females (Jennings et al., 2014b). However, once mating began, both females mated for longer with Colorado males.

Premating isolation of the second mating

There was a significant effect of cross-type on the probability of remating for Colorado females (binomial GLM: $X^2 = 18.55$, df = 3, $p < 0.001$). Colorado females were more likely to remate with a Colorado male if their first mate was a Vancouver male (CVC; Table 3.1). Vancouver females were equally likely to remate regardless of male identity (binomial GLM: $X^2 = 6.68$, df = 3, p = 0.083; Table 3.1). Although a formal statistical test was not appropriate as Colorado and Vancouver females were never tested together, Vancouver females had a higher remating rate than Colorado females on average (Table 3.1).

Table 3.1. Total number of females used in mating trials, number mating and remating. Cross-type denotes the population of the female followed by her first and second male mate. C, Colorado, V, Vancouver.

Cross-type (F x M1 x M2)	**CCC**	**CCV**	**CVC**	**CVV**	**VVV**	**VVC**	**VCV**	**VCC**
Total	149	119	120	150	142	116	117	150
Mating	127	93	99	116	116	98	87	96
Remating	77	51	83	75	82	77	67	76
% Remating	61%	55%	84%	65%	71%	79%	77%	79%

Colorado females that mated with a Vancouver male in the second position did not mate for as long as females mating with two Colorado males (quasipoisson GLM: $F_{3,286} = 9.19$, $p < 0.001$; CCV: $\beta = -0.23 \pm 0.08$, CVV: $\beta = -0.18 \pm 0.05$). Vancouver females copulated for longer with Colorado males after mating a Vancouver male than vice versa (quasipoisson GLM: $F_{3,298} = 4.22$, $p = 0.006$; VVC: $\beta = 0.11 \pm 0.06$; VCV: $\beta = -0.06 \pm 0.07$).

Postmating prezygotic isolation after the first mating

In our nonirradiated crosses, after the first mating, hatching success rates were similar to those reported previously for within- and between- population crosses (Garlovsky and Snook, 2018; Jennings et al., 2014b). Colorado females had reduced hatching success when mating with males from Vancouver (quasibinomial GLM: $F_{1,183} = 380.09$, $p < 0.001$). Likewise, Vancouver females had reduced hatching success when mating with males from Colorado (quasibinomial GLM: $F_{1,174} = 60.00$, $p < 0.001$) (Fig. S3.1).

Postmating prezygotic isolation after the second mating

In our control (double nonirradiated) crosses, after the second mating, cross-type had a significant effect on the proportion of eggs that hatched for Colorado females (quasibinomial GLM: $F_{3,115} = 92.82$, $p < 0.001$) and Vancouver females (quasibinomial GLM: $F_{3,115} = 35.11$, $p < 0.001$). In both populations, females mating with a between-population male followed by a within-population male had hatching success similar to mating two within-population males (Tukey's HSD, all $p > 0.116$). Females mating with two between-population males had lower hatching success than other groups (Tukey's HSD, all $p < 0.001$). Females mating with a within-population male followed by a between-population male had lower hatching success than females mating with two within-population males, but higher hatching success than females mating with two between-population males (Tukey's HSD, all $p < 0.015$). Therefore, females mating with either two within-population males, or a within-population in the second position had equal fertility, whereas females mating with two between-population males, or a between-population male in the second position had reduced fertility (Fig. 3.3).

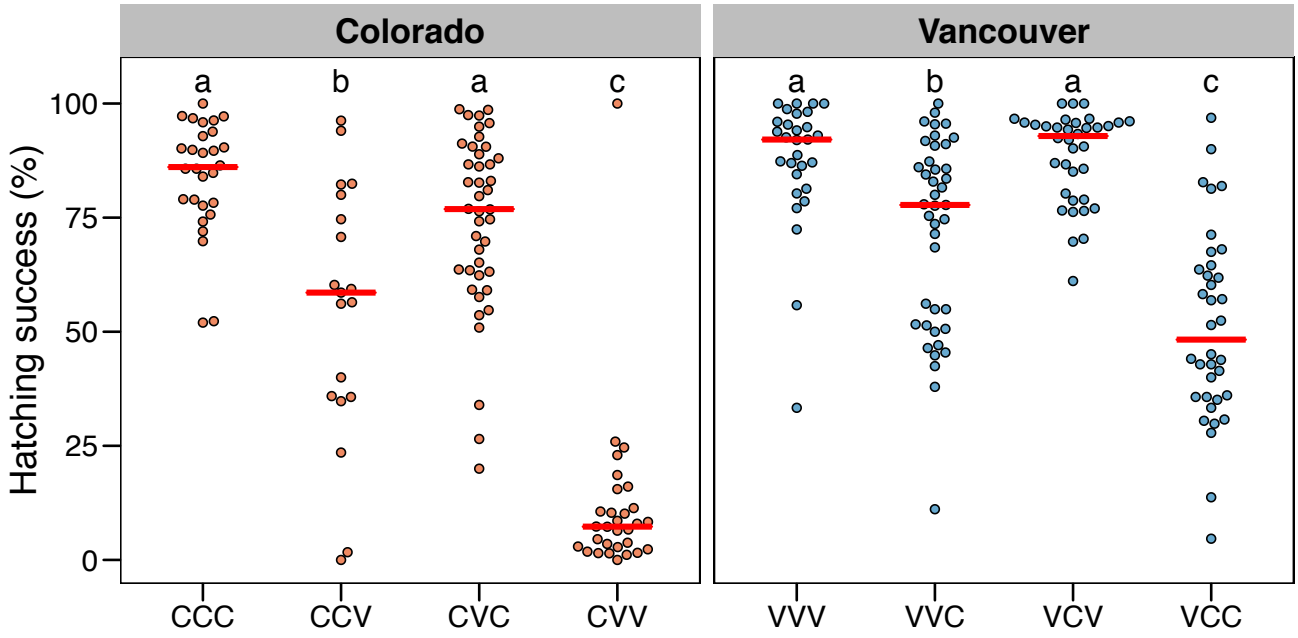

Figure 3.3. Hatching success (% eggs laid that hatched) of females that mated two nonirradiated males. Points are observations and red crossbars show the median. Cross-type denotes female population followed by first and second male mate. C, Colorado; V, Vancouver. Different letters above the points indicate significant differences from post-hoc Tukey's HSD. Letters are recycled in each panel.

Con-population sperm precedence

Females from both populations showed last-male sperm precedence when mating with two within-population males (Colorado (CCC), $P_2 = 0.69 \pm 0.17$; Vancouver (VVV), $P_2 = 0.66 \pm 0.18$). There was a significant effect of cross-type on P_2 in both Colorado (quasibinomial GLM: $F_{2,89} = 74.12$, $p < 0.001$) and Vancouver (quasibinomial GLM: $F_{3,118} = 4.96$, $p = 0.003$). Colorado females showed paternity bias towards Colorado males in both the first (CCV) and second (CVC) mating position, indicating con-population sperm precedence in Colorado female reproductive tracts (Fig. 3.4). Conversely, paternity was not biased towards Vancouver males in Vancouver female reproductive tracts (Fig. 3.4). Vancouver females mating with a Colorado male followed by a Vancouver male showed P_2 values that were not different from within-population Vancouver matings (VCV, $P_2 = 0.70 \pm 0.22$). Vancouver females mating with a Colorado male in the second position used sperm equally from the first and second male (VVC, $P_2 = 0.46 \pm 0.18$; VCC, $P_2 = 0.63 \pm 0.21$).

In Colorado there was a significant effect of irradiation ($F_{1,88} = 24.66$, $p < 0.001$), and the cross-type x irradiation interaction on P_2 ($F_{2,86} = 13.98$, $p < 0.001$). Irradiated males mating in the second position had a greater P_2 than irradiated males mating in the first position in the CCV and CVC crosses, but not the CCC cross (Fig. S3.2). In Vancouver the irradiation main effect was not significant ($F_{1,117} = 1.31$, $p = 0.255$) but there was a significant cross-type x irradiation interaction ($F_{3,114} = 7.45$, $p < 0.001$). In the VVV cross, irradiated males in the second position had a lower P_2, whereas in the other crosses irradiated males in the second position had a greater P_2 (Fig. S3.2).

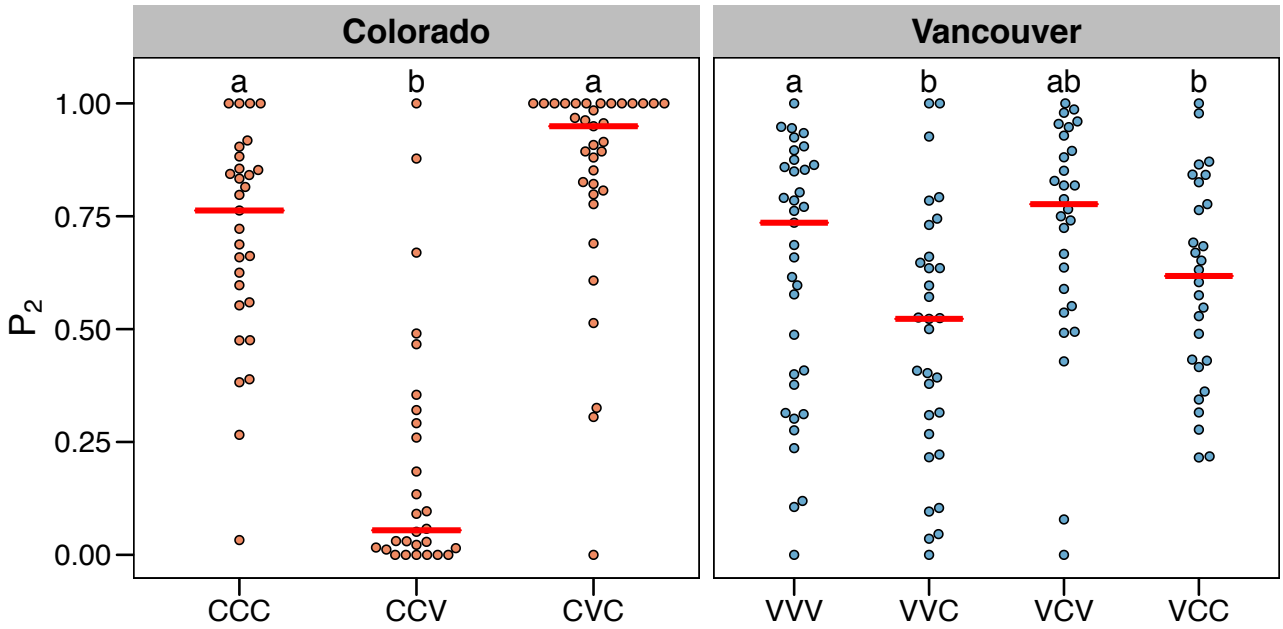

Figure 3.4. Proportion of offspring sired by the second male to mate (P_2). Points are observations and red crossbars show the median. Cross-type denotes female population followed by first and second male mate. C, Colorado; V, Vancouver. Different letters above points indicate significant differences from post-hoc Tukey's HSD. Letters are recycled in each panel.

DISCUSSION

Little understanding exists of whether PMPZ barriers to gene flow are the product of intrinsic incompatibilities between populations or are generated as a direct consequence of postcopulatory sexual selection (PCSS) acting within populations. Furthermore, little is known about how premating and PMPZ isolation might interact or coevolve. Here we assessed whether the strength of conspecific sperm precedence acting between populations reflected the strength of PCSS acting within populations, and whether premating and PMPZ isolation cooccurred or counteracted each other. In line with previous studies, sexual isolation was stronger in Vancouver, as Vancouver females mated faster with Vancouver males, whereas Colorado females showed no difference in mating latency. We found support for the prediction that precopulatory sexual selection and sexual isolation would weaken PMPZ isolation in Vancouver as con-population sperm precedence (CSP) was stronger in Colorado than in Vancouver. Colorado males sired the majority of offspring when mating with Colorado females, regardless of mating order. In Vancouver we found a similar, albeit weaker pattern of paternity bias towards males from their own population, as paternity was shared equally or biased towards the second male regardless of population identity. Therefore, CSP is evident in both populations, but to a greater extent in Colorado. Given the asymmetry in both competitive and non-competitive PMPZ isolation, we predicted that Colorado males would exhibit greater investment in traits associated with heightened PCSS. Contrary to predictions, our measures assessing the risk of sperm competition faced by males within populations showed no difference between populations, in either male relative reproductive investment or male mating capacity. There was some evidence of mate guarding differences, as Colorado males mated for longer with females from both populations.

As a proxy for the strength of PCSS we assessed whether the risk of sperm competition experienced by males differed between Colorado and Vancouver. While Vancouver males were larger bodied on average, we found that relative investment in reproductive tissue did not differ between populations (Tomkins and Simmons, 2002). Sperm competition theory predicts that in populations experiencing an increased risk of sperm competition, males will invest more in the ejaculate to increase their mating capacity (Wedell et al., 2002). Males from Colorado and

Vancouver showed a similar trend in per-mating investment, indicated by the equal slopes of the regression of progeny production against mating number. Vancouver males sired more progeny on average than Colorado males, which may be explained by the larger body size of Vancouver females, often associated with increased fecundity (Blanckenhorn et al., 2007). Colorado males did mate for longer with females from both Colorado and Vancouver, which is potential evidence of differences between populations in mate guarding behaviour associated with increased risk of sperm competition faced in Colorado (Stockley, 1997).

While overall reproductive tract mass did not differ between populations, it may be that we did not capture the relevant metric for differential investment. Both comparative and experimental evidence indicate the evolution of longer sperm in response to an increased risk of sperm competition (Miller and Pitnick, 2002; Pizzari and Parker, 2009; Snook, 2005). If Colorado males possess longer sperm that has evolved in response to heightened PCSS (see above), males may be differentially investing in sperm length, rather than sperm number (Snook, 2005). Further, overall reproductive tract mass did not differ, but it could be that accessory gland size differs between populations (Crudgington et al., 2009). Larger accessory glands that produce a greater volume of seminal fluids may act as a 'cheap filler' allowing males to increase mating capacity and potentially delay female remating. Thus, contrary to our predictions, neither proxy for the risk of sperm competition within populations showed evidence for divergent reproductive tactics that would contribute to PMPZ isolation.

Mating two between-population males showed a similar strength of PMPZ isolation (reduced hatching success) to that of a single mating (Garlovsky and Snook, 2018; Jennings et al., 2014b). In both populations, mating with a within-population male second restored fertility to that of within-population mating, while if a female's first mate was from the foreign population, fertility was reduced. We used the irradiated male technique (Boorman and Parker, 1976) to determine second male paternity share (P_2) in a separate set of crosses. Within-population crosses both showed last-male sperm precedence ($P_2 > 0.66$) in accordance with previous studies in *D. montana* (Ala-Honkola et al., 2016; Aspi and Lankinen, 1992). However, when mating with both a within- and between-population male, Colorado females almost exclusively used Colorado male sperm to fertilise eggs, regardless of mating order. Therefore, we show con-

population sperm precedence can act as a PMPZ barrier to gene flow in Colorado. Vancouver females did not show the same skew in paternity towards males from their own population. If Vancouver females mated with a Colorado male followed by a Vancouver male (VCV), last-male sperm precedence persisted. However, if the mating order was reversed (VVC), Vancouver females used sperm equally from their first and second mate. This pattern suggests Vancouver ejaculates are able to maintain offensive sperm competition ability against a Colorado male ejaculate in Vancouver female reproductive tracts but cannot maintain a sperm defensive role. In *D. melanogaster* paternity is determined by numerical representation of sperm in storage (Lüpold et al., 2012). Longer and slower sperm are better able to retain representation in the fertilisation set, resisting displacement and displacing resident sperm more successfully (Lüpold et al., 2012). Together with our results of asymmetrical CSP, this suggests that Colorado males have relatively longer sperm than Vancouver males such that Vancouver sperm are unable to displace Colorado male sperm especially in a Colorado female reproductive tract. Other mechanisms can also bias representation in the fertilisation set e.g. biased use of sperm storage organs or preferential dumping of sperm (Manier et al., 2013). An obvious next step will be to quantify the relative representation of different males' sperm in storage to assess the mechanisms biasing paternity in this system.

Combined, the results from our double matings offer some insights into the mechanisms of PMPZ isolation acting between populations of *D. montana*. First, asymmetrical CSP indicates postcopulatory sexual selection biases paternity towards co-evolved males in Colorado but not in Vancouver. This could be due to Colorado males possessing more competitive ejaculates or cryptic female choice in both Colorado and Vancouver favouring "Colorado-like" ejaculates. Second, the proportion of first and second male sperm used inferred from our P_2 analysis indicates the mechanism resulting in reduced fertilisation success, whereby females lay unfertilised eggs (Garlovsky and Snook, 2018; Jennings et al., 2014b), is an inability of between-population male sperm to penetrate ova, despite attempts to be used, rather than sperm not being released from storage for instance. For example, in the CCV cross, the 20% of eggs fertilised by the second (Vancouver) male, results in a corresponding reduction in the number of eggs laid that hatch. Three possible mechanisms causing this incompatibility are currently under investigation in the lab. First, mismatches between the male ejaculate and the female

reproductive tract might not elicit the correct postmating female responses necessary for the release of sperm from storage coordinated with ovulation (Mattei et al., 2015; Singh et al., 2018). Seminal fluid proteins, essential to elicit the correct physiological responses for proper ejaculate-female reproductive tract interactions, evolve rapidly and thus might generate PMPZ incompatibilities between even closely related taxa (Ahmed-Braimah et al., 2017; McDonough et al., 2016; Pitnick et al., 2009). Second, fertilisation failure could result from failure of sperm to physically enter through the micropyle. This could again be due to females not receiving the correct physiological responses from a divergent male ejaculate, resulting in mistiming of sperm and egg release, or direct mechanical or biochemical interactions between the gamete cell surfaces (Karr et al., 2009; Loppin et al., 2015). Third, morphological differences between sperm and female reproductive tract morphology could impede efficient fertilisation (Miller and Pitnick, 2003).

In addition to measures of PMPZ isolation, we also found that mating and remating behaviours differed between populations. Colorado females were equally likely to mate with Colorado or Vancouver males as virgins, whereas Vancouver females were more likely to mate with males from their own population. This is in agreement with previous findings that sexual isolation is stronger in Vancouver (Jennings et al., 2014b). The observation that the Vancouver population exhibits stronger sexual isolation but weaker PMPZ isolation, while Colorado shows the opposite pattern, suggests that the evolution of different modes of prezygotic isolation (sexual vs. postmating prezygotic) might not cooccur. Barriers to gene flow that act earlier in the reproductive cycle (e.g. sexual isolation) will lower the probability of proceeding to later stages and so might impede the evolution of later acting barriers (e.g. postmating prezygotic isolation) (Coyne and Orr, 2004). Thus, strong sexual isolation in Vancouver might have curtailed the evolution of strong PMPZ isolation. Both sexual isolation and PMPZ isolation evolve rapidly (Turissini et al., 2018). PMPZ isolation could emerge before sexual isolation, given the rapid evolution of seminal fluid proteins (Ahmed-Braimah et al., 2017; Findlay et al., 2009). Which form of prezygotic isolation evolves first, and potentially plays a more important role in reducing gene flow between taxa, may depend on the biology of the taxa involved, or could perhaps be serendipitous.

If PMPZ isolation evolves first, it may circumvent the evolution of sexual isolation. For instance, conspecific sperm precedence will slow the evolution of sexual isolation if females remate frequently, as the costs of mating a heterospecific will be reduced (Marshall et al., 2002). Colorado females that had previously mated a Vancouver male were more likely to remate with a Colorado male. Tactical remating behaviour could be a mechanism reducing gene flow, whereby Colorado females employ a bet-hedging strategy, mating with available low quality (i.e. hetero-specific/population) males, and subsequently 'trade-up' given the opportunity to mate with a higher quality (i.e. con-specific/population) male (Kokko and Mappes, 2005). *D. montana* are thought to be the predominant virilis clade drosophilid in Vancouver, with its close relative, *D. flavomontana* having recently migrated north with warming temperatures (Poikela et al., 2019). In Colorado, *D. montana* has a longer history of sympatry with at least two closely related species (*D. borealis* and *D. flavomontana*) (Routtu et al., 2007). Frequent interspecific interactions in Colorado may have resulted in the 'rarer female effect', where females experience selection against hybridisation and the evolution of prezygotic isolation, which could reinforce PMPZ isolation (Yukilevich, 2012).

In conclusion, we found asymmetry in the strength of conspecific sperm precedence acting between populations in the opposite direction to the strength of asymmetry in sexual (premating) isolation. While the asymmetry in sexual isolation between populations mirrors the strength of sexual selection acting on precopulatory traits within populations, we found that the asymmetry in PMPZ isolation did not coincide with stronger postcopulatory sexual selection acting in Colorado. Our results suggest that how postmating prezygotic isolation evolves may be fundamentally different to how sexual isolation evolves. Further, our study suggests that a more nuanced understanding of the types of sexual interactions individuals encounter in their local environment will be key to understanding the evolution of prezygotic reproductive isolation in nature.

4. Characterisation of the *Drosophila montana* seminal fluid proteome

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CONTRIBUTIONS

MDG and CE collected the data. MDG analysed the data and wrote the manuscript with input from all authors.

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ABSTRACT

Seminal fluid proteins (Sfps) are among the fastest evolving in the animal kingdom, diverging rapidly between even closely related species. For internally fertilising taxa, the rapid evolution of Sfps within populations is predicted to result in mismatches between the male ejaculate and female reproductive tract, and the emergence of postmating prezygotic (PMPZ) isolation between populations. Using liquid chromatography mass spectrometry (LC-MS/MS) we investigate whether populations of the malt fly, *Drosophila montana*, that exhibit PMPZ isolation, differ in the proteomes in the seminal fluid protein producing organs. Here we show

differential abundance between populations in a number of proteins found in the two major male seminal fluid secretory organs in *D. montana*: the paired accessory glands, and ejaculatory duct and bulb. Several of these proteins have orthologues with known *D. melanogaster* seminal fluid proteins. Our proteomics analysis is also one of the first to describe the proteome of both the accessory glands and ejaculatory duct and bulb in any species, and we show these tissues may provide some discrete functions to reproduction. Our analysis offers insights into the evolution of Sfps and implicates the rapid evolution of Sfps in the early emergence of reproductive isolation between populations.

KEYWORDS: Postcopulatory sexual selection, seminal fluid proteins, postmating prezygotic isolation, speciation, proteomics, tandem mass-spectrometry.

INTRODUCTION

For internally fertilising species, components of the male ejaculate and the female reproductive tract interact and are subject to sexual selection and sexual conflict (Birkhead and Pizzari, 2002). Ejaculate traits, such as sperm length and seminal fluid proteins (Sfps), can be subject to strong postcopulatory sexual selection (Ramm et al., 2009; Rowe et al., 2015). In the zebra finch, *Taeniopygia guttatta*, males with longer sperm sire a greater proportion of offspring (Bennison et al., 2014) and, in *Drosophila melanogaster*, longer sperm are better competitors as they both resist displacement and displace competing males sperm from the female sperm storage organs (Lüpold et al., 2012). Likewise, Sfps that are transferred along with sperm in the ejaculate can influence the outcome of sperm competition (Fedorka et al., 2011; Holman, 2009; Wigby et al., 2009). For instance, Acp29AB is necessary for retention of sperm in storage and is associated with improved sperm defence (P1) (Wong et al., 2008), and Acp36DE influences sperm storage dynamics and improves sperm offence (P2) (Avila and Wolfner, 2009). Molecular evidence suggests Sfps are subject to strong sexual selection, exhibiting among the fastest rates of positive selection of any genes or proteins in the animal kingdom (Findlay et al., 2009).

The rapid evolution and turnover of reproductive genes may have implications for the emergence of reproductive isolation between populations. Postmating prezygotic (PMPZ) isolation involves incompatibilities between the male ejaculate and the female reproductive tract, or incompatibilities at the gamete cell surfaces. As the male ejaculate and female reproductive tract co-evolve within populations, a non-coevolved ejaculate that is, for example, either deficient of particular Sfps or results in incorrect protein-protein interactions and subsequent cell signalling, may not elicit an optimal fertility response in mated females (Howard et al., 2009; McDonough et al., 2016; Pitnick et al., 2009). In *D. melanogaster*, over 200 Sfps have been identified (Findlay et al., 2009, 2008; Mueller et al., 2005). A handful of these Sfps have known functions, including aiding in sperm transfer, transport and storage in the female after mating, and inducing postmating female responses, including increased fecundity and feeding, and reduced remating (Avila et al., 2010; Ravi Ram and Wolfner, 2007; Wolfner, 2009). Gene knockouts in *D. melanogaster* investigating the role of known Sfps produce phenotypes similar to PMPZ isolation phenotypes, such as reduced fecundity or problems with sperm storage (Ravi Ram and Wolfner, 2007). Thus, the rapid evolution and turnover of Sfps along with their fundamental role during reproduction suggests Sfps as promising targets to study the evolution of PMPZ isolation (McDonough et al., 2016).

As ejaculate-female reproductive tract interactions evolve rapidly, the male ejaculate is predicted to diverge between even closely related populations. Here we test whether populations of the malt fly, *Drosophila montana*, that exhibit PMPZ isolation differ in the proteomic composition of the male seminal fluid producing organs: the paired accessory glands, and ejaculatory duct and bulbs. Females from populations within North America and Finland mate with foreign males and appear to store sperm normally. However, between-population crosses result in reduced fertility, as females lay unfertilised eggs (Garlovsky and Snook, 2018; Jennings et al., 2014). Furthermore, crosses (within North America) exhibit conspecific sperm precedence (chapter 4) (Price, 1997). To test whether populations differ in the abundance of Sfps which might be involved in PMPZ isolation, we compared the abundance of Sfps produced by the two main *Drosophila* male Sfp secretory organs, the paired accessory glands and the ejaculatory duct and bulb (Fig. 4.1). We first provide the first description of the *D. montana* accessory glands and ejaculatory duct and bulb proteomes using liquid chromatography mass

spectrometry (LC-MS/MS) and performed GO analyses and differential abundance analyses. We constructed both an accessory gland proteome (AgP) and ejaculatory duct and bulb proteome (EbP), as recent studies have shown that these tissues may provide discrete functions and contribute different Sfps to the ejaculate (Sepil et al., 2019).

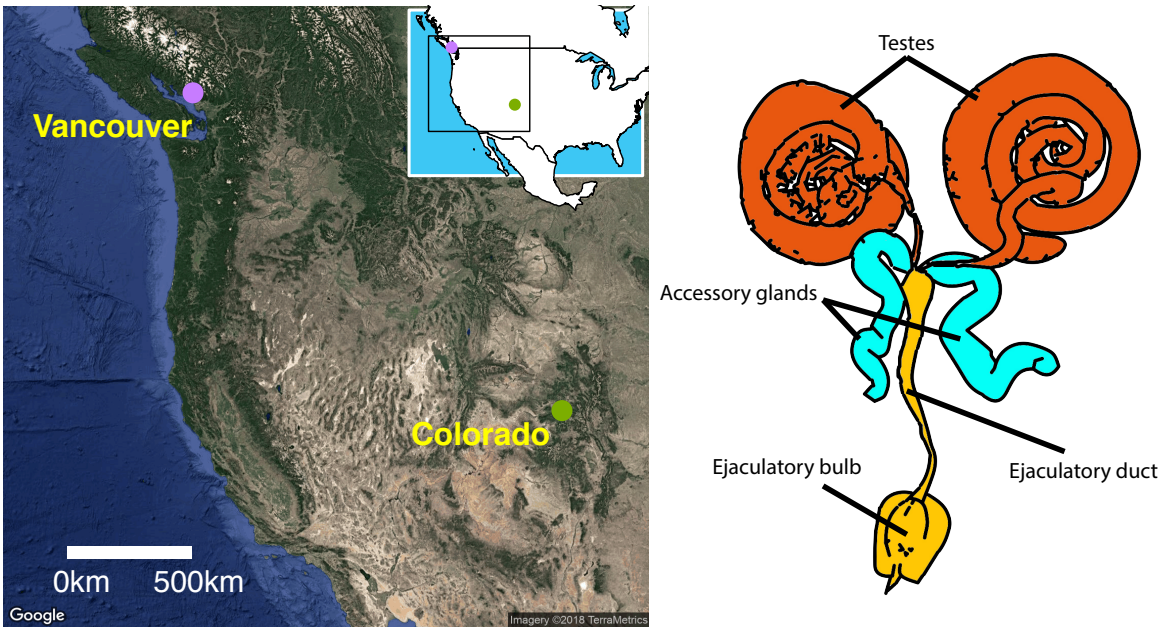

Figure 4.1. Left: Collection locations of *Drosophila montana* populations. Right: male *D. montana* reproductive tract highlighting tissues harvested; paired accessory glands (blue) and ejaculatory duct and bulb (yellow). The testes (orange) were discarded. Image modified from flybase.org after Patterson (1943).

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; C), and Vancouver, British Columbia, Canada (48°55'N, 123°48'W) in 2008 (referred to as Vancouver; V) (Fig. 4.1). Stocks were subsequently cultured on Lakovaara malt media (Lakovaara, 1969) in overlapping generations in constant light at 19^{o}C. Flies used for dissections were collected within 3 days of eclosion and housed in groups of between 10-20 same sex individuals in food vials until reproductively mature at 21 days old.

Tissue collection

Twenty-one-day old males were anaesthetised with ether, the abdomen removed with insect pins, and placed in a drop of phosphate buffered saline (PBS). The whole reproductive tract (testes, accessory glands, and ejaculatory duct and bulb) was moved to a second drop of PBS, rinsed, the testes removed from the rest of the reproductive tract and discarded (Fig. 4.1). The ejaculatory duct and bulb were separated from the accessory glands, intact where possible. Each tissue was washed in a third drop of PBS and placed in a LoBind Eppendorf tube containing 15µl lysis buffer (5% SDC; 1% SDS) and protease inhibitor cocktail kept on ice. After reproductive tissues were harvested from 15 males the combined sample was freeze/thawed three times; placed on dry ice for 5 minutes, then placed in a water bath at 20^{o}C for 30 seconds and vortexed for 30 seconds. Each sample was then centrifuged at 20000G for 5 minutes at 4^{o}C, supernatant collected and placed in a new Eppendorf and stored at -80^{o}C until further processing.

Replicate information

In total we collected three biological replicates for each tissue and each population. Biological replicate 1 consisted of 30 individuals per population (collected over two days), replicates 2 and 3 were 15 individuals each. Biological replicate 1 was divided in to two equal volumes after tissue collection and processed separately to give a technical replicate of sample processing (see below). Two equal volumes of biological replicate 2 were loaded on to the mass spectrometer

to give a technical replicate of the LC-MS/MS data acquisition. Biological replicate 3 was run in singlet. This gave a total of 5 LC-MS/MS runs for each tissue from each population (Fig. S4.1).

Protein purification and quantification

We performed Bradford assays to quantify protein concentration in our samples for downstream SDS-PAGE, comparing absorbance readings of 1µl of each sample to Bovine Serum Albumin (BSA) standard (0.15µg/µl) at 595nm with 20µl of Bio-Rad® protein assay reagent and diluted to a final volume of 100µl. To quantify protein concentration to standardise loading volumes on to the mass spectrometer we ran 1µl of each sample with Lammeli buffer on an SDS-PAGE gel (Fig. S4.2) and performed densitometry in GelAnalyzer (www.gelanalyzer.com).

Protein samples were processed with a HiPPR™ detergent removal kit (Thermo Fisher™, Catalogue number: 88305) and reduced in 2µl tris(2-carboxyethyl)phosphine (TCEP) (50mM) and incubated at 60^{o}C for 1 hour, allowed to cool then alkylated by addition of 1µl methyl methanethiosulfonate (MMTS) (200 mM) for 10 minutes at room temperature. Samples were then treated with a 1:20 trypsin:protein dilution in NH_4HCO_3 (100mM) overnight at 37^{o}C and then dried to completion by vacuum centrifugation. All samples were then resuspended in 20µl AMBIC (3% v/v acetonitrile, 0.1% v/v trifluoroacetic acid) ready for analysis by LC-MS/MS.

To assess protein recovery after sample processing with the HiPPR™ detergent removal kit we harvested and processed whole male reproductive tracts (accessory glands and ejaculatory ducts and bulbs) from the Vancouver 2008 cage as described above. We then compared SDS-PAGE gel bands of 30µl of protein before and after HiPPR™ detergent removal as per the manufacturer's instructions. This control showed good recovery of protein after detergent removal (Fig. S4.3).

Liquid chromatography mass spectrometry (LC-MS/MS) data acquisition

A 105-minute data dependent acquisition (DDA) method was set up on the QExactive HF (Thermo Fisher™). The full MS scan was from 375-1500 m/z acquired in the Orbitrap at a resolution of 120,000 in profile mode. Subsequent fragmentation was Top 10 in the HCD cell,

with detection of ions in the Orbitrap using centroid mode, resolution 30,000. The following MS method parameters were used for MS1: Automatic Gain Control (AGC) target 1e6 with a maximum injection time (IT) of 60 ms and MS2: Automatic Gain Control (AGC) target 1e5, maximum injection time (IT) of 60 ms and isolation window 2 Da. The intensity threshold was 3.3e4, normalized collision energy 27, charge exclusion was set to unassigned, 1, exclude isotopes was on, apex trigger deactivated. The peptide match setting was preferred with dynamic exclusion of 20 seconds.

Protein identification

Quantitative proteomic analysis for label free quantification was performed using the MaxLFQ algorithm (Cox et al., 2014) in MaxQuant (Tyanova et al., 2016) with mass spectra matched to the *D. montana* predicted proteome. Detailed description of the proteome construction can be found in Parker et al. (2018). Briefly, the proteome was generated using gene predictions from the Maker2 pipeline (Holt and Yandell, 2011) reciprocally blasted against *D. virilis* proteins.

Gene ontology (GO) and functional analysis

We retrieved *D. melanogaster* orthologs for peptide sequences identified from the *D. montana* predicted proteome using BLASTp (NCBI Resource Coordinators, 2016). We then successfully converted NCBI accession numbers to flybase gene numbers (FBgns) for 85% (1459/1711) of proteins using the uniprot.org web interface. We imported these FBgns in to Cytoscape (Shannon et al., 2003) and performed network analyses separately for the AgP and EbP using the ClueGO plugin (Bindea et al., 2009). We set the following settings for network groups. For biological processes we set GO tree levels min = 1, max = 2; GO term restriction min number of genes = 50; min percentage = 10%; Kappa score threshold = 0.4. For cellular components; GO tree levels min = 1 min, max = 4; GO term restriction min number of genes = 50; min percentage = 0%; Kappa score threshold = 0.5. For molecular functions: GO tree levels min = 1, max = 4; GO term restriction min number of genes = 20; min percentage = 0%; Kappa score threshold = 0.5. For all networks, groups consisted of two GO terms minimum and $\geq$ 50% sharing of terms and GO term fusion only showing pathways with $p \leq 0.01$. For GO enrichment we used a right-sided hypergeometric test with Benjamini-Hochberg multiple test

correction. For determine GO grouping differences between Colorado and Vancouver in the AgP and EbP we used default settings. We also assigned gene names to the *D. montana* predicted proteome using Blast2GO (Götz et al., 2008).

Differential abundance analysis

Statistical analyses were performed in R (v.3.5.1) (R Core Team, 2018). To test for differential protein abundance between populations we restricted the data sets to include only proteins identified in all 5 replicates for each tissue and each population. We fitted linear mixed effects models for each protein using *lmer* from 'lme4' (Bates et al., 2017). We tested for differential abundance between populations using the $\log_2$(ion intensity) values with population (Colorado or Vancouver) as the only fixed effect and a random effect to account for repeated measures of biological replicates. We obtained p-values using likelihood ratio tests, comparing full models to a null (intercept only) model. We corrected the resulting p-values for multiple testing using the Benjamini-Hochberg procedure.

RESULTS

In total, we identified 1703 proteins from the *D. montana* predicted proteome. Both sets of technical replicates (sample processing and LC-MS/MS) were highly correlated (all adjusted $R^2 > 0.71$; Fig. S4.4) and the majority of proteins identified in each tissue were found in all 5 technical replicates of that tissue (CAG: 773/1080 = 72%; CEB: 993/1288 = 77%; VAG: 804/1131 = 71%; VEB: 1011/1457 = 69%) (Fig. S4.5). The majority of proteins identified, 60% (1022/1703), were shared between the accessory gland proteome (AgP) and the ejaculatory duct and bulb proteome (EbP), while 175 (10%) proteins were unique to the AgP, and 506 (30%) were unique to the EbP (Fig. 4.2). Proteins only identified in the AgP had a less than 1x fold decrease in abundance compared to all proteins, and proteins only identified in the EbP had a 5-fold decrease in abundance compared to all proteins (Fig. 4.2). Furthermore, visual inspection of the abundances of proteins with annotations retrieved from Blast2GO showed protein annotations associated with one tissue were more highly abundant in that tissue (Fig. S4.6). Thus at least some of these differences between the AgP and EbP in protein identity likely represent real differences.

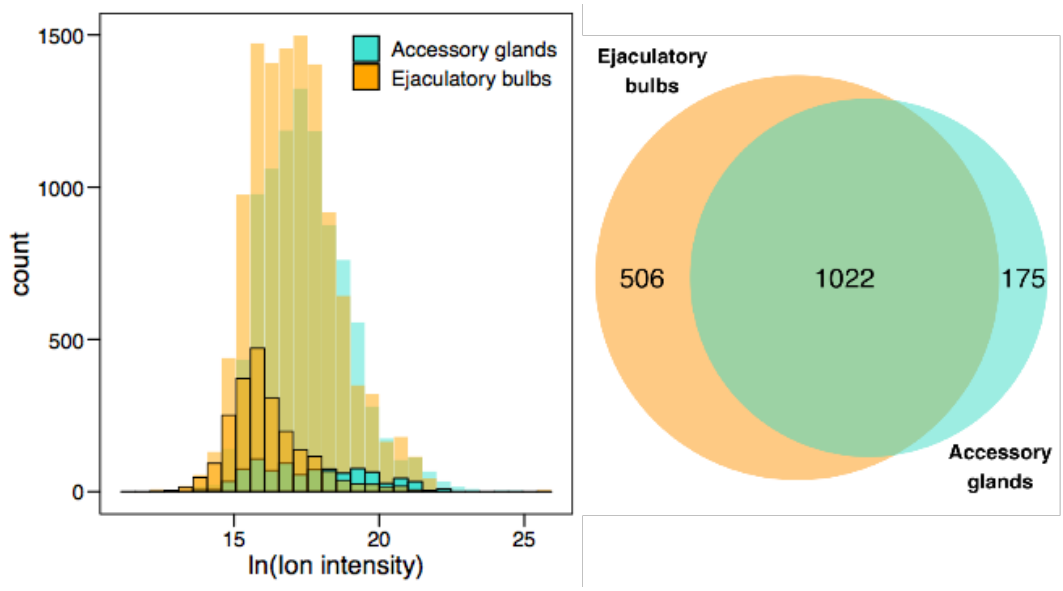

Figure 4.2. The AgP and EbP share a large proportion of proteins but also have unique components. Left: Total abundance of proteins (n = 1703) identified in the AgP (blue) and EbP (yellow). Distribution of unique proteins found in the AgP (n = 175) and the EbP (n = 506) shown in black outline. Right: Venn diagram of total number of proteins across all replicates.

The D. montana *AgP and EbP include known Sfps*

A subset of the proteins we identified overlapped with previously identified *Drosophila* Sfps from the literature (Findlay et al., 2009, 2008; Mueller et al., 2005). We identified 31, and 25 (out of 212) *Drosophila* Sfps in the AgP, and the EbP respectively. These proteins included four Sfps that comprise part of the Sex Peptide (SP) network, but not SP itself (Singh et al., 2018). In both the AgP and EbP we identified three of the eight SP network proteins: Seminase (CG10586), lectin-46Cb (CG1652), and CG17575, and unique to the AgP we also identified aquarius (CG14061).

Gene ontology and functional analysis of the AgP and EbP

Our gene ontology (GO) analyses identified both the AgP and EbP as having functions predicted for highly metabolically active secretory organs. For the AgP, GO analysis using biological processes resulted in 93.4% of FlyBase gene numbers (FBgns) being annotated, comprising a 40-node network in 12 major groups. The dominant GO categories were single-organism cellular process, developmental process involved in reproduction, single-organism (metabolic) processes, and macromolecule localisation (Fig 4.3). For cellular components GO annotation was achieved for 89.4% of FBgns in a 37-node network in 14 groups. The major categories were: mitochondrion, organelle membrane, intracellular organelle, and intracellular non-membrane-bounded organelle (Fig. 4.3). For molecular function 91.1% of FBgns were annotated in a 34-node network in 10 categories. The major groups were: small molecule binding, active ion transmembrane transport activity, actin binding, and structural constituent of ribosome (Fig. 4.3).

For the EbP, GO analysis for biological processes resulted in 91.4% of FBgns annotated in a 38-node network consisting of 13 major categories. The top categories were; cellular component organisation or biogenesis, single-organism metabolic process, macromolecule localisation, and anatomical structure morphogenesis (Fig. 4.4). For cellular components 87% of FBgns were annotated in a 42-node network consisting of 16 major GO categories. The top categories were organelle membrane, mitochondrion, endomembrane system, and membrane protein complex (Fig. 4.4). For molecular function, GO annotation was achieved for 88.7% of FBgns, resulting in a 38-node network in 22 major categories. The major categories were: nucleotide binding,

purine nucleoside binding, cofactor binding, active ion transmembrane transported activity, actin binding (Fig. 4.4). Thus, while both the AgP and EbP have functions expected for organs with secretory function, there is some different functionality between tissues.

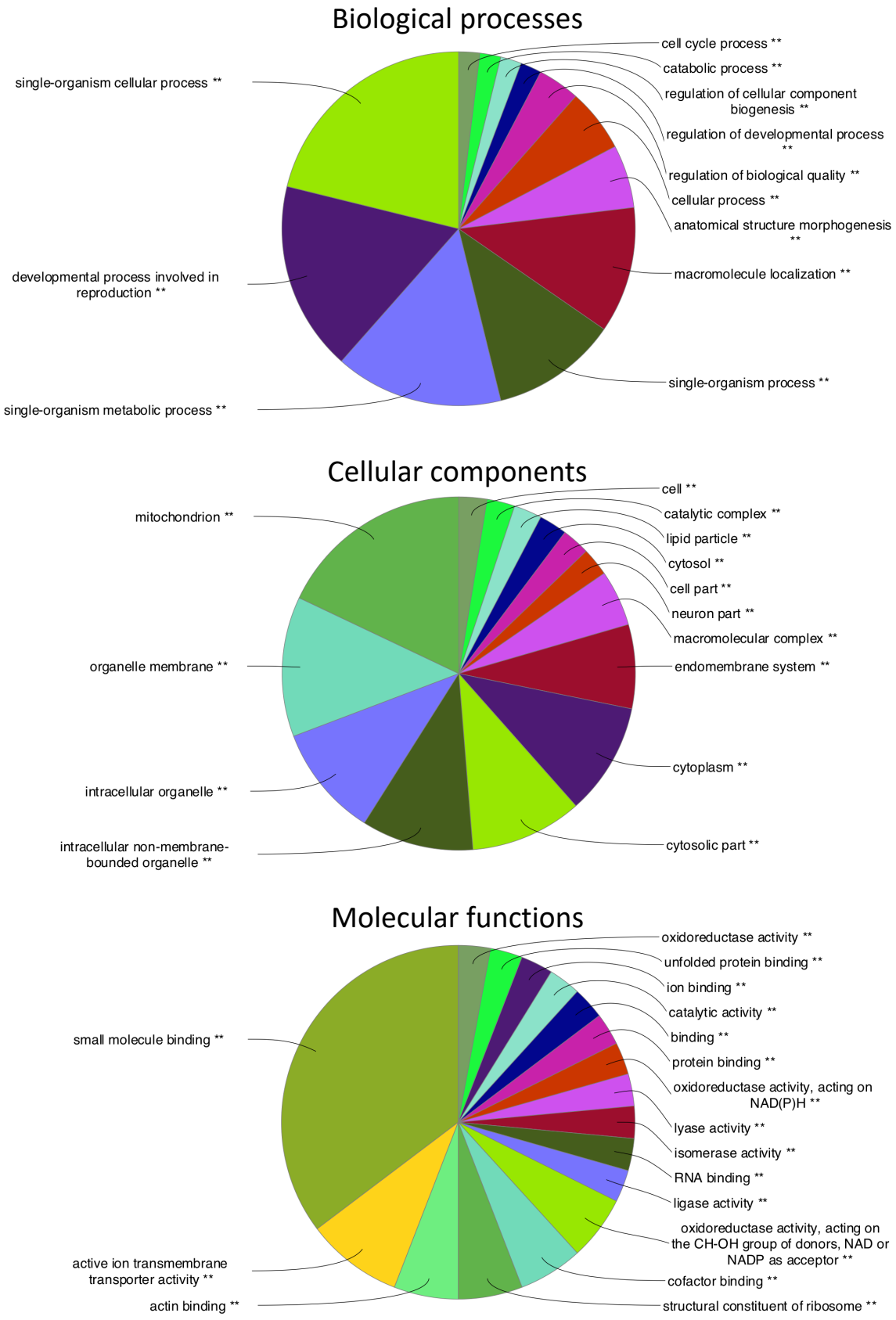

Figure 4.3. ClueGO gene ontology (GO) major categories for genes identified in the accessory gland proteome (AgP). See supplementary information for network views.

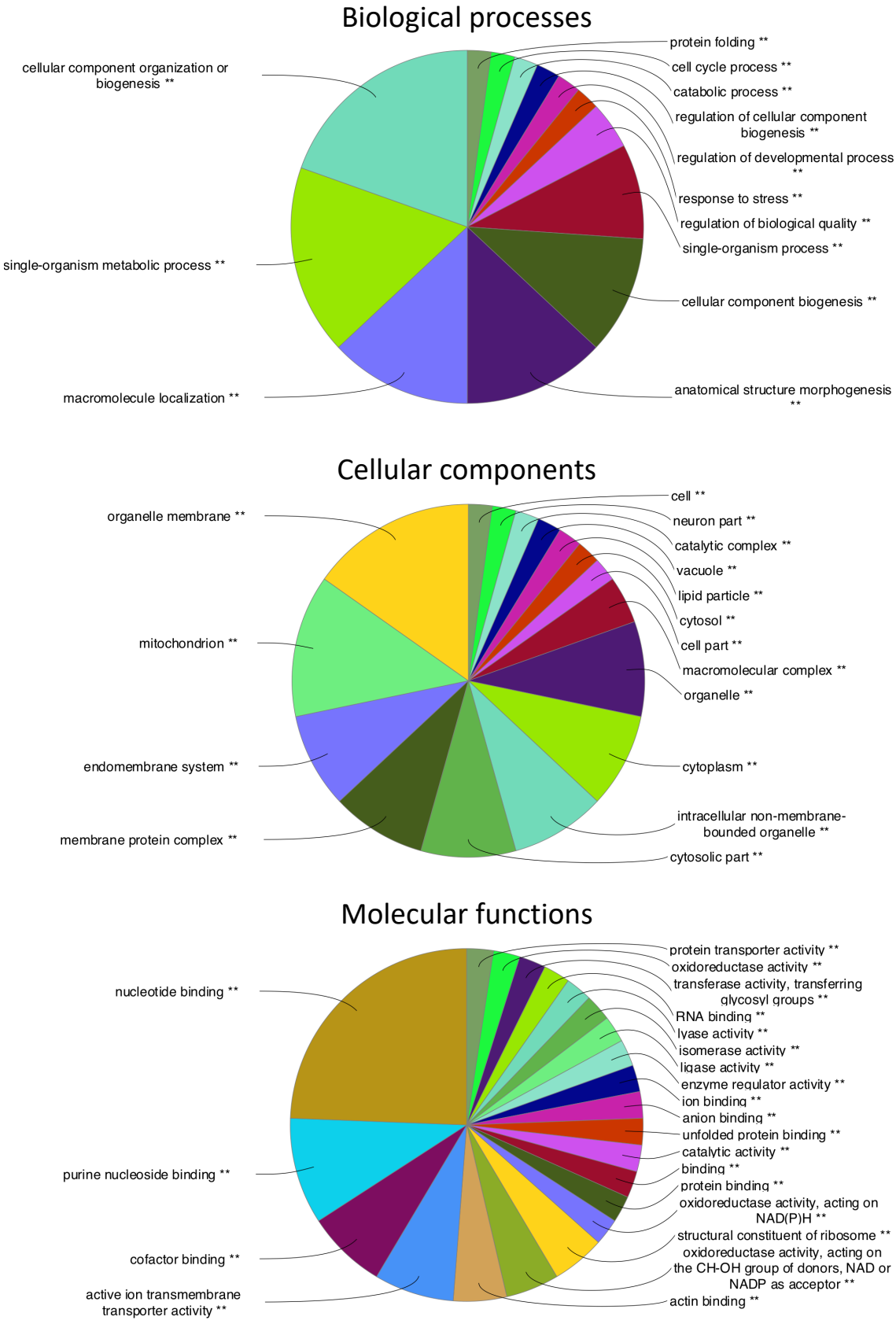

Figure 4.4. ClueGO gene ontology major categories for genes identified in the ejaculatory duct and bulb proteome (EbP). See supplementary information for network views.

Differential abundance analysis

For quantitative analysis we considered the AgP and EbP separately. In the AgP 1014 (84.7%) proteins were shared between Colorado and Vancouver, 66 (5.5%) proteins were unique to Colorado, and 117 (9.8%) proteins were unique to Vancouver (Fig. S4.7). In the EbP 1217 (79.7%) proteins were shared between populations, 71 (4.6%) were unique to Colorado, and 240 (15.7%) were unique to Vancouver (Fig. S4.7). Proteins only identified in one population showed a 20-fold reduction in abundance in the AgP (Colorado = 20-fold, Vancouver = 19-fold) and a more than 24-fold reduction in the EbP (Colorado = 38-fold, Vancouver = 25-fold) (Fig. S4.7). Therefore, low abundance proteins only identified in one population were likely missed rather than truly unique. To test for differential protein abundance, we restricted the data sets to proteins identified in all 5 replicates for each tissue and each population (Fig. S4.5). For the AgP the high confidence data set comprised 729 proteins and for the EbP 933 proteins (Fig. 4.5).

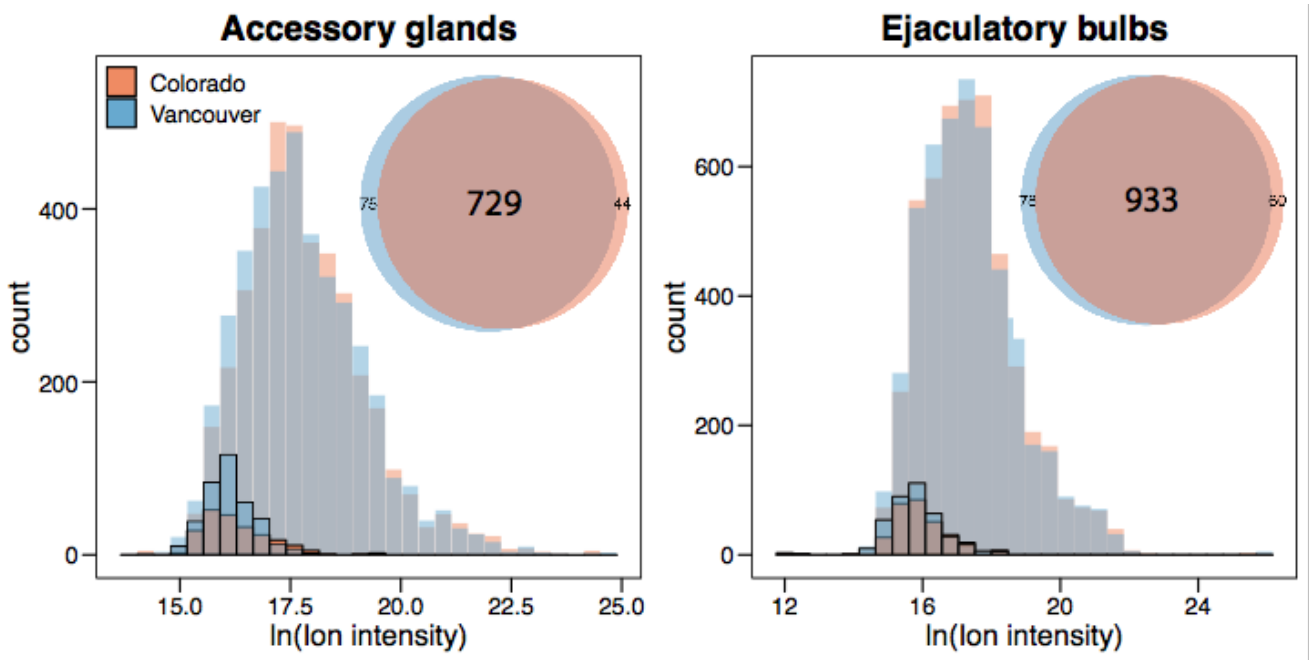

Figure 4.5. Protein abundance for proteins found in all 5 replicates for each tissue and each population. Distribution of proteins identified in only one population shown in black outline.

In the AgP we identified 90 differentially abundant proteins between Colorado and Vancouver (Fig. 4.6). Five of the proteins we identified (CG4815, CG6461, CG8050, CG10363, CG1803) overlapped with known *D. melanogaster* Sfps. The full list of differentially abundant AgP

proteins can be found in Table S4.1. Of these, 41 (46%) were more abundant in Colorado, and 49 (54%) were more abundant in Vancouver. GO analysis for differentially abundant proteins in the AgP showed enrichment for biological processes including translational initiation, cellular lipid catabolic processes, lipid oxidation, and carboxylic catabolic processes (Table 4.1). To determine the function and cellular location of these differentially abundant proteins between the two populations we performed GO grouping in Cytoscape and ClueGO which showed genes involved in translational initiation (biological processes) and translational initiation factor activity (molecular function) were enriched in Vancouver, and aminopeptidase activity were enriched in Colorado (Fig. S4.8).

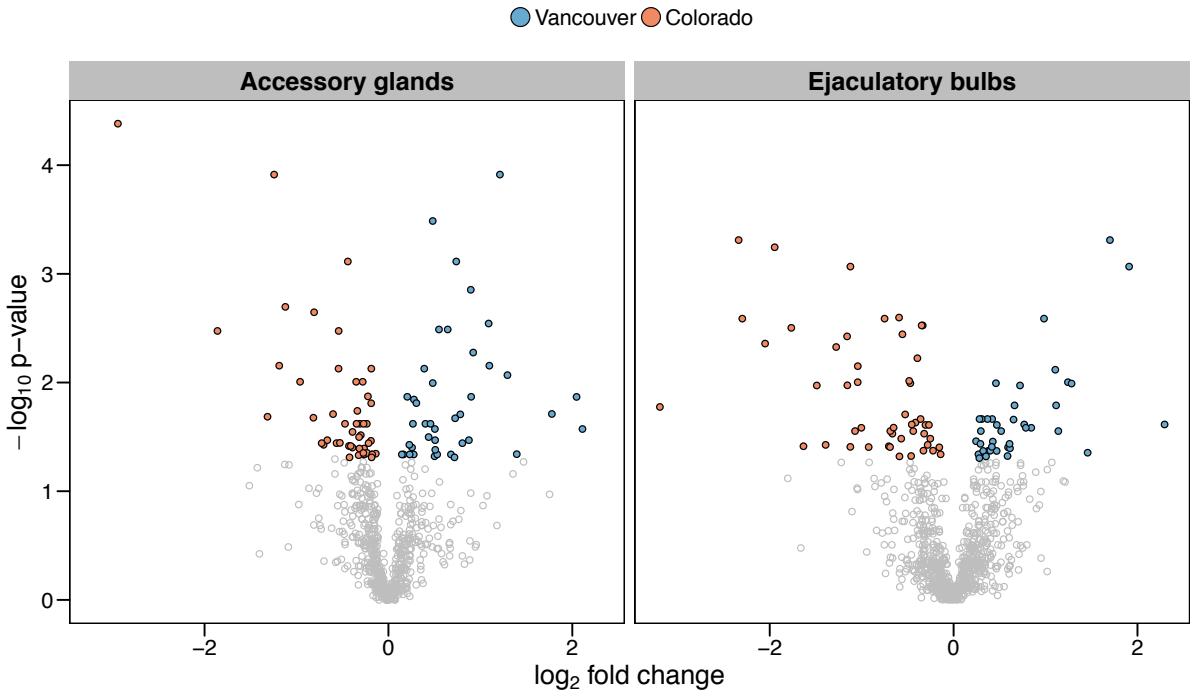

Figure 4.6. Volcano plots showing differentially abundant proteins between Colorado and Vancouver in the AgP (left) and the EbP (right). Proteins showing significant differential abundance after correction for multiple testing are coloured based on the population showing significantly higher abundance. Colorado, red; Vancouver, blue.

Table 4.1. Enriched gene ontology (GO) terms for differentially abundant proteins found in the accessory gland proteome (AgP).

	GO term	No. Genes	P-value*
Biological processes	translational initiation	5	<0.001
	cellular lipid catabolic process	5	<0.001
	lipid oxidation	5	<0.001
	carboxylic acid catabolic process	5	<0.001
	fatty acid catabolic process	5	<0.001
	fatty acid oxidation	5	<0.001
	monocarboxylic acid catabolic process	5	<0.001
	fatty acid beta-oxidation	5	<0.001
Molecular functions	translation initiation factor activity	3	0.01
	oxidoreductase activity, acting on the CH-OH donors, NAD or NADP as acceptor	5	<0.001
	peptide binding	3	0.01
	aminopeptidase activity	4	<0.001
	metalloexopeptidase activity	3	0.01

*Benjamini-Hochberg corrected

In the EbP, 89 proteins were differentially abundant, of which 40 (45%) were more abundant in Colorado and 49 (55%) more abundant in Vancouver (Fig. 4.6). The five known Sfps identified as differentially abundant in the AgP were all also identified in the EbP. The full list of differentially abundant EbP proteins can be found in Table S4.2. GO analysis GO analysis for differentially abundant proteins in the EbP showed enrichment for biological processes including organic acid catabolic processes, cellular lipid catabolic processes, lipid oxidation, and carboxylic acid catabolic processes (Table 4.2). GO groupings showed genes involved in monocarboxylic acid catabolic processes (biological processes) and hydro-lyase activity (molecular function) were enriched in Vancouver, and NAD binding (molecular function) were enriched in Colorado (Fig. S4.9).

Table 4.2. Enriched gene ontology (GO) terms for differentially abundant proteins found in the ejaculatory duct and bulb proteome (EbP).

	GO term	No. genes	P-value*
Biological processes	organic acid catabolic process	4.00	<0.001
	cellular lipid catabolic process	4.00	<0.001
	lipid oxidation	3.00	<0.001
	carboxylic acid catabolic process	4.00	<0.001
	fatty acid catabolic process	3.00	<0.001
	fatty acid oxidation	3.00	<0.001
	monocarboxylic acid catabolic process	3.00	<0.001
	fatty acid beta-oxidation	3.00	<0.001
Molecular functions	oxidoreductase activity, acting on the CH-OH donors, NAD or NADP as acceptor	6.00	<0.001
	NAD binding	4.00	<0.001
	carbon-oxygen lyase activity	3.00	<0.001
	hydro-lyase activity	3.00	<0.001

*Benjamini-Hochberg corrected

DISCUSSION

Seminal fluid proteins (Sfps) are predicted to be involved in the emergence of PMPZ isolation given their rapid evolution may generate mismatched ejaculate x female reproductive tract interactions. Here we test whether populations of *D. montana* that exhibit PMPZ isolation vary in their production of proteins found in the Sfp secretory organs - the accessory glands and the ejaculatory duct and bulb. To test this, we first had to identify putative Sfps. We accomplished this using a high throughput LC-MS/MS approach, followed by GO analysis, which supported secretory functions for these tissues. We identified over 1700 proteins in the accessory gland proteome (AgP) and the ejaculatory duct and bulb proteome (EbP), of which a subset are known Sfps. Our analyses also identified some differences between tissues in function. Using these data, we determined whether proteins in the AgP and the EbP shared between populations showed differential abundance. We found a subset of proteins, about 100 in each tissue, that were differentially abundant.

The primary goal of this work was to determine whether the male Sfp secretory organs differed in composition between populations exhibiting PMPZ isolation. We identified 90, and 89 differentially abundant proteins between Colorado and Vancouver in the AgP, and the EbP, respectively. Five of the differentially abundant proteins we identified, all five of which were found in both the AgP and EbP, are known *D. melanogaster* Sfps. All but one of these proteins, regucalcin (CG1803), were also identified in a recent study which characterised the accessory gland proteome of *D. pseudoobscura* (Karr et al., 2019). Thioester-containing protein 4 (Tep4, CG10363) is involved in immunity and inflammation response in *D. melanogaster* (Shokal et al., 2018). CG4815, a serine protease, may function as a digestive enzyme (Ross et al., 2003). γ-glutamyl transpeptidase (Gtg-1, CG6461), a secreted peptidase, may function to maintain a protective redox environment for sperm (Walker et al., 2006). Cystatin-like (Cys, CG8050), is a protease inhibitor (Delbridge and Kelly, 1990). Finally, regucalcin (CG1803) is involved in cold acclimation in *D. montana* (Vesala et al., 2012) and is currently being investigated for its role in sperm aging in *D. melanogaster* (T. L. Karr, unpublished data). Future research should aim to investigate these, and other, of our list of differentially abundant putative Sfps for their potential role in mediating ejaculate-female reproductive tract interactions and PMPZ isolation.

A secondary aim of our analysis was to characterise differences between the paired accessory glands and the ejaculatory duct and bulb, the two major seminal fluid secretory organs in *Drosophila*. Few studies have characterised the ejaculatory duct or bulb proteome (Ahmed-Braimah et al., 2017). Unfortunately, data was not publicly available from the only relevant study to make comparisons with our own. For both the AgP and EbP, GO analyses showed enrichment for terms consistent with the recognised function of these tissues as secretory organs involved in reproduction. This is consistent with other recent work investigating the seminal fluid proteome in *Drosophila spp.* (Karr et al., 2019; Sepil et al., 2019) and other taxa (Bayram et al., 2019; Rowe et al., 2018). We also found the two tissues possibly provide different functions, evidenced by the unique proteins identified in each tissue, and the associated differences in GO terms between them. Thus, our data support other recent findings that suggest the ejaculatory duct and bulb, as well as the accessory glands, may provide important, and perhaps distinct reproductive functions (Sepil et al., 2019).

We found only a fraction of proteins in the AgP and EbP overlapped with previously identified Sfps in *D. melanogaster*, perhaps unsurprising given the rapid evolution of Sfps (Findlay et al., 2009). Included in our data sets were some members of the Sex Peptide (SP) network, but notably not SP itself (Findlay et al., 2014; Singh et al., 2018). We may not have identified SP due to the bias against small proteins and stochastic nature inherent in LC-MS/MS data acquisition. However, SP was also not found in the *D. pseudoobscura* seminal fluid proteome (Karr et al., 2019). A recent study found significant variation in female postmating responses after receipt of SP between wild-type *D. melanogaster* populations, suggesting rapid co-evolution and turnover within species (Wensing and Fricke, 2018). Although beyond the scope of the current study, future work will test the molecular evolutionary rates of Sfp divergence between populations using our data sets.

To conclude, the rapid co-evolution of ejaculate-female reproductive tract interactions is expected to result in the emergence of PMPZ isolation early during speciation. We identified a number of differentially abundant proteins in the male Sfp secretory organs between populations of *D. montana* which have previously shown PMPZ isolation via both the inability of between-population sperm to fertilise eggs, and conspecific sperm precedence. Our list of

differentially abundant proteins included several known Sfps in *D. melanogaster*. Future work will target some of the putative Sfps we identified to determine if they contribute to PMPZ isolation in *D. montana*. We also provide the first description and analyses of the *D. montana* accessory glands and the ejaculatory duct and bulb proteomes, which exhibit different reproductive functionality. Overall, this work provides novel insights into the evolution of PMPZ isolation and the function and evolution of seminal fluid proteins generally.

5. Sexual selection shapes the evolution of physiological and life history traits

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CONTRIBUTIONS

ALB, MDG, RRS and ZKN collected data on metabolite composition, juvenile development time, desiccation and starvation resistance and metabolic rates, respectively. RRS and GA designed the experiments. MDG and GA performed statistical analyses. MDG and RRS wrote the manuscript.

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ABSTRACT

Many non-sexual traits underpin organismal performance and reproductive success. Yet, few studies have directly tested how physiological and life history traits respond to the strength of sexual selection. Here we show coordinated trait divergence in multiple physiological and life history traits using experimental sexual selection. Males evolving under enforced monogamy had lower metabolic rates than males evolving under polyandry and were more reliant on carbohydrates as metabolic fuel. In contrast, polyandrous individuals invested in lipid and glycogen storage associated with more efficient energy metabolism and regulation. Despite increased energy storage, polyandrous males were less stress resistant than monogamous males, suggesting a trade-off with investment in current reproduction and sexually selected traits. Development time was extended under polyandry relative to monogamy in both sexes, potentially due to additional investment in traits linked to sexual selection and sexual conflict. Overall, males evolving under enforced monogamy had inefficient energy metabolism and resource management whereas polyandrous males had increased energy efficiency but at a cost to development time and stress resistance. Our results show sexual selection and sexual conflict can impact multiple aspects of organismal performance beyond those traits directly involved in reproduction.

KEYWORDS: Sexual selection, polyandry, metabolism, life history evolution, trade-offs, experimental evolution.

INTRODUCTION

Variation in the operational sex ratio dictates the strength of sexual selection and sexual conflict acting within populations, with important consequences for the evolution of traits involved in sexual interactions and reproduction (Arnqvist and Rowe, 2005; Orr and Garland, 2017; Zera and Harshman, 2001). Many aspects of organismal performance underpin reproductive success, such that traits under sexual selection will also capture components of non-sexual fitness (Lailvaux and Irschick, 2006). Female multiple mating in polyandrous mating systems increases the opportunity for pre- and post-copulatory sexual selection and sexual conflict. These forces can shape the evolution of traits contributing to mating and fertilisation success (Andersson,

1994; Birkhead and Pizzari, 2002). Polyandry may have direct benefits to males and females (Slatyer et al., 2012). Sexual selection can also benefit the population, "purging the genome" of deleterious mutations (Rowe and Houle, 1996; Tomkins et al., 2004; Whitlock and Agrawal, 2009). Thus, under polyandry, heightened inter- and intra- sexual selection is expected to weed out unfit males, resulting in fitter, more competitive individuals, while relaxed sexual selection will allow poor-quality males to persist in populations. For instance, experimental removal of sexual selection can lead to reduced competitive or cognitive abilities (Bacigalupe et al., 2007; Firman et al., 2015; Hollis and Kawecki, 2014).

The expression of sexually selected traits often depends upon the underlying condition of an individual, reflecting their physiological state, contingent upon a broad range of genetic and environmental factors (Martinossi-Allibert et al., 2017). Energetically costly traits are often favoured by both inter- and intra- sexual selection (Clark, 2012; Kotiaho, 2001) and secondary sexual traits often require increased energy expenditure (Basolo and Alcaraz, 2003) and/or resource allocation towards growth and maintenance (Emlen et al., 2012). Sexual selection may also favour repeated bouts of sustained locomotor activity, for example during scramble competitions, elaborate courtship displays, and competition for access to mates or territories (Debelle et al., 2017; Gyulavári et al., 2014; Hunt et al., 2004). Similarly, postcopulatory sexual selection favouring ejaculate investment (Linklater et al., 2007; Lüpold et al., 2016) may pose a significant metabolic cost to males (Immonen et al., 2016). Conflicts over courtship, mating, and fertilisation can also lead to the evolution of persistence and resistance traits that require increased energy expenditure (Córdoba-Aguilar and González-Tokman, 2011; Franklin et al., 2012; Watson et al., 1998). Thus, sexual selection and sexual conflict should favour physiological adaptations such as metabolic machinery, respiratory substrate use, and energy storage systems that efficiently provide metabolic energy (Gyulavári et al., 2014; Montooth et al., 2003).

Investment in sexually selected traits may also result in trade-offs with other aspects of fitness and changes in life history strategies (Simmons et al., 2017; Zera and Harshman, 2001). For instance, males possessing exaggerated traits, or investing more in sexual displays, may incur survival costs (Hunt et al., 2004; Romiti et al., 2015) or delayed reproductive maturity (Pitnick

et al., 1995). Sexual conflict also plays an important role in the evolution of life history strategies (Arnqvist and Rowe, 2005; Wedell et al., 2006). For instance, intralocus sexual conflict over body size and development time due to different selection pressures acting on females and males (e.g. fecundity selection vs. sexual selection) can lead to one or both sexes deviating from their phenotypic optimum (Blanckenhorn et al., 2007).

Despite these individual studies, experimental integrative studies assessing how the sexual selection landscape impacts the evolution of physiological mechanisms underlying organismal performance and life history strategies has not been performed. Here, we implemented an experimental evolution approach to investigate how sexual selection and sexual conflict affect the evolution of physiological and life history traits. We used replicate populations of *Drosophila pseudoobscura* subjected to either elevated polyandry (P) or enforced monogamy (M). In the wild, female *D. pseudoobscura* mate multiply (Anderson, 1974; Cobbs, 1977), prompting bouts of inter- and intra- sexual selection, both before and after mating. Snook and colleagues have shown divergence between sexual selection treatments in a number of traits subject to pre- and post-copulatory sexual selection. Polyandrous males produce more abundant and complex chemical signals (cuticular hydrocarbons) (Hunt et al., 2012), perform a faster and more vigorous courtship song (Debelle et al., 2017), and have larger accessory glands (Crudgington et al., 2009). The male biased sex ratio in the polyandrous lines also intensifies sexual conflict; polyandrous females are courted more frequently (Crudgington et al., 2010) and are more resilient to male harm and coercion (Crudgington et al., 2005; Debelle et al., 2014). Sex-specific gene expression has diverged between treatments. Polyandrous females show greater enrichment for genes with reproductive function while monogamous females show enrichment for somatic tissue function (Immonen et al., 2014). Enforced monogamy also resulted in masculinisation of sex-biased genes in the transcriptome with variation in response both across different tissues and in mating context (e.g. virgin or mated) (Veltsos et al., 2017). Thus, differential investment between experimental evolution treatments in traits subject to sexual selection and sexual conflict has altered traits from gene expression to morphology to behaviour. This evolutionary response provides an excellent opportunity to test predictions about how physiological and life history traits underpinning whole organismal fitness respond to changes

in the intensity of sexual selection. To that end, we tested whether and to what extent sexual selection influenced a suite of key physiological and life history traits.

METHODS

Establishment and maintenance of experimental evolution lines

Detailed description of the establishment and maintenance of the experimental evolution lines can be found in Crudgington et al. (2005). Briefly, the ancestral population was established from 50 wild-caught, inseminated female *Drosophila pseudoobscura* collected in Tucson, Arizona in 2001. From the ancestral population four replicate populations for each of the sexual selection treatments were established in successive generations (except replicate 4 which was established two generations after replicate 3). The opportunity for sexual selection and sexual conflict was manipulated by housing one male with one female (enforced monogamy treatment, M), or six males with one female (elevated polyandry treatment, P). The number of families (vials containing the appropriate ratio of males:females for the corresponding treatment) was altered between the M and P treatments to control for variation in autosomal effective population size (Snook et al., 2009). In each generation, males and females were allowed to interact for 5 days in 'interaction vials' (IVs) before transfer to 'oviposition vials' (OVs) for a further 5 days to reduce potential larval competition and ensure sufficient opportunity for episodes of pre- and post-copulatory sexual selection. To ensure representation of all families in the next generation, all offspring within each replicate population were collected en masse and a random sample of the offspring housed using the appropriate sex ratio to establish the next generation. Flies were kept at 22^{o}C on a 12:12 light:dark cycle on standard cornmeal-agar-molasses media with added live yeast, with a generation time of 15 days.

Experimental individuals

Prior to all experimental protocols (described below) flies were taken out of selection. Newly eclosed individuals were collected from the OVs within each replicate population en masse. A random sample of these flies were allowed to mate and oviposit for two days. From these eggs, we set up controlled density vials (CDVs), picking 100 first instar larvae in to vials containing food. Flies eclosing from CDVs were collected as virgins, stored in same-sex food vials, and

used for experiments at between 3-5 days old. Thus, experimental flies experienced the same "common garden" environmental conditions.

Statistical analysis

All statistical analyses were performed in R version 3.5.1 (R Core Team, 2018). Linear mixed effects models (LMMs) were fitted using the 'nlme' package (Pinheiro et al., 2018); survival analyses using the 'coxme' and 'survival' packages (Therneau, 2018, 2015); and partial least squares regression using the 'matrixpls' package (Rönkkö, 2017).

Juvenile development time

We measured juvenile development time at generations 180, 179, 178 and 176 for replicates 1-4, respectively. For each replicate of the M and P treatments, we seeded 6 CDVs (see above) on three consecutive days (i.e. 600 larvae per replicate population per seeding day = 14,400 larvae total). On the day of eclosion, emerging flies were CO_2 anaesthetised and killed in ethanol. We continued collecting until no individuals eclosed for two consecutive days. The number of flies emerging each day from each vial were later counted and sexed. We analysed development time with mixed effects Cox proportional hazards models. Time (days elapsed since seeding day) to event (eclosion) was used as the response. We included sexual selection treatment, sex, and the treatment x sex interaction as fixed effects, and sex nested in experimental evolution line, seeding day, and vial ID as random effects. Flies that did not eclose within the observation period were right censored on the last collection day. Sex was assigned to censored individuals by calculating the observed sex ratio of eclosees from each vial and assigning the appropriate sex ratio to the remaining uneclosed individuals of the 100 larvae initially seeded (assuming an equal 50:50 sex ratio of larvae). Four vials were excluded from analysis due to overseeding.

We used the length of wing vein IV as a proxy for body size (Crudgington et al., 2005; Gilchrist et al., 2001). We measured a random subsample of individuals (n = 15 per sex per replicate per seeding day where available) that emerged on the peak eclosion day. The left wing was removed from flies preserved in ethanol using fine forceps and mounted on a microscope slide in a drop of phosphate-buffered saline (PBS) and dried at room temperature overnight. Digital

photographs of wings were taken using a Motic camera and Motic Images Plus 2.0 software (Motic Asia, Hong Kong). Image files were anonymised prior to measurement using a custom Python script. The length of wing vein IV was measured using ImageJ software (Schneider et al., 2012). We used a LMM to analyse body size differences. Sexual selection treatment, sex, and the treatment x sex interaction were included as fixed effects and sex nested in experimental evolution line and seeding day as random effects.

Metabolic rates

We measured metabolic rates at generations 196, 195, 194 and 192 for replicates 1-4, respectively. Within each selection line, flies were placed in groups of three same sex, same age triads. Each triad, representing a sample in our design (n = 3 per sex per replicate), was weighed to the nearest 0.1 mg (Sartorius Genius ME 235P-OCE) before transfer to a respirometry chamber (a glass cylinder; 17mm x 70 mm).

Metabolic rate was measured using a Sable Systems (Las Vegas, NV, USA) respirometry system (Lighton, 2008). This system pumps air at a precisely regulated flow rate through a sealed chamber containing animals with a known weight. Downstream gas analysers measure the amount of CO_2 produced and O_2 consumed, providing estimates of metabolic parameters. Briefly, the respirometry system was set up in stop-flow mode (Lighton, 2008), in which each chamber was sealed for 60 min and then flushed for 2.5 min. Each cycle (through all 24 chambers) lasted for 62.5 min and each measuring session resulted in four consecutive cycles with four readings of CO_2 produced and O_2 consumed in each individual chamber, of which the first was discarded as a wash-out and the second – fourth were used for analyses. Each respirometry chamber was placed in an activity detector (AD-2, Sable Systems) connected to a data acquisition interface (Quick-DAQ, National Instruments, Coleman Technologies, Newton Square, US), which uses reflective infrared-light technology to provide a precise and continuous measure of locomotor activity of the subjects in each chamber during the entire session. One of the 24 chambers was left empty and used as a baseline to control for any drift of the gas analysers during each session (washed out twice in each cycle). Thus, each observation consisted of three consecutive readings of the amount of CO_2 produced and O_2 consumed during 62.5 minutes by a triad of flies, under dark conditions, with a known weight

and total amount of activity performed. The ratio of CO_2 produced:O_2 consumed (the respiratory quotient; RQ), which indicates the oxidative fuel used for respiration, was used to test for differences in the use of metabolic substrates. Respiratory quotients of 0.7 indicate fatty acid oxidation, 0.8-0.9 indicate protein oxidation, and 1.0 indicate pure carbohydrate oxidation. We analysed metabolic metrics (mean values of CO_2 production, O_2 consumption, or RQ, recorded during the three measurement cycles for each triad of flies) using LMMs. Sexual selection treatment, sex, activity, body weight, all two-way and three-way interactions were used as fixed effects and sex nested in experimental evolution line as a random effect. We mean centred activity within each sexual selection treatment as polyandrous flies were significantly more active than monogamous flies (LMM; $F_{1,6} = 29.08$, $p = 0.002$; Fig. S5.1), and body weight was mean centred within each sex as females were larger than males (LMM; $F_{1,6} = 37.19$, $p < 0.001$; Table S5.1) (Schielzeth, 2010).

Metabolite extractions

We measured metabolite composition at generations 199, 198, 197 and 195 for replicates 1-4, respectively. For each replicate of the M and P treatments, single sex triads of mature flies eclosing from CDVs were weighed to the nearest 1µg (METTLER TOLEDO® UMX2 ultra-microbalance) and flash frozen in liquid nitrogen (n = 3 per sex per replicate). Each triad was then placed in a 0.35ml glass vial insert (SUPELCO Analytical®) of known weight, dried at 55ºC overnight and re-weighed to obtain a dry weight.

Lipids

To extract lipids, 200µl of hexane (Fisher scientific®) was added to each sample, which was then vacuum infiltrated and incubated at room temperature overnight. The supernatant was discarded, and samples dried overnight at 55ºC. The lipid content was determined by subtracting the dry weight after hexane extraction from the initial dry weight.

Soluble carbohydrates

After hexane extraction, samples were placed in 200µl of 80% ethanol (Fisher scientific®), vacuum infiltrated, and incubated at room temperature overnight. The supernatant was discarded, and samples dried overnight at 55ºC. The soluble carbohydrate content was

calculated as the dry weight after hexane extraction minus the dry weight after ethanol extraction.

Soluble protein

After ethanol extraction, dried samples were transferred to a screwcap tube (SUPELCO Analytical®) and ground before adding 200µl of Tris buffer (20mM, pH 7.0; Fisons Analytical Reagents®) and centrifuged at 16000G. A 10µl aliquot of supernatant from each sample was loaded on a 96-well plate containing 200µl bicinchoninic acid protein assay reagent (Bio-Rad®). Protein concentrations were determined using standards of bovine serum albumin (SIGMA-Aldrich®) at an absorbance of 562nm (FLUOstar OPTIMA® plate reader, BMG labtech).

Glycogen

Glycogen extraction protocol was modified from Caporn et al. (Caporn et al., 1999). Remaining samples in Tris buffer were autoclaved before adding 100µl of MES (500mM, pH 4.5; SIGMA-Aldrich®), containing 4 units of α-amylase from *Aspergillus oryzae* (SIGMA-Aldrich®) and 14 units of Amylglucosidase from *A. niger* (SIGMA-Aldrich®) and incubated at 37^{o}C for 4 hours. Samples were centrifuged at 16000G and 50µl of supernatant loaded on a 96 well-plate containing 200µl of 100mM HEPES (pH 7.4; Roche®), 5mM magnesium chloride (Fisons Analytical Reagents®), 1.6mM NAD (SIGMA-Aldrich®), 4mM ATP, 0.5 U glucose-6-phospate dehydrogenase (Roche®). Glucose concentration was determined by the addition of 0.5 units of hexokinase (Roche®) taking readings at 340nm.

Chitin

After centrifugation in Tris buffer, the pellet was incubated at 100^{o}C for two hours with KOH (BDH Laboratory Reagents®) to remove enzyme contaminants, centrifuged again, and the pellet then washed three times in ddH20. The remaining residue was transferred into the original vial insert (used to obtain dry weight), dried and reweighed. Chitin content was determined by subtracting the final dry weight from the initial dry weight.

Multivariate data analysis

Data on metabolite composition is inherently multivariate and we characterized metabolite composition in our samples using the following five variables collectively: lipids (mg/mg), sugars (mg/mg), protein (µg/mg), glycogen (µg/mg), and chitin (mg/mg). We analysed variation in metabolite composition by inspecting the multivariate vector describing differences in metabolite composition across (1) monogamous and polyandrous lines and (2) males and females, using the following analytical strategy. We first performed an omnibus test using a multivariate analysis of variance (MANOVA), with sexual selection treatment, sex, and the treatment x sex interaction as factors and the matrix of metabolites (mean values per line and sex) as response variables. We then used partial least squares (PLS) regression to examine whether and how multivariate metabolite composition differed between sexual selection treatments. A PLS model aims to find the multivariate relationships between two matrices, in our case the metabolite matrix and the classifier matrix, by modelling their covariance structures in a latent variable approach (Carrascal et al., 2009). To characterize differences in metabolite composition across sexual selection treatments, we fitted PLS models using all available data for each sex separately, as males and females differ markedly in metabolite composition (Marron et al., 2003). The PLS models were evaluated by bootstrapping (10k bootstrap replicates) following correction for axis-reversal using the *matrixpls.boot* function from the 'matrixpls' package (Rönkkö, 2017). Differences between male and female vectors were tested with t-tests of the bootstrap mean loadings and standard errors and 6 degrees of freedom to reflect the number of selection lines (df = 8 – 2 = 6).

Desiccation and starvation resistance

We measured desiccation and starvation resistance at generations 199, 198, 197 and 195 for replicates 1-4, respectively. For each replicate of the M and P treatments, single sex triads of mature flies eclosing from CDVs (n = 20 per sex per replicate) were housed in 8-dram plastic vials stoppered with cotton balls and covered with Parafilm®. For the desiccation resistance assay, vials contained no food and between the cotton and Parafilm® we placed a packet of silica gel beads. For the starvation resistance assays, vials contained an agar solution that provided moisture but no food. Vials were checked every 2 hours and any deaths recorded until all flies perished. Flies were scored as dead if they were not able to right themselves or no

movement was observed (e.g. (Folk et al., 2001)). Preliminary analysis indicated violation of the proportional hazard assumption due to crossing hazards. Therefore, we used accelerated failure time models with a Weibull distribution to model survival. Time (in hours) to event (death) was used as the response, with sexual selection treatment, sex, and the treatment x sex interaction as fixed effects. We included experimental evolution line as a frailty term (random effect) with 6 degrees of freedom to reflect the number of selection lines (df = 8 – 2 = 6).

RESULTS

Juvenile development time

Sexual selection treatment had a significant effect on juvenile development time (Cox proportional hazards model; $X_1^2 = 3944.12$, $p < 0.001$) as did sex ($X_1^2 = 20.04$, $p < 0.001$). Development time was significantly longer in the polyandrous treatment in both sexes (Hazard ratio = 0.38; 95% confidence intervals [CI] = 0.23 – 0.63) and males took longer to eclose than females (Hazard ratio = 0.82; 95% CI = 0.78 – 0.88) (Fig. 5.1). There was a significant effect of sex on body size (LMM; $F_{1,6} = 989.7$, $p < 0.001$); as expected, females were larger than males (females: 2329 ± 3.70 µm (mean ± standard error), n = 270; males: 2106 ± 3.83 µm, n = 281); but there was no effect of sexual selection treatment (LMM; $F_{1,6} = 0.17$, $p = 0.69$) or the treatment x sex interaction (LMM; $F_{1,6} = 0.09$, $p = 0.77$) (Table S5.2). Therefore, the effect of sexual selection treatment on development time cannot simply be attributed to differences between treatments in body size.

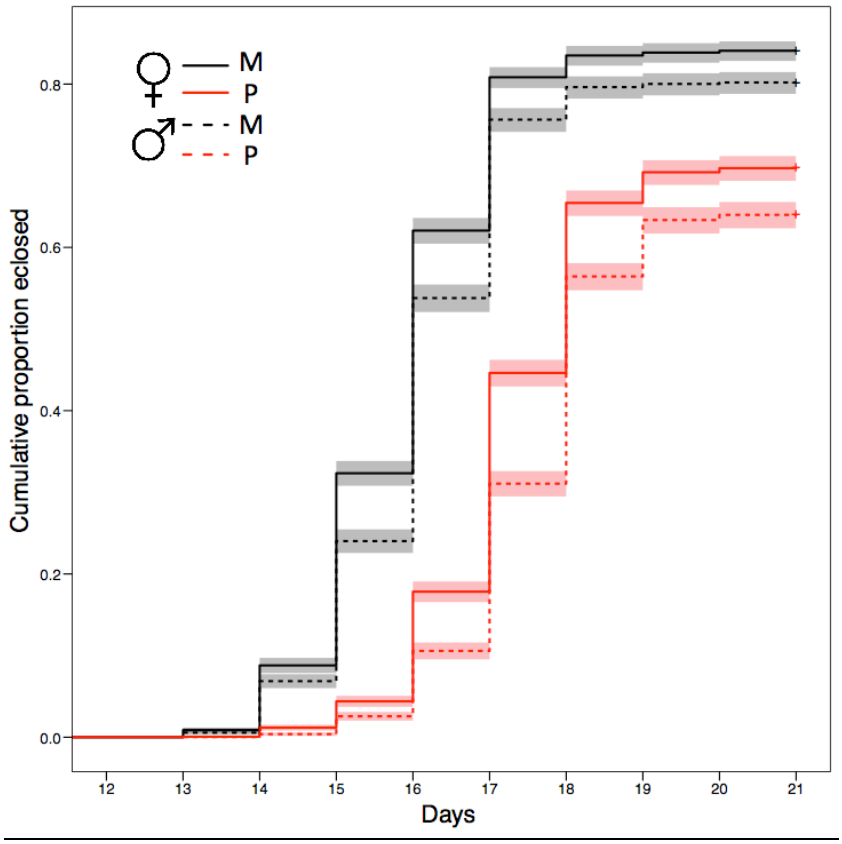

Figure 5.1. Kaplan-Meier survival curves (± 95% confidence intervals) for time (in days) elapsed from 1st instar larvae until eclosion. M, monogamy (black lines); P, polyandry (red lines); females, solid lines; males, dashed lines. Crosses indicate right censored individuals (n = 3552).

Metabolic rates

Linear mixed-effects models with sex nested in experimental evolution line and sample ID as random effects showed activity and body weight were both significant predictors of CO_2 production (activity: $F_{1,95} = 34.96$, $p < 0.001$; body weight: $F_{1,31} = 5.04$, $p = 0.032$) and O_2 consumption (activity: $F_{1,95} = 35.52$, $p < 0.001$; body weight: $F_{1,31} = 4.95$, $p = 0.034$) but not of RQ (activity: $F_{1,95} = 0.78$, $p = 0.379$; body weight: $F_{1,31} = 0.35$, $p = 0.561$). CO_2 production and O_2 consumption were highly correlated ($r = 0.96$) and analyses of each metabolic metric yielded similar results; here we present results for CO_2 production only. There was a significant three-way interaction effect between sexual selection treatment x sex x body weight (LMM; $F_{1,24} = 9.305$, $p = 0.006$; Table 5.1). Monogamous males had relatively low metabolic rates independent of body size, while metabolic rate increased with body size in polyandrous males (Fig. 5.2). In females, metabolic rate increased with body size under monogamy, while

polyandrous females had relatively high metabolic rates independent of body size (Fig. 5.2). In sum, sexual selection treatment had contrasting effects on metabolic rates in males and females with metabolic rates lower in monogamous males while polyandry selected for high metabolic rates in both sexes.

Table 5.1. Results from linear mixed-effects models investigating the effects of predictors on metabolic rate (mean volume of CO_2 produced) and the respiratory quotient (RQ).

			Metabolic rate		RQ	
Source	**numDF**	**denDF**	***F***	***p***	***F***	***p***
Selection	1	6	27.786	0.002	0.184	0.683
Sex	1	6	9.018	0.024	1.290	0.299
Activity	1	24	17.310	< 0.001	3.338	0.080
Body weight	1	24	0.764	0.391	0.043	0.837
Selection x sex	1	6	2.270	0.183	5.405	0.059
Selection x activity	1	24	0.313	0.581	1.380	0.252
Selection x body weight	1	24	1.972	0.173	0.079	0.781
Sex x activity	1	24	0.621	0.439	0.295	0.592
Sex x body weight	1	24	0.013	0.911	1.330	0.260
Selection x sex x activity	1	24	1.423	0.245	2.552	0.123
Selection x sex x body weight	1	24	9.305	0.006	0.370	0.549
Random effects			σ^2		σ^2	
Line			<0.001		0.001	
Sex			<0.001		<0.001	
Residual			<0.001		0.007	

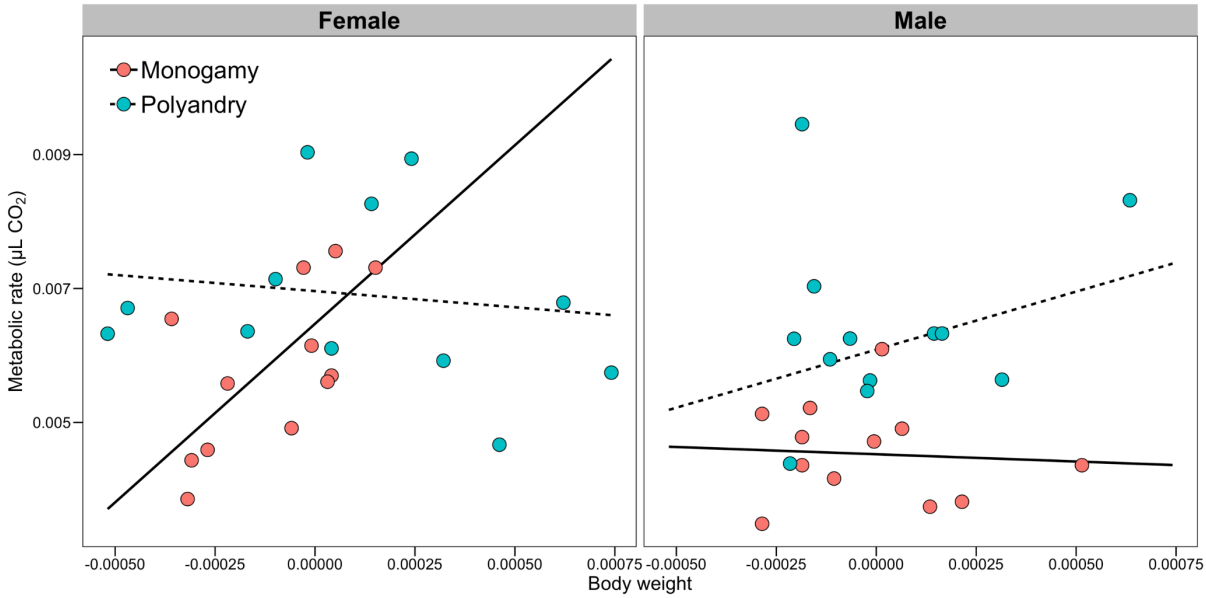

Figure 5.2. Metabolic rate (mean volume of CO_2 produced) in females (left) and males (right) and model predicted lines. Monogamy, red circles and solid lines; Polyandry, blue circles and dashed lines.

Monogamy males were relatively more reliant on carbohydrates as metabolic fuel than other groups (selection x sex interaction; $p = 0.059$; monogamy males: RQ = 0.95 ± 0.028 (mean ± SE); polyandry males: 0.88 ± 0.03; monogamy females: 0.89 ± 0.04; polyandry females: 0.90 ± 0.03; Fig. 5.3; Table 5.1;).

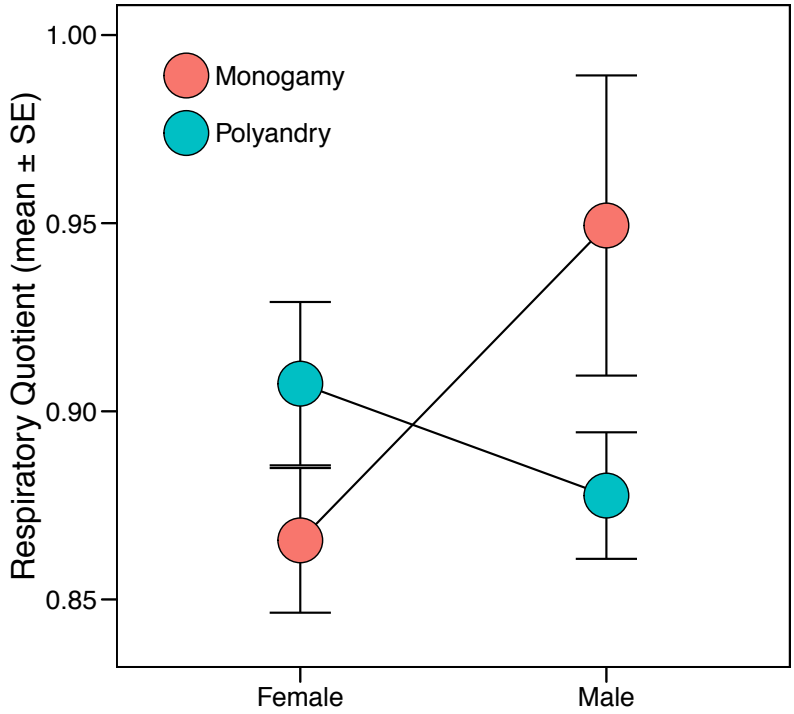

Figure 5.3. Respiratory quotient (mean ± standard error). M, monogamy (red); P, polyandry (blue).

Metabolite composition

Females were larger than males (LMM: $F_{1,6} = 278.1$, $p < 0.001$; females: 1.68 ± 0.03 mg [wet weight], n = 24; males: 1.05 ± 0.02 mg, n = 24) and males and females differed significantly in metabolite composition, primarily due to females having relatively more lipids and males more chitin in the metabolite pool (MANOVA; Wilks' $\lambda = 0.253$, $F_{5,8} = 4.72$, $p = 0.026$). This analysis also identified a significant effect of sexual selection treatment (Wilks' $\lambda = 0.286$, $F_{5,8} = 3.99$, $p = 0.041$), such that flies from polyandrous lines showed more lipids than flies from monogamous lines (Table S5.3 & S5.4, Fig. S5.2).

Inspection of the sex-specific PLS models showed the vector that best differentiated between flies from polyandrous and monogamous lines was dominated by the relative amount of lipids in the pool of metabolites in both sexes, validating the results of the MANOVA. Sexual selection significantly affected sex specific metabolite composition (Fig. 5.4). Glycogen content was positively associated with polyandry in males but not in females ($t = 2.43$, df = 6, $p = 0.025$) and protein content was positively associated with monogamy in females but not in males ($t = 2.15$, df = 6, $p = 0.037$).

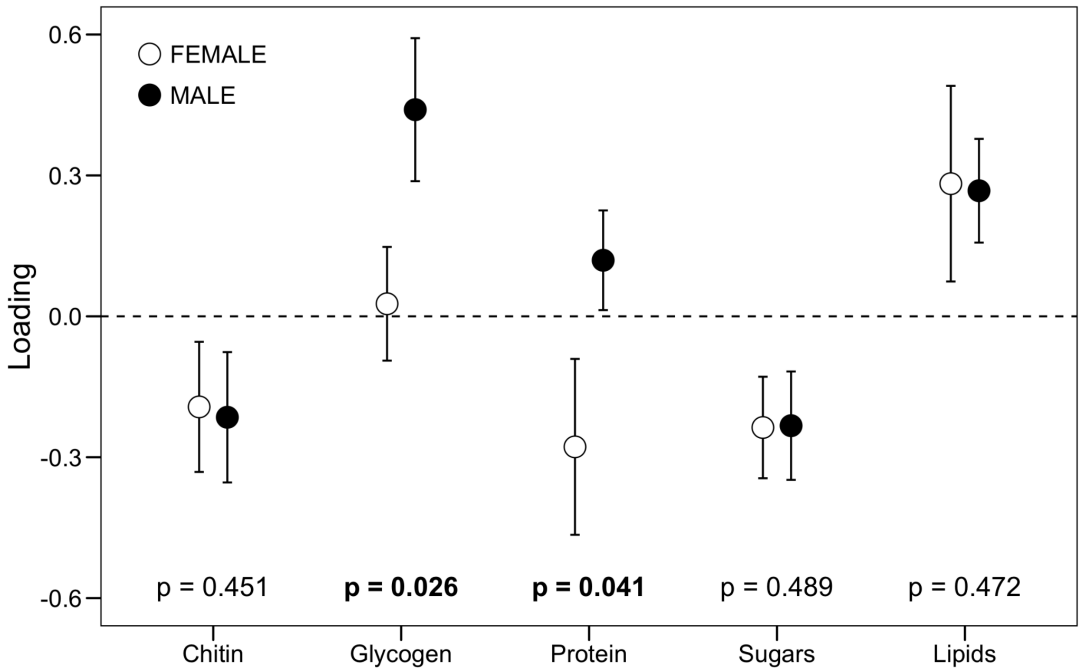

Figure 5.4. Loadings on the latent PLS variable that best separates monogamous from polyandrous lines in males (closed circles) and females (open circles). Shown are bootstrap mean loadings (± 95% confidence intervals) for the sex-specific multivariate vectors. Positive loadings indicate higher values in polyandrous lines; negative loadings indicate higher values in monogamous lines. P-values represent *t*-tests of a difference in loading between males and females.

Desiccation and starvation resistance

Desiccation resistance showed a significant effect of sexual selection treatment ($X^2 = 3.85$, df = 1, $p = 0.050$), sex ($X^2 = 154.90$, df = 1, $p < 0.001$), and the treatment x sex interaction ($X^2 = 8.68$, df = 1, $p = 0.003$). Polyandrous flies had a lower mortality risk than monogamous flies (Hazard ratio = 0.74 ± 0.35 [estimate ± standard error]) and males had a higher mortality risk than females (Hazard ratio = 2.87 ± 0.87). To investigate the treatment x sex interaction, we analysed each sex separately, revealing a significant effect of sexual selection treatment on desiccation resistance in females ($X^2 = 5.00$, df = 1, $p = 0.025$) and in males ($X^2 = 5.03$, df = 1, $p = 0.025$) but in opposite directions; polyandrous females had a lower mortality risk than monogamous females (Hazard ratio = 0.73 ± 0.22), while polyandrous males had a higher mortality risk than monogamous males (Hazard ratio = 1.48 ± 0.20) (Fig. 5.5a).

Starvation resistance showed no significant main effect of sexual selection treatment ($X^2 = 2.31$, df = 1, p = 0.129), but there was a significant effect of both sex ($X^2 = 210.44$, df = 1, $p < 0.001$) and the treatment x sex interaction ($X^2 = 20.91$, df = 1, $p < 0.001$). Males had a higher mortality risk than females (Hazard ratio = 3.89 ± 0.88). To investigate the treatment x sex interaction, we analysed each sex separately, which again showed a significant effect of sexual selection treatment on starvation resistance in females ($X^2 = 5.86$, df = 1, p = 0.015) and males ($X^2 = 8.72$, df = 1, p = 0.003) in opposite directions; polyandrous females had a lower mortality risk than monogamous females (Hazard ratio = 0.60 ± 0.26), while polyandrous males had a higher mortality risk than monogamous males (Hazard ratio = 1.42 ± 0.33) (Fig. 5.5b).

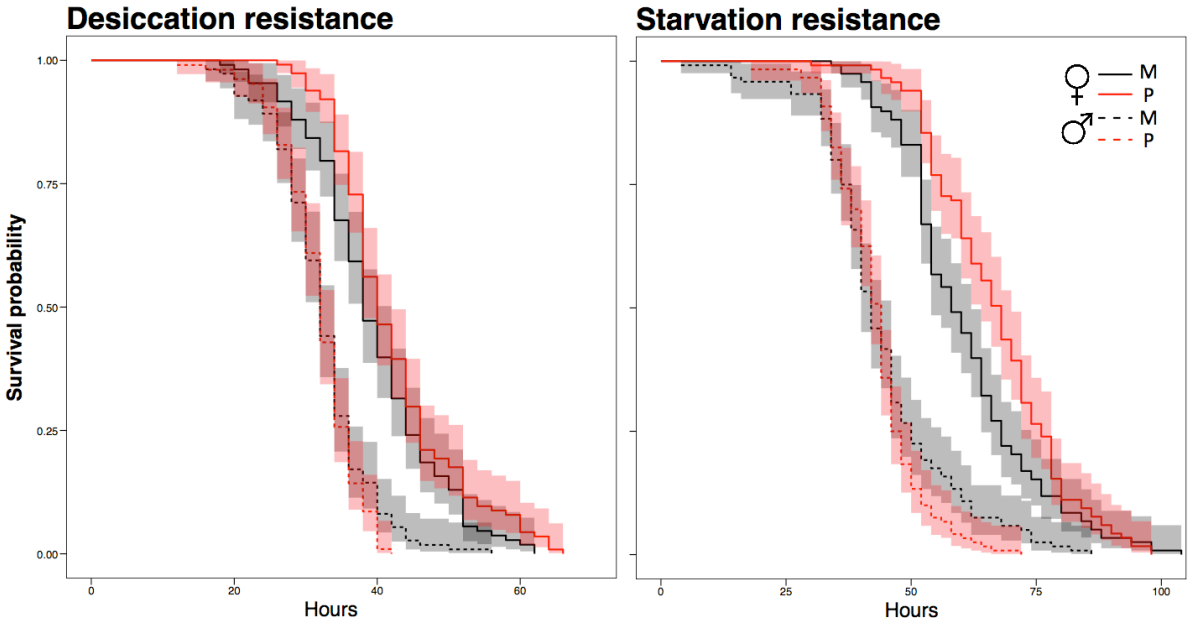

Figure 5.5. Kaplan-Meier survival curves (± 95% confidence intervals) for flies under desiccation (left) and starvation (right) conditions. M, monogamy, black lines; P, polyandry. red lines; females, solid lines; males, dashed lines. Note different time scales on the x axes.

DISCUSSION

We directly showed divergence in a number of key physiological and life history traits under divergent experimental sexual selection and sexual conflict. Juvenile development time shifted substantially such that polyandrous males and females took longer to develop than their monogamous counterparts. Metabolic rates and oxidative substrate use diverged such that monogamous flies had lower metabolic rates and monogamous males were more reliant on energy-poor carbohydrates as fuel. Along with reduced general activity and courtship, this indicates monogamy males have evolved into "couch potatoes". For females, metabolic rate increased with body size under monogamy, whereas polyandrous females had high metabolic rates independent of body size. Thus, higher metabolic rates are selected for under polyandry. Why higher metabolic rates are selected for under polyandry is currently not well understood. However, some possible explanations are discussed below. Metabolite composition showed divergence between sexual selection treatments, with lipid content positively associated with polyandry, perhaps providing energy stores for sustained activity, while chitin and sugar content were positively associated with monogamy. There were also sex-specific effects on metabolite composition. Glycogen, the primary fuel used during intense activity, was positively associated with polyandry in males, while protein content was positively associated with monogamy in females. Finally, polyandrous females were more resistant to desiccation and starvation than monogamous females, whereas polyandrous males were less resistant to these stressors than monogamous males.

Experimental evolution revealed divergence between sexual selection treatments in metabolism and the abundance and types of respiratory fuel available for respiration. Both signalling and competitive traits have diverged between sexual selection treatments. Compared to monogamous males, polyandrous males produce a faster and more vigorous courtship song (Debelle et al., 2017, 2014), more cuticular hydrocarbons (Hunt et al., 2012), and have larger accessory glands (Crudgington et al., 2009). These traits may be energetically costly to produce and maintain, demanding greater metabolic activity (Immonen et al., 2016; Reinhold, 1999). Correspondingly, the physiological demands necessary to invest in sexually selected traits may drive changes in the underlying metabolic machinery (Montooth et al., 2003). We calculated

the respiratory quotient, which indicated monogamous males were more reliant on carbohydrates as metabolic fuel than other energy sources. Carbohydrates are the main fuel used during intense aerobic activities, such as flight (Wigglesworth, 1949) and courtship (Bertram et al., 2011), however, monogamous males are less active and invest less in metabolically challenging behaviour than polyandrous males (Crudgington et al., 2010, 2009; Debelle et al., 2017). This pattern suggests relaxed sexual selection and sexual conflict has resulted in monogamy males evolving into couch potatoes. Monogamous males mobilise energy resources normally used for strenuous activity during even minimal effort, while polyandrous males have evolved compensatory physiological mechanisms to offset the metabolic costs of investment in sexually selected traits (Husak and Swallow, 2011).

Sexual selection may affect biochemical pathways and resource acquisition and allocation decisions that provide energy more efficiently (Montooth et al., 2003). For instance, sexual selection acting on endurance capacity may select for greater lipid respiration and fat storage (Gyulavári et al., 2014). Indeed, we found lipid content was associated with polyandry in both sexes. Fatty acids also serve as precursors for signalling molecules (i.e. eicosanoids and pheromones), which act as contact pheromones and have previously shown to respond to sexual selection in our lines (Hunt et al., 2012). Glycogen content was also positively associated with polyandry in males. Although carbohydrates provide less energy per unit than lipids (Arrese and Soulages, 2010), glycogen is an important energy reserve marshalled during intense activity (Beenakkers et al., 1984). The higher association of both lipids and glycogen in polyandrous males suggests polyandry has selected for enhanced energy storage for use during bouts of inter- and intra- sexual selection (Crudgington et al., 2009; Debelle et al., 2017, 2014). While circulating haemolymph sugars, such as trehalose, were associated with monogamy in both sexes, stored glycogen provides the main source of trehalose (Becker et al., 1996). Based on this pattern, we suggest polyandry has selected for increased storage and regulation of energy in the form of glycogen to meet metabolic demands, whereas under monogamy this regulation is relaxed. Despite greater lipid and glycogen content, which buffer against desiccation and starvation (Marron et al., 2003), polyandrous males were less stress tolerant than monogamous males, expending more stored energy to invest in current reproduction at a cost to later life survival (Hunt et al., 2004; Kotiaho, 2001). In contrast, we found no such trade-off in

polyandrous females. Even though polyandrous females invest more in reproduction than monogamous females (Crudgington et al., 2009; Immonen et al., 2014), polyandrous females were more stress resistant than monogamous females.

Investment in physiological mechanisms underlying sexually selected traits, and the traits themselves, is predicted to necessitate trade-offs and consequently changes in life history strategies. In addition to a potential trade-off between stress tolerance and sexual selection, we found a significant shift in juvenile development time between sexual selection treatments with polyandrous flies taking longer to emerge as adults than monogamous flies. This result is in contrast to a previous study that found male *D. melanogaster* evolving under enforced monogamy emerged later than males from control polyandry populations (Hollis et al., 2017). The difference between our study and these previous results may be that the two species fundamentally differ in how sexual selection and sexual conflict operate (Veltsos et al., 2017). Two non-mutually exclusive hypotheses could explain the pattern we show here. First, nutrients essential for the expression of costly sexually selected traits are acquired during juvenile development in *Drosophila* (Morimoto and Wigby, 2016). For a given development time, constraints on resource acquisition and allocation mean individuals must make strategic decisions and trade-offs between different aspects of fitness (Morimoto and Wigby, 2016; Simmons et al., 2017; Tomkins et al., 2005). Thus, despite the benefits of early reproductive maturity (Kingsolver and Huey, 2008), longer development time may have evolved to allow polyandrous males to invest more in resource acquisition and subsequent growth of sexually selected traits. Second, intralocus sexual conflict over development time could drive its divergence. For instance, if longer development time is favoured in males evolving under polyandry, heightened sexual selection and sexual conflict may shift development time towards a male-biased optimum in both sexes (Blanckenhorn et al., 2007).

In conclusion, sexual selection is expected to affect whole organismal performance, not only those traits directly involved in reproduction. We have directly shown using experimental evolution that divergent sexual selection can simultaneously shape several physiological mechanisms underlying organismal performance, and consequently shift life history strategies. Released from selection to invest in energetically costly activity and metabolism/reproduction,

evolving under enforced monogamy produced slothful males, reliant on low quality carbohydrates as metabolic fuel with inefficient metabolic machinery whereas males exposed to multiple rivals have more abundant and higher quality energy reserves. Our findings highlight that sexual selection results in coordinated evolution of fundamental physiological and non-reproductive life history traits, implicating a broad role of sexual selection in the evolution of life history strategies.

Discussion

In this thesis I have explored the role of postmating prezygotic (PMPZ) isolation during the evolution of reproductive isolation, and the role of sexual selection and sexual conflict in the evolution of physiological and life history traits. In chapter 1 I reviewed the literature relating to postmating prezygotic isolation and show that it can be an important barrier to gene flow that evolves early during speciation. In chapters 2 and 3 I investigated patterns of PMPZ isolation between populations of *Drosophila montana* within North American, including the presence of strong asymmetrical PMPZ isolation in the form of fertilisation failure, and con-population sperm precedence. In chapter 4, I tested whether evolution of the male ejaculate my contribute to PMPZ isolation and provide the first description of the *D. montana* seminal fluid proteome. Finally, in chapter 5, using experimental evolution I showed how sexual selection and sexual conflict can have far reaching implications, leading to the evolution and divergence of key traits that underlie differences in reproductive investment.

In chapter 1 I reviewed the literature on PMPZ isolation in metazoans published over the past 15 years. Several key patterns emerged from this survey. First, the majority of studies investigated PMPZ isolation between recently diverged taxa, supporting the prediction that PMPZ isolation emerges early during speciation. Second, I highlighted a number of relatively unexplored areas of research which could prove fruitful for the study of PMPZ isolation and speciation, and perhaps reproductive biology more generally. Extrinsic PMPZ isolation affecting fertility may be of interest to biologists interested in understanding how populations will respond in the face of climate change. Studies investigating PMPZ isolation in birds and mammals are challenging but will improve our understanding of factors which result in fertilisation failure, of interest to biologists interested in improving fertility treatments. Third, the fuzzy border between barriers to gene flow acting shortly before or after mating needs further study to understand how these barriers might be influenced by one-another, and what role PMPZ isolation has in reinforcement – acting as either the agent, or target, of reinforcement. Finally, more empirical tests are needed to assess whether PMPZ isolation evolves more readily via sexual selection and sexual conflict than via natural selection or drift.

As more studies investigate PMPZ isolation, we may be able to answer questions such as whether PMPZ isolation emerges more often due to natural or sexual selection; in what kinds of mating systems (polyandrous vs. monandrous) does PMPZ isolation more often arise; what are the common mechanisms across groups or to specific types of organism that are likely to result in the emergence of PMPZ isolation; and when does PMPZ isolation emerge during taxonomic divergence. However, there are still relatively few empirical studies of PMPZ isolation such that a meta-analysis was not appropriate.

In chapter 2, I showed a persistent pattern of PMPZ isolation between populations of *Drosophila montana* within North America that is not limited to particular strains, or mitigated or exacerbated by remating in either sex (Garlovsky and Snook, 2018). Future work should aim to dissect the precise mechanism that causes fertilisation failure and collect further populations from within North America. Identifying other populations that show patterns of PMPZ isolation similar to the Colorado population will allow comparisons to identify common factors which contribute to the evolution of PMPZ isolation. Detailed field studies to provide information of the local biology and ecology of different populations, such as variation in the types and frequency of inter- and intra- specific interactions, the operational sex ratio, and the incidence of predation and parasitism, will help to reveal the evolutionary processes acting within populations that may contribute to the evolution of reproductive isolation between populations. Finally, collaborators are currently investigating patterns of ongoing or historical gene flow between populations and/or other species, to improve our understanding of speciation in this system (M.G. Ritchie, pers. comms.).

In chapter 3 I tested whether sexual isolation circumvents the evolution of PMPZ isolation and vice versa, and whether the strength of PMPZ isolation reflects the strength of postcopulatory sexual selection (PCSS) acting within populations. This analysis suggests a more intrinsic role of reproductive physiology rather than divergent selection *per se* in the emergence of PMPZ isolation. However, it should be noted that the male and female traits involved in PMPZ isolation may evolve in perhaps arbitrary directions. The male ejaculate can be considered a multivariate trait, on which selection could act along any number of axes of differentiation. The two metrics used in this study which measured the risk of sperm competition perhaps did

not capture the relevant metric of PCSS. While overall reproductive tract mass did not vary, perhaps accessory gland size alone would have shown a significant difference between populations. Work in the Snook lab and with collaborators elsewhere continues to parse out the mechanisms and evolution of PMPZ isolation in *D. montana*. Differences between populations in sperm-seminal receptacle length co-variation may be a promising avenue for future research (Miller and Pitnick, 2002; Pitnick et al., 2003).

In chapter 4 I tested whether seminal fluid proteins (Sfps), which are predicted to evolve rapidly and thus generate incompatibilities early during population divergence, have differentiated between populations of *D. montana* that show PMPZ isolation. Using high throughput shotgun proteomics, this study identified a subset of proteins that showed evidence of differential abundance between Colorado and Vancouver. This work also identified the two main Sfp producing organs, the accessory glands and the ejaculatory duct/bulb, contribute differently towards Sfp production and may hold some distinct roles in reproduction. The next steps will be to characterise the proteomes in more detail and measure the rates of molecular evolution of these proteins to test if they are rapidly evolving between populations.

A number of the differentially abundant proteins identified are known Sfps in *D. melanogaster*. Future research should aim to manipulate some of these candidate genes to explore their function in PMPZ isolation. Collaborators are currently developing CRISPR/Cas9 gene editing in *D. montana* (M. G. Ritchie and Y. H. Ahmed-Braimah, pers. comms.) which will allow manipulation and validation of proposed PMPZ isolation genes/proteins. Due to resource limitations I was only able to investigate differences in the male ejaculate proteome between populations. Future research investigating the role of ejaculate-female reproductive tract interactions would benefit from also examining the female reproductive tract proteome and changes in female postmating physiology after mating with within- vs. between- population males. Recent studies have used heavy labelling techniques or compared mated vs. virgin flies to distinguish the male and female contribution to the female postmating reproductive environment (Bayram et al., 2019; Degner et al., 2019; Sepil et al., 2019).

Finally, in chapter 5 I used experimental evolution to investigate how changes in the mating system can shape the evolution of physiological and life history traits. This study highlights that more integrated studies are needed to explore the impacts of sexual selection and sexual conflict across a broad range of traits, not only those directly involved in reproduction. This study was not conducted specifically in the context of how sexual selection might contribute to the evolution of reproductive isolation; however, it does show that by simply altering the mating system a broad range of physiological and life history traits can diverge between populations. Divergent life history strategies can be a first step towards incipient speciation, for instance, causing assortative mating as populations become separated in time or space (Filchak et al., 2000; Rice and Salt, 1990). Changes in the physiological machinery underlying reproductive traits may also indirectly generate incompatibilities between populations (Mendelson et al., 2014). It should be noted however, that experimental tests have not found evidence of reproductive isolation between the M and P lines (Bacigalupe et al., 2007). Despite divergent female preferences for male song, polyandrous males court more vigorously, and so win more often in contests between P and M males which would act to erode linkage disequilibrium between populations preventing divergence (Debelle et al., 2016).

Concluding remarks

Overall, this work offers novel insights into the rapid evolution of postmating traits and their role in reproductive isolation, and how sexual selection and sexual conflict may generally play important roles for the evolution of populations and traits within them. How different episodes of sexual selection might interact and impact the evolution of different prezygotic barriers to gene flow will be important for future work investigating sexual selection and speciation. The integrated study of pre- and post-copulatory sexual selection is only recently coming to the fore (Evans and Garcia-Gonzalez, 2016; McDonald and Pizzari, 2018). Furthermore, how inter- and intra-specific interactions shape the evolution of sexually selected traits has only recently begun to be appreciated (McDonald et al., 2019). Understanding the extent of inter- and intra-specific interactions in the wild will be necessary to determine how traits involved in assortative mating and fertilisation evolve. Considering eco-evolutionary dynamics will also provide greater insights in to the interaction between natural and sexual selection, and their contribution to speciation (Miller and Svensson, 2014; Perry et al., 2017; Svensson, 2018).

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Appendices

Chapter 2.

Table S2.1. Location and year of collection of *Drosophila montana* strains used in this study.

	Location	Coordinates	Altitude (m)	Year	Strain
Population cages	Crested Butte, Colorado, USA	38°49'N, 107°04'W	2868	2013	Co13PC†‡
	Vancouver, British Columbia, Canada	48°55'N, 123°48'W	142	2008	Vn08PC†‡
		49°11'N, 123°10'W	4	2014	Vn14PC
Iso-female lines	Ashford, Washington, USA	46°45'N, 121°57'W	573	2013	As13F9
				2013	A13F13
	Crested Butte, Colorado, USA	38°49'N, 107°04'W	2868	2009	Co7CC4
				2009	C29CC4
	Jackson, Wyoming, USA	43°26'N, 110°50'W	1857	2013	Jx13F3‡
	Vancouver, British Columbia, Canada	49°15'N, 123°10'W	4	2014	Vn14F1

†Strains used in experiments testing female genotype x male genotype interactions and female and male mating history effects on PMPZ.

‡ Strains used for DAPI staining eggs to confirm prezygotic egg hatch failure.

Table S2.2. Progeny counts and sex ratio (female:male) in crosses between Co13PC and Vn08PC population cages.

Cross-type	Total progeny	Total female	Total male	Sex ratio (f:m)
CC	815	420	395	1.06
CV	83	32	51	0.63
VC	409	198	211	0.94
VV	582	298	284	1.05

Table S2.3. Post-hoc Tukey's HSD tests for PMPZ reproductive barriers (fecundity and hatching success) in crosses between Ashford and Colorado.

ASHFORD X COLORADO				
Fecundity				
Linear Hypotheses:	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
AC - AA	-0.041	0.132	-0.314	0.989
CA - AA	-0.071	0.131	-0.543	0.948
CC - AA	-0.238	0.144	-1.650	0.350
CA - AC	-0.030	0.120	-0.248	0.995
CC - AC	-0.197	0.132	-1.496	0.439
CC - CA	-0.167	0.131	-1.276	0.577
Hatching success				
Linear Hypotheses:	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
AC - AA	-1.993	0.463	-4.308	**< 0.001**
CA - AA	-3.597	0.462	-7.780	**< 0.001**
CC - AA	-0.388	0.493	-0.788	0.860
CA - AC	-1.604	0.430	-3.728	**0.001**
CC - AC	1.605	0.463	3.467	**0.003**
CC - CA	3.209	0.462	6.950	**< 0.001**

Table S2.4. Post-hoc Tukey's HSD tests for PMPZ reproductive barriers (fecundity and hatching success) in crosses between Ashford and Jackson.

ASHFORD X JACKSON				
Fecundity				
Linear Hypotheses:	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
AJ - AA	0.310	0.106	2.915	**0.019**
JA - AA	-0.044	0.122	-0.363	0.983
JJ - AA	0.159	0.124	1.291	0.567
JA - AJ	-0.354	0.117	-3.022	**0.013**
JJ - AJ	-0.150	0.119	-1.266	0.583
JJ - JA	0.204	0.133	1.532	0.417

Hatching success				
Linear Hypotheses:	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
AJ - AA	0.713	0.726	0.981	0.759
JA - AA	0.476	0.817	0.583	0.937
JJ - AA	0.625	0.834	0.749	0.876
JA - AJ	-0.236	0.797	-0.296	0.991
JJ - AJ	-0.088	0.815	-0.107	1.000
JJ - JA	0.149	0.896	0.166	0.998

Table S2.5. Post-hoc Tukey's HSD tests for PMPZ reproductive barriers (fecundity and hatching success) in crosses between Ashford and Vancouver.

ASHFORD X VANCOUVER				
Fecundity				
Linear Hypotheses:	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
AV - AA	-0.072	0.084	-0.856	0.827
VA - AA	0.171	0.077	2.224	0.116
VV - AA	-0.119	0.082	-1.451	0.466
VA - AV	0.243	0.087	2.796	**0.026**
VV - AV	-0.047	0.091	-0.518	0.955
VV - VA	-0.291	0.085	-3.422	**0.003**

Hatching success				
Linear Hypotheses:	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
AV - AA	-0.072	0.084	-0.856	0.827
VA - AA	0.171	0.077	2.224	0.116
VV - AA	-0.119	0.082	-1.451	0.466
VA - AV	0.243	0.087	2.796	**0.026**
VV - AV	-0.047	0.091	-0.518	0.955
VV - VA	-0.291	0.085	-3.422	**0.003**

Table S2.6. Post-hoc Tukey's HSD tests for PMPZ reproductive barriers (fecundity and hatching success) in crosses between Colorado and Jackson.

COLORADO X JACKSON				
Fecundity				
Linear Hypotheses:	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
CJ - CC	-0.204	0.124	-1.641	0.355
JC - CC	-0.112	0.126	-0.888	0.811
JJ - CC	-0.185	0.127	-1.463	0.460
JC - CJ	0.093	0.128	0.723	0.888
JJ - CJ	0.019	0.129	0.147	0.999
JJ - JC	-0.074	0.130	-0.565	0.942

Hatching success				
Linear Hypotheses:	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
CJ - CC	-4.156	0.335	-12.419	**< 0.001**
JC - CC	-1.813	0.316	-5.729	**< 0.001**
JJ - CC	0.450	0.332	1.356	0.527
JC - CJ	2.344	0.337	6.962	**< 0.001**
JJ - CJ	4.606	0.351	13.119	**< 0.001**
JJ - JC	2.263	0.336	6.744	**< 0.001**

Table S2.7. Post-hoc Tukey's HSD tests for PMPZ reproductive barriers (fecundity and hatching success) in crosses between Colorado and Vancouver.

COLORADO X VANCOUVER				
Fecundity				
Linear Hypotheses:	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
CV - CC	-0.137	0.091	-1.507	0.432
VC - CC	0.109	0.089	1.226	0.609
VV - CC	0.032	0.097	0.325	0.988
VC - CV	0.247	0.079	3.103	**0.010**
VV - CV	0.169	0.087	1.943	0.209
VV - VC	-0.078	0.086	-0.904	0.802

Hatching success				
Linear Hypotheses:	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
CV - CC	-3.655	0.292	-12.520	**< 0.001**
VC - CC	-1.720	0.285	-6.035	**< 0.001**
VV - CC	0.076	0.321	0.237	0.995
VC - CV	1.935	0.247	7.827	**< 0.001**
VV - CV	3.731	0.277	13.479	**< 0.001**
VV - VC	1.796	0.275	6.541	**< 0.001**

Table S2.8. Post-hoc Tukey's HSD tests for PMPZ reproductive barriers (fecundity and hatching success) in crosses between Jackson and Vancouver.

JACKSON X VANCOUVER				
Fecundity				
Linear Hypotheses:	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
JV - JJ	-0.168	0.093	-1.817	0.264
VJ - JJ	0.187	0.111	1.690	0.327
VV - JJ	0.013	0.094	0.137	0.999
VJ - JV	0.355	0.111	3.213	**0.007**
VV - JV	0.181	0.094	1.934	0.212
VV - VJ	-0.174	0.111	-1.562	0.399

Hatching success				
Linear Hypotheses:	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
JV - JJ	-0.729	0.296	-2.462	0.065
VJ - JJ	0.081	0.366	0.220	0.996
VV - JJ	-0.421	0.302	-1.391	0.502
VJ - JV	0.810	0.363	2.233	0.113
VV - JV	0.309	0.298	1.036	0.726
VV - VJ	-0.501	0.368	-1.363	0.520

Table S2.9. Summary of models testing between-male variance in hatching success after mating between three and five within- or between- population females over consecutive days. Each cross-type was modelled separately as to not artificially inflate the between-group variance. All models included mating day as the only fixed effect. Estimates of random effects variance excluding male identity random effect or observation level random effect (OLRE) in each cross-type are shown and model Akaike information criterion corrected for small sample size (AICc). Cross-types are abbreviated with the female population given first. C, Colorado; V, Vancouver.

Cross-type	Random effects	AICc	Variance	
			Male	OLRE†
CC	Male + OLRE	320.159	0.000	0.647
	Male	384.475	0.056	-
	OLRE	317.427	-	0.647
CV	Male + OLRE	251.387	0.000	0.807
	Male	303.678	0.154	-
	OLRE	248.655	-	0.807
VC	Male + OLRE	234.192	0.000	0.564
	Male	316.843	0.179	-
	OLRE	230.592	-	0.564
VV	Male + OLRE	343.720	0.000	1.078
	Male	635.476	0.186	-
	OLRE	340.948	-	1.078

†Higher OLRE values indicate more overdispersion in the dataset (Harrison, 2015).

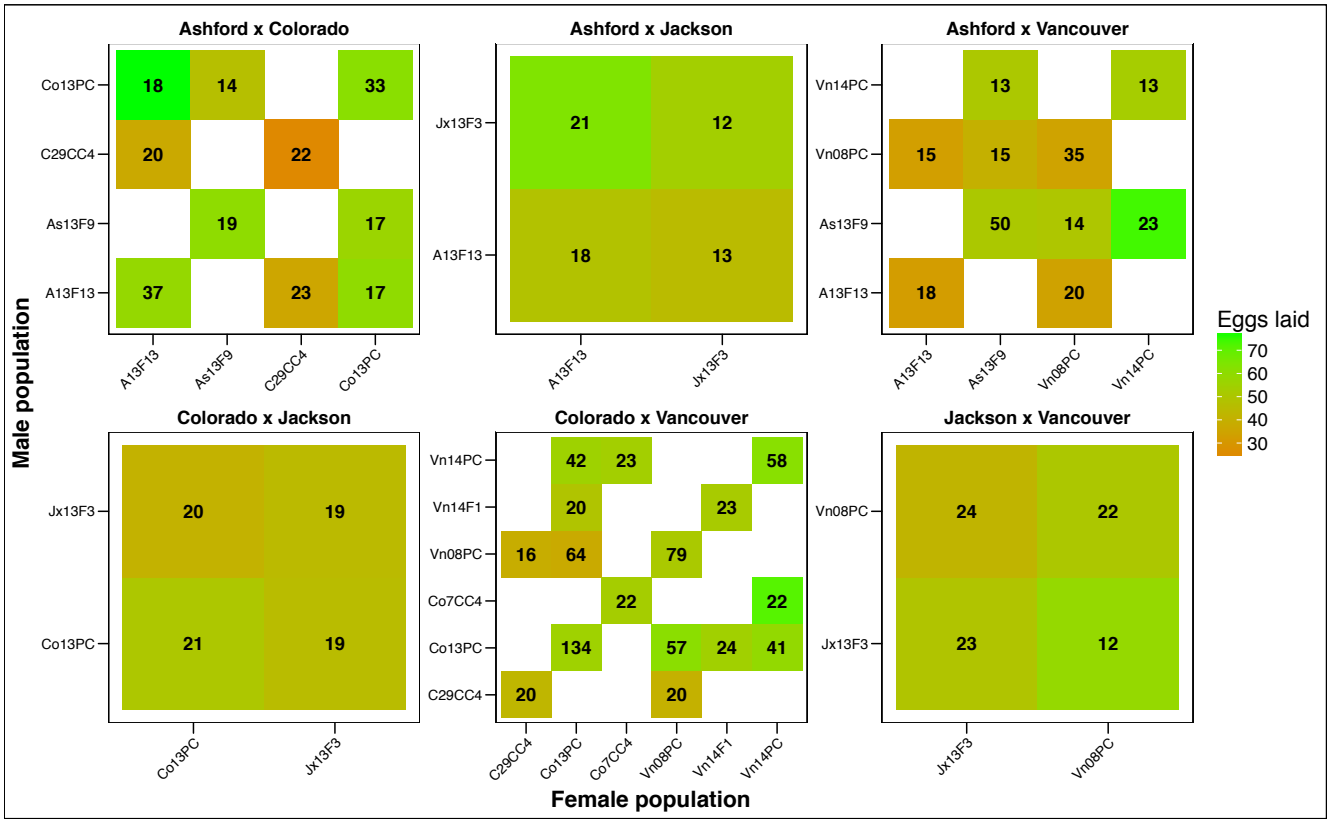

Figure S2.1. Mean number of eggs laid in each specific strain x strain cross-type. Brighter green colours represent higher fecundity, redder colours represent lower fecundity. Numbers in each square show total number of successfully mating pairs in each cross-type over all experimental blocks. See Table S2.1 for full description of strain abbreviations.

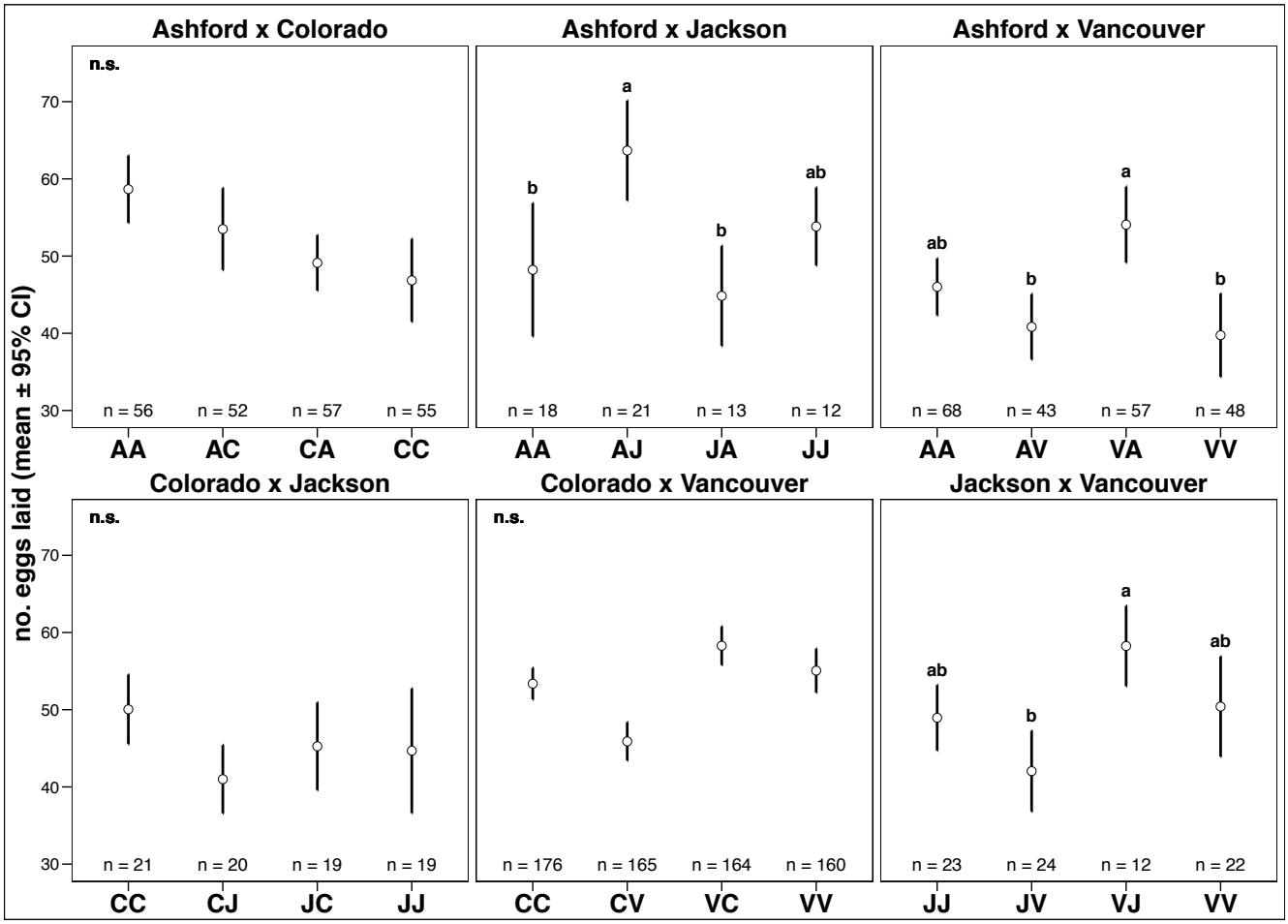

Figure S2.2. Number of eggs laid (mean ± 95% CI) in each cross between populations. Within each panel different letters above points indicate significant differences from posthoc Tukey's HSD; letters are recycled in each panel. n.s., non-significant. N = number of mating pairs over all experimental blocks.

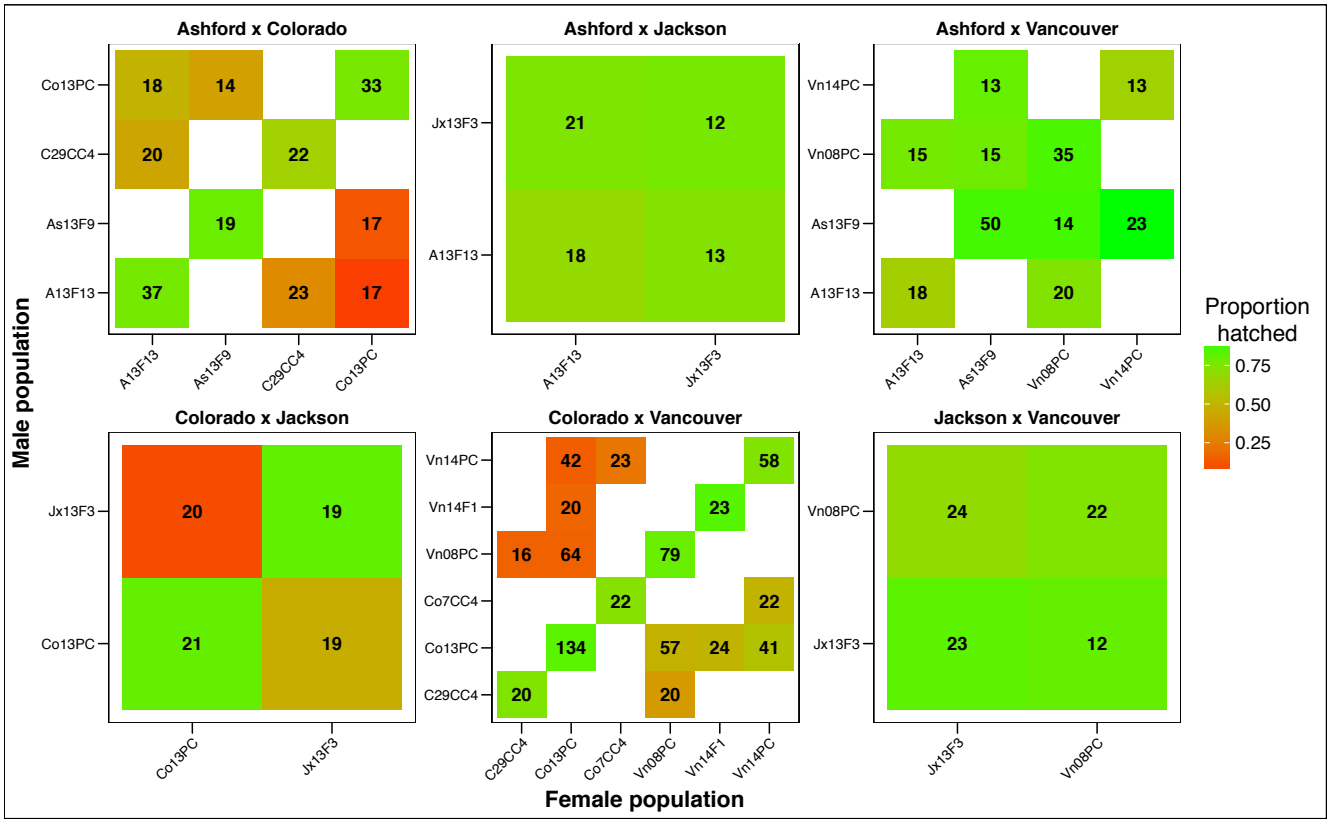

Figure S2.3. Mean proportion of eggs hatched in each specific strain x strain cross-type. Brighter green colours represent higher hatching success; more red colours represent lower hatching success. Numbers in each square show total number of successfully mating pairs in each cross-type over all experimental blocks. See Table S2.1 for full description of strain abbreviations.

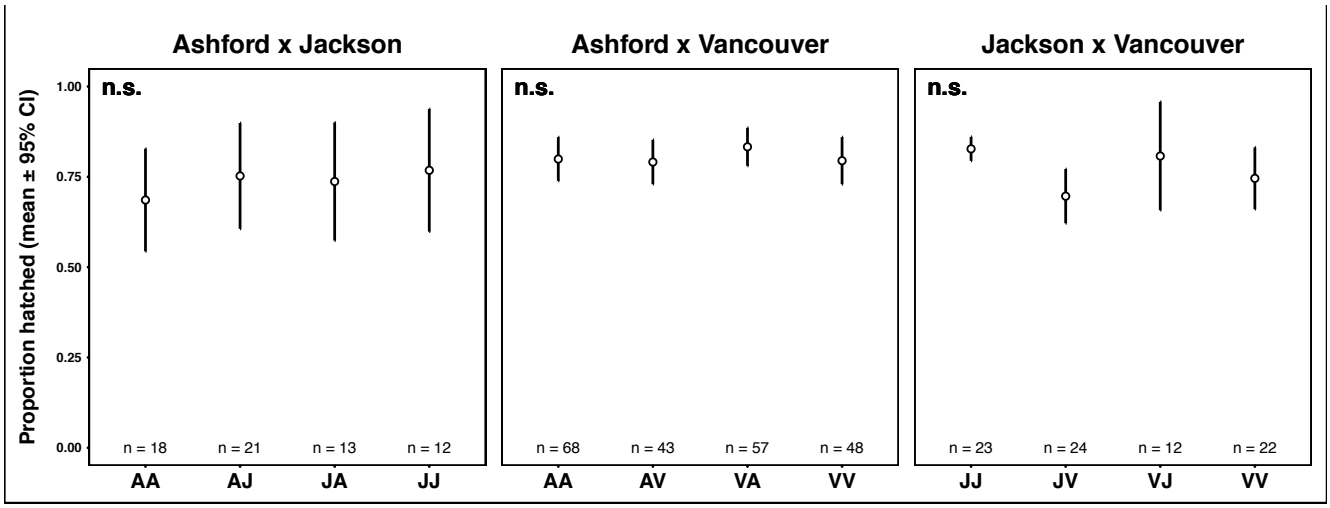

Figure S2.4. Proportion of eggs hatching (mean ± 95% CI) in crosses not showing PMPZ isolation. n.s., non-significant. N = number of mating pairs over all experimental blocks.

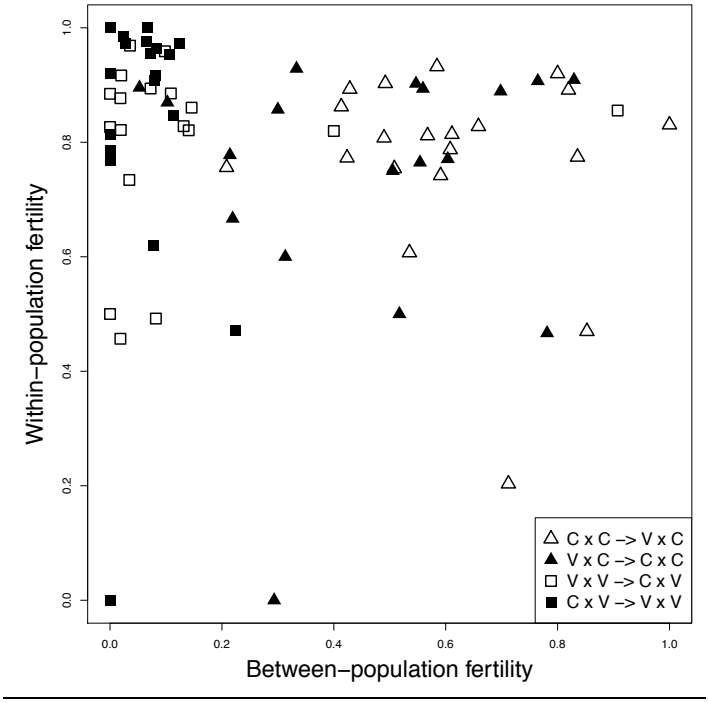

Figure S2.5. Correlation in fertility (proportion of eggs hatching) between the first and second mating for males mated to both a within- and between-population female. Triangles, Colorado males; Squares, Vancouver males. Open points, first mating = within-population female; filled points, first mating = between-population female.

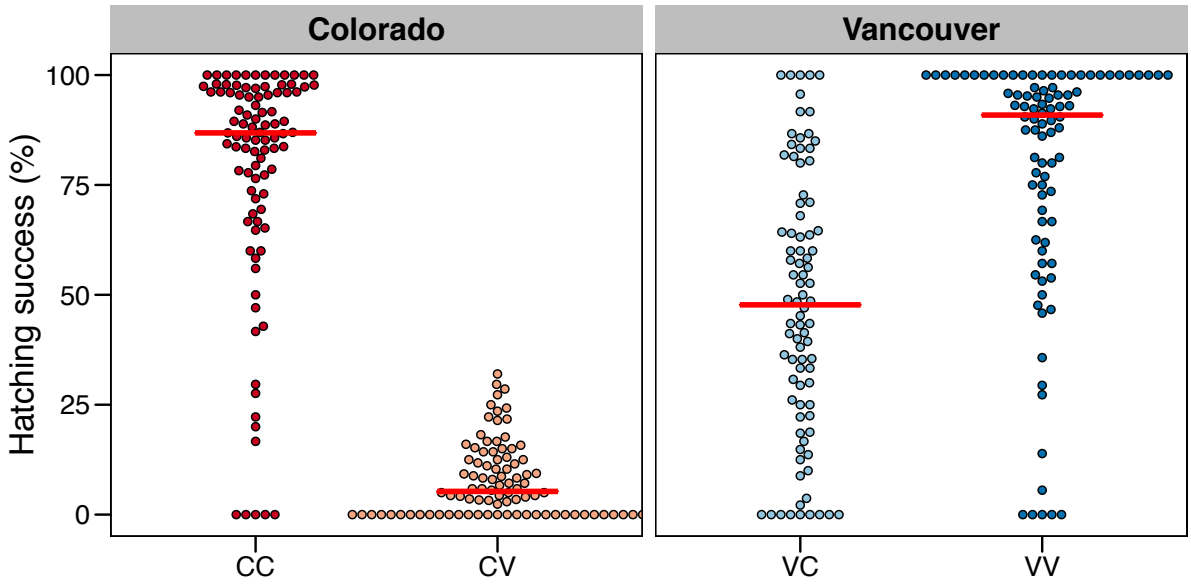

Figure S3.1. Hatching success (% eggs laid that hatched) after the first mating for females that mated nonirradiated males. Points are observations and red crossbars show the median. Cross-type denotes female population followed by male. C, Colorado; V, Vancouver.

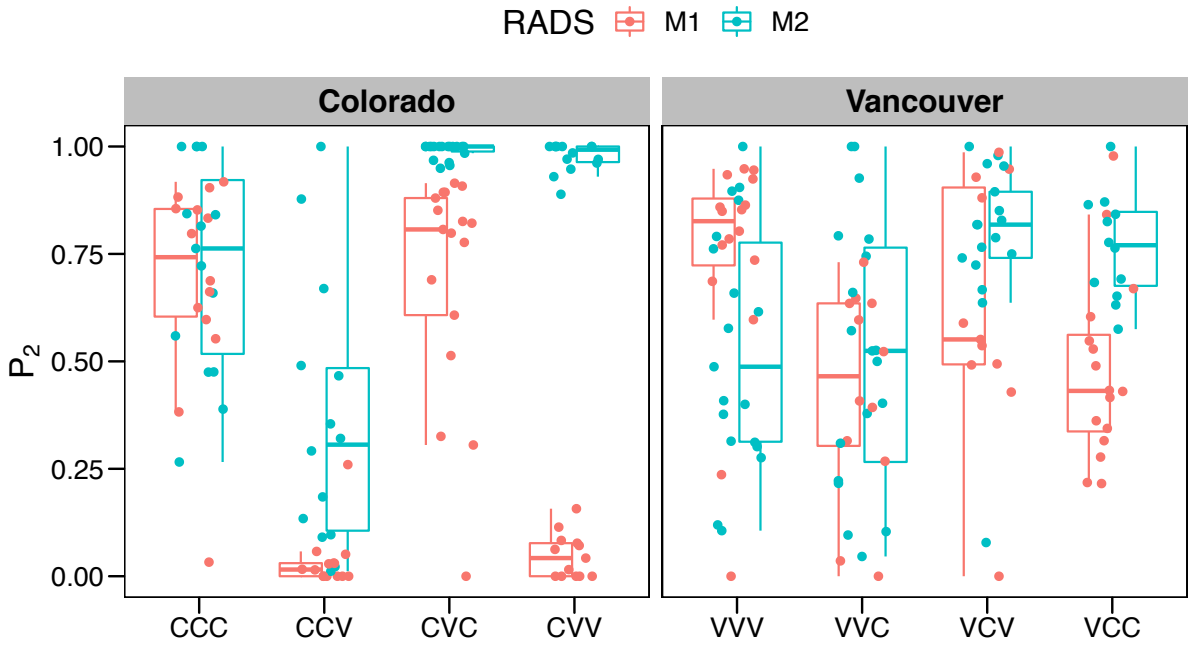

Figure S3.2. Proportion of offspring sired by the second male to mate (P_2). Points are observations. Colours represent irradiation order (RADS), where either the first (M1, red) or second (M2, blue) male to mate was irradiated. Cross-type denotes female population followed by first and second male mate. C, Colorado; V, Vancouver.

Table S4.1. Differentially abundant *D. melanogaster* orthologues in the AgP obtained from flybase.com. Previously identified seminal fluid proteins in bold.

FBgn ID	Annotation symbol	Gene name	Gene symbol
FBgn0040064	CG4600	yippee interacting protein 2	yip2
FBgn0004003	CG7225	windbeutel	wbl
FBgn0010516	CG8996	walrus	wal
FBgn0005671	CG17369	Vacuolar H[+]-ATPase 55kD subunit	Vha55
FBgn0035147	CG12030	UDP-galactose 4'-epimerase	Gale
FBgn0035402	CG12082	Ubiquitin specific protease 5	Usp5
FBgn0041180	**CG10363**	**Thioester-containing protein 4**	**Tep4**
FBgn0010213	CG8905	Superoxide dismutase 2 (Mn)	Sod2
FBgn0014028	CG3283	Succinate dehydrogenase, subunit B (iron-sulfur)	SdhB
FBgn0022359	CG4649	Sorbitol dehydrogenase-2	Sodh-2
FBgn0038810	CG5434	Signal recognition particle protein 72	Srp72
FBgn0028983	CG10913	Serpin 55B	Spn55B
FBgn0033339	CG8266	Secretory 31	Sec31
FBgn0263006	CG3725	Sarco/endoplasmic reticulum Ca(2+)-ATPase	SERCA
FBgn0034743	CG4046	Ribosomal protein S16	RpS16
FBgn0000100	CG7490	Ribosomal protein LP0	RpLP0
FBgn0285949	CG1821	Ribosomal protein L31	RpL31
FBgn0030362	**CG1803**	**regucalcin**	**regucalcin**
FBgn0261243	CG1009	Puromycin sensitive aminopeptidase	Psa
FBgn0022382	CG15862	Protein kinase, cAMP-dependent, regulatory subunit type 2	Pka-R2
FBgn0038570	CG7217	Peroxiredoxin 5	Prx5
FBgn0037718	CG8286	P58IPK	P58IPK
FBgn0031589	CG3714	Nicotinate phosphoribosyltransferase	Naprt
FBgn0029155	CG5889	Malic enzyme b	Men-b
FBgn0262782	CG5362	Malate dehydrogenase 1	Mdh1
FBgn0263594	CG14648	lost	lost
FBgn0011296	CG4533	lethal (2) essential for life	l(2)efl
FBgn0027338	CG9423	karyopherin alpha3	Kap-alpha3
FBgn0026415	CG1780	Imaginal disc growth factor 4	Idgf4
FBgn0040493	CG7340	granny smith	grsm
FBgn0000053	CG31628	GART trifunctional enzyme	Gart

FBgn ID	Annotation symbol	Gene name	Gene symbol
FBgn0030932	**CG6461**	**gamma-glutamyl transpeptidase**	**Ggt-1**
FBgn0013954	CG11001	FK506-binding protein 12kD	Fkbp12
FBgn0023213	CG10811	eukaryotic translation initiation factor 4G1	eIF4G1
FBgn0015834	CG8882	eukaryotic translation initiation factor 3 subunit i	eIF3i
FBgn0037249	CG9805	eukaryotic translation initiation factor 3 subunit a	eIF3a
FBgn0284245	CG8280	eukaryotic translation elongation factor 1 alpha 1	eEF1alpha1
FBgn0033879	CG6543	Enoyl-CoA hydratase, short chain 1	Echs1
FBgn0033663	CG8983	Endoplasmic reticulum p60	ERp60
FBgn0050104	CG30104	Ecto-5'-nucleotidase 2	NT5E-2
FBgn0027835	CG5170	Dodeca-satellite-binding protein 1	Dp1
FBgn0037580	CG7415	Dipeptidyl aminopeptidase III	DppIII
FBgn0037138	CG7145	delta-1-Pyrroline-5-carboxylate dehydrogenase 1	P5CDh1
FBgn0031830	CG11015	Cytochrome c oxidase subunit 5B	COX5B
FBgn0004629	**CG8050**	**Cystatin-like**	**Cys**
FBgn0037240	CG1084	Contactin	Cont
FBgn0030521	CG10992	Cathepsin B1	CtsB1
FBgn0000261	CG6871	Catalase	Cat
FBgn0263231	CG9748	belle	bel
FBgn0004587	CG10851	B52	B52
FBgn0283494	CG3140	Adenylate kinase 2	Adk2
FBgn0022343	CG3760	-	CG3760
FBgn0023537	CG17896	-	CG17896
FBgn0030060	CG2004	-	CG2004
FBgn0030245	CG1637	-	CG1637
FBgn0030447	CG2200	-	CG2200
FBgn0031320	CG5126	-	CG5126
FBgn0031418	CG3609	-	CG3609
FBgn0032350	CG6287	-	CG6287
FBgn0032453	CG6180	-	CG6180
FBgn0032721	CG10602	-	CG10602
FBgn0032787	CG10195	-	CG10195
FBgn0033312	CG8642	-	CG8642
FBgn0035911	CG6638	-	CG6638
FBgn0037279	CG1129	-	CG1129

FBgn ID	Annotation symbol	Gene name	Gene symbol
FBgn0037432	CG10298	-	CG10298
FBgn0039568	**CG4815**	**-**	**CG4815**
FBgn0039616	CG11828	-	CG11828
FBgn0039737	CG7920	-	CG7920
FBgn0040503	CG7763	-	CG7763
FBgn0042138	CG18815	-	CG18815
FBgn0086254	CG6084	-	CG6084

Table S4.2. Differentially abundant *D. melanogaster* orthologues in the EbP.

FBgn ID	Ann. symbol	Gene name	Gene symbol
FBgn0040064	CG4600	yippee interacting protein 2	yip2
FBgn0004003	CG7225	windbeutel	wbl
FBgn0004003	CG7225	windbeutel	wbl
FBgn0010516	CG8996	walrus	wal
FBgn0005671	CG17369	Vacuolar H[+]-ATPase 55kD subunit	Vha55
FBgn0005671	CG17369	Vacuolar H[+]-ATPase 55kD subunit	Vha55
FBgn0035147	CG12030	UDP-galactose 4'-epimerase	Gale
FBgn0035147	CG12030	UDP-galactose 4'-epimerase	Gale
FBgn0035402	CG12082	Ubiquitin specific protease 5	Usp5
FBgn0041180	CG10363	Thioester-containing protein 4	Tep4
FBgn0010213	CG8905	Superoxide dismutase 2 (Mn)	Sod2
FBgn0010213	CG8905	Superoxide dismutase 2 (Mn)	Sod2
FBgn0014028	CG3283	Succinate dehydrogenase, subunit B (iron-sulfur)	SdhB
FBgn0022359	CG4649	Sorbitol dehydrogenase-2	Sodh-2
FBgn0038810	CG5434	Signal recognition particle protein 72	Srp72
FBgn0028983	CG10913	Serpin 55B	Spn55B
FBgn0033339	CG8266	Secretory 31	Sec31
FBgn0263006	CG3725	Sarco/endoplasmic reticulum Ca(2+)-ATPase	SERCA
FBgn0034743	CG4046	Ribosomal protein S16	RpS16
FBgn0034743	CG4046	Ribosomal protein S16	RpS16

FBgn ID	Annotation symbol	Gene name	Gene symbol
FBgn0000100	CG7490	Ribosomal protein LP0	RpLP0
FBgn0000100	CG7490	Ribosomal protein LP0	RpLP0
FBgn0285949	CG1821	Ribosomal protein L31	RpL31
FBgn0030362	CG1803	regucalcin	regucalcin
FBgn0261243	CG1009	Puromycin sensitive aminopeptidase	Psa
FBgn0022382	CG15862	Protein kinase, cAMP-dependent, regulatory subunit type 2	Pka-R2
FBgn0038570	CG7217	Peroxiredoxin 5	Prx5
FBgn0037718	CG8286	P58IPK	P58IPK
FBgn0031589	CG3714	Nicotinate phosphoribosyltransferase	Naprt
FBgn0031589	CG3714	Nicotinate phosphoribosyltransferase	Naprt
FBgn0029155	CG5889	Malic enzyme b	Men-b
FBgn0262782	CG5362	Malate dehydrogenase 1	Mdh1
FBgn0263594	CG14648	lost	lost
FBgn0011296	CG4533	lethal (2) essential for life	l(2)efl
FBgn0011296	CG4533	lethal (2) essential for life	l(2)efl
FBgn0027338	CG9423	karyopherin alpha3	Kap-alpha3
FBgn0026415	CG1780	Imaginal disc growth factor 4	Idgf4
FBgn0026415	CG1780	Imaginal disc growth factor 4	Idgf4
FBgn0040493	CG7340	granny smith	grsm
FBgn0000053	CG31628	GART trifunctional enzyme	Gart
FBgn0040064	CG4600	yippee interacting protein 2	yip2
FBgn0000053	CG31628	GART trifunctional enzyme	Gart
FBgn0030932	CG6461	gamma-glutamyl transpeptidase	Ggt-1
FBgn0013954	CG11001	FK506-binding protein 12kD	Fkbp12
FBgn0023213	CG10811	eukaryotic translation initiation factor 4G1	eIF4G1
FBgn0015834	CG8882	eukaryotic translation initiation factor 3 subunit i	eIF3i
FBgn0037249	CG9805	eukaryotic translation initiation factor 3 subunit a	eIF3a
FBgn0284245	CG8280	eukaryotic translation elongation factor 1 alpha 1	eEF1alpha1
FBgn0033879	CG6543	Enoyl-CoA hydratase, short chain 1	Echs1
FBgn0033663	CG8983	Endoplasmic reticulum p60	ERp60
FBgn0050104	CG30104	Ecto-5'-nucleotidase 2	NT5E-2
FBgn0027835	CG5170	Dodeca-satellite-binding protein 1	Dp1
FBgn0037580	CG7415	Dipeptidyl aminopeptidase III	DppIII
FBgn0037138	CG7145	delta-1-Pyrroline-5-carboxylate dehydrogenase 1	P5CDh1

FBgn ID	Annotation symbol	Gene name	Gene symbol
FBgn0031830	CG11015	Cytochrome c oxidase subunit 5B	COX5B
FBgn0004629	CG8050	Cystatin-like	Cys
FBgn0037240	CG1084	Contactin	Cont
FBgn0030521	CG10992	Cathepsin B1	CtsB1
FBgn0000261	CG6871	Catalase	Cat
FBgn0263231	CG9748	belle	bel
FBgn0004587	CG10851	B52	B52
FBgn0004587	CG10851	B52	B52
FBgn0283494	CG3140	Adenylate kinase 2	Adk2
FBgn0022343	CG3760	-	CG3760
FBgn0023537	CG17896	-	CG17896
FBgn0030060	CG2004	-	CG2004
FBgn0030245	CG1637	-	CG1637
FBgn0030447	CG2200	-	CG2200
FBgn0030447	CG2200	-	CG2200
FBgn0031320	CG5126	-	CG5126
FBgn0031418	CG3609	-	CG3609
FBgn0032350	CG6287	-	CG6287
FBgn0032453	CG6180	-	CG6180
FBgn0032721	CG10602	-	CG10602
FBgn0032787	CG10195	-	CG10195
FBgn0033312	CG8642	-	CG8642
FBgn0035911	CG6638	-	CG6638
FBgn0037279	CG1129	-	CG1129
FBgn0037279	CG1129	-	CG1129
FBgn0037432	CG10298	-	CG10298
FBgn0039568	CG4815	-	CG4815
FBgn0039616	CG11828	-	CG11828
FBgn0039737	CG7920	-	CG7920
FBgn0040503	CG7763	-	CG7763
FBgn0042138	CG18815	-	CG18815
FBgn0086254	CG6084	-	CG6084

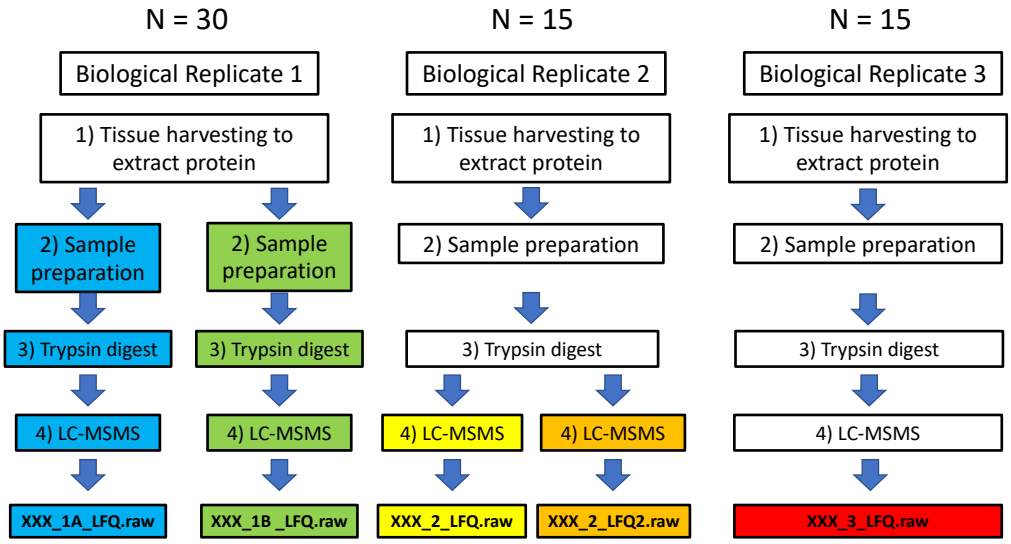

Figure S4.1. LC-MS/MS data acquisition experimental design. "XXX" refers to population (C, Colorado or V, Vancouver) followed by tissue type (AG, accessory gland or EB, Ejaculatory bulb). "XXX_1A/1B" = sample processing technical replicates of biological replicate 1; "XXX_2_1/2" = LC-MS/MS technical replicates of biological replicate 2; "XXX_3" = biological replicate 3, ran in singlet.

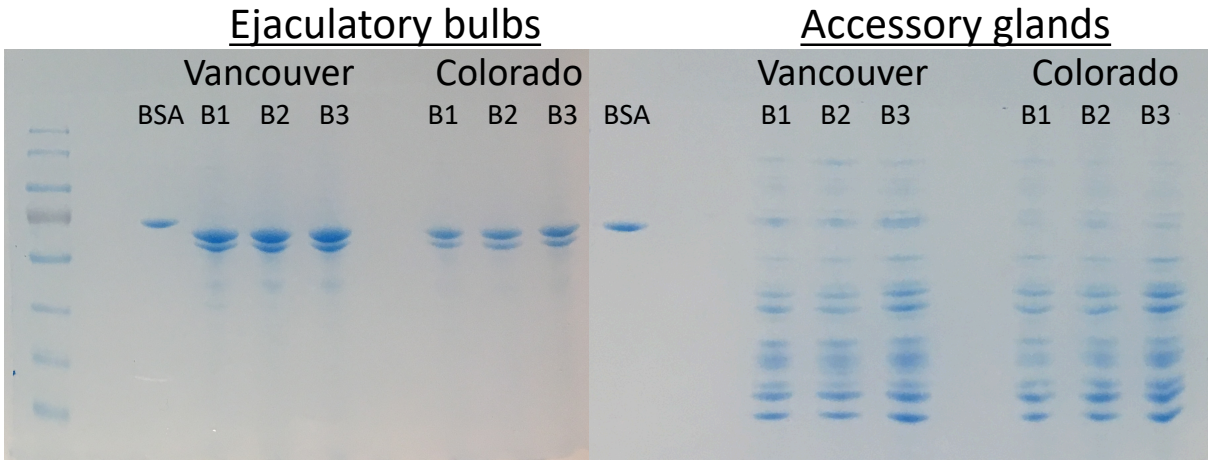

Figure S4.2. SDS-PAGE gel images of 1µl of each tissue sample diluted in Lammeli buffer and 1µl Bovine Serum Albumin (BSA) standard (0.15µg/µl). B1-3; Biological replicates 1-3. Images have been cropped and merged to exclude the molecular weight marker from the accessory gland gel (right). Molecular weight ladder: Pierce™ Prestained Protein Molecular Weight Marker Thermo Scientific™ 26612.

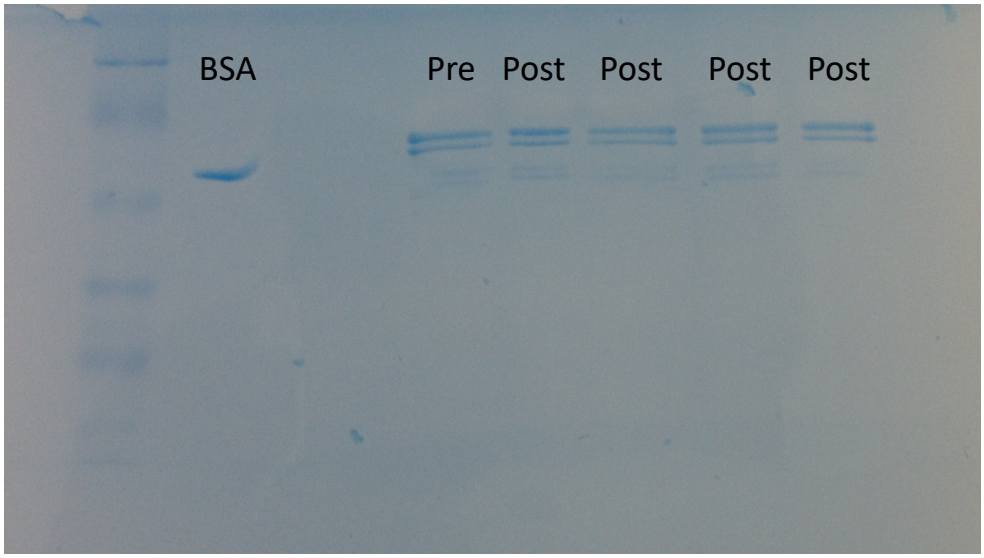

Figure S4.3. SDS-PAGE gel image comparing 1µl protein extracted from whole Vancouver 2008 cage male reproductive tract before (Pre) and after (Post) HiPPR™ detergent removal x 4 and 1µl BSA standard (0.15µg/µl). Molecular weight ladder: Pierce™ Prestained Protein Molecular Weight Marker Thermo Scientific™ 26612.

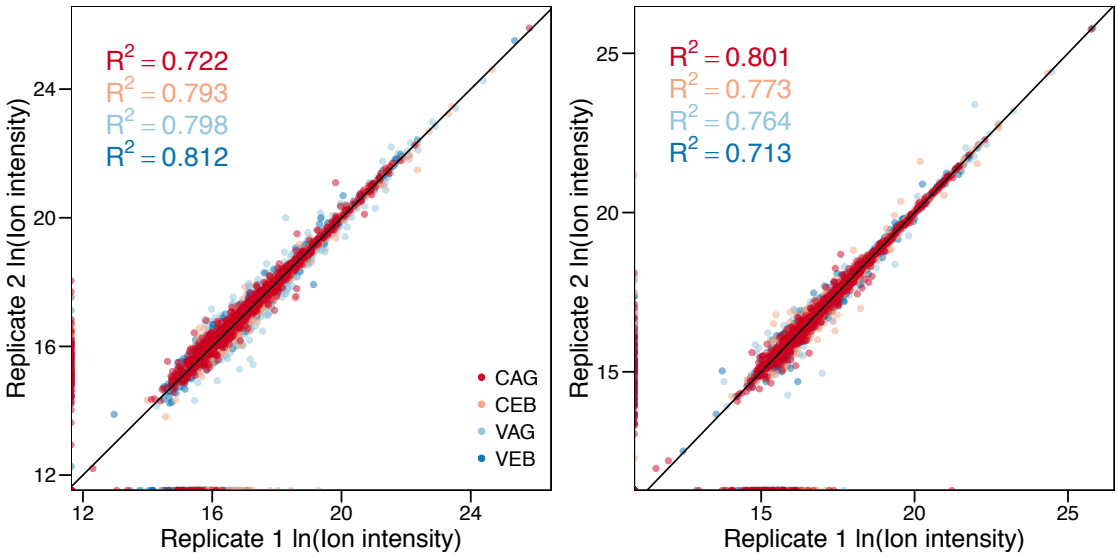

Figure S4.4. Correlation between sample processing technical replicates of biological replicate 1 (left) and between LC-MS/MS technical replicates of biological replicate 2 (right). Adjusted R^2 in top left of each panel for each tissue. C, Colorado; V, Vancouver; AG, Accessory Glands; EB, Ejaculatory Bulbs. Black line = 1:1 line.

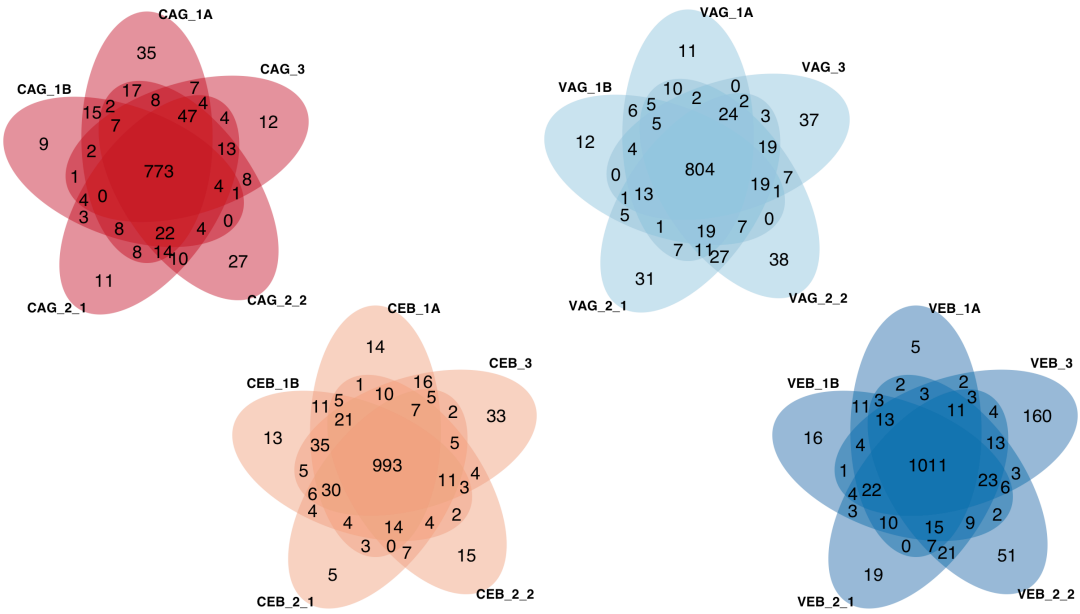

Figure S4.5. Total number of proteins identified in each replicate of each tissue. C, Colorado; V, Vancouver; AG, Accessory Glands; EB, Ejaculatory Bulbs; Replicate labels refer to Figure S4.1; "XXX_1A/1B" = sample processing technical replicates of biological replicate 1; "XXX_2_1/2" = LC-MS/MS technical replicates of biological replicate 2; "XXX_3" = biological replicate 3, ran in singlet.

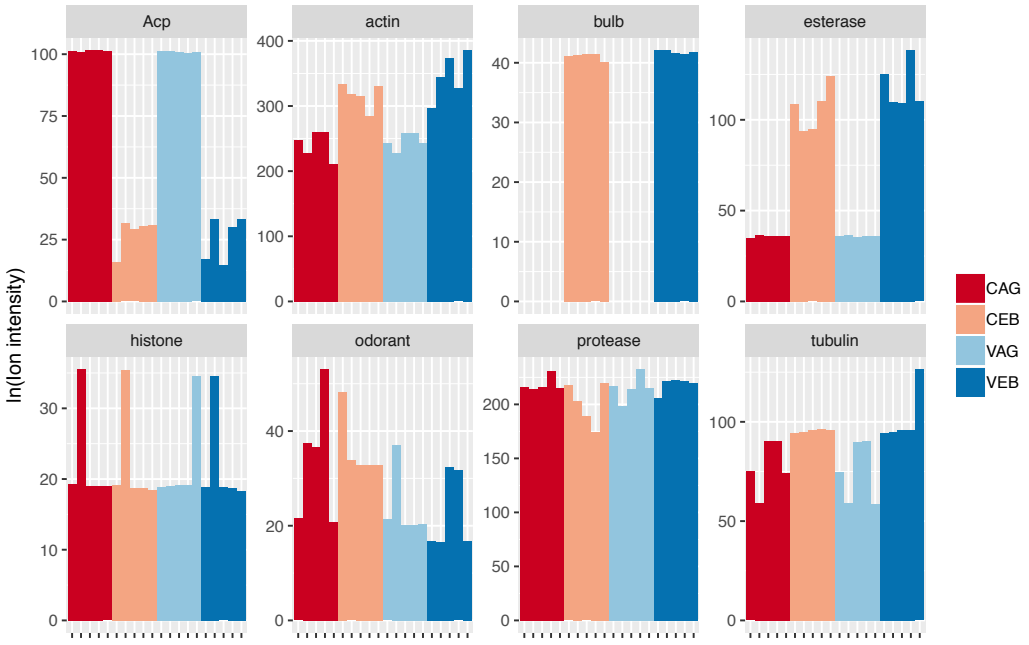

Figure S4.6. Representative protein abundances showing proteins with annotations associated with the accessory gland proteome (e.g. "Acp") or ejaculatory duct/bulb (e.g. "bulb") are more abundant in samples of the respective tissue. Note y-axes vary in each panel. C, Colorado; V, Vancouver; AG, Accessory Glands; EB, Ejaculatory Bulbs.

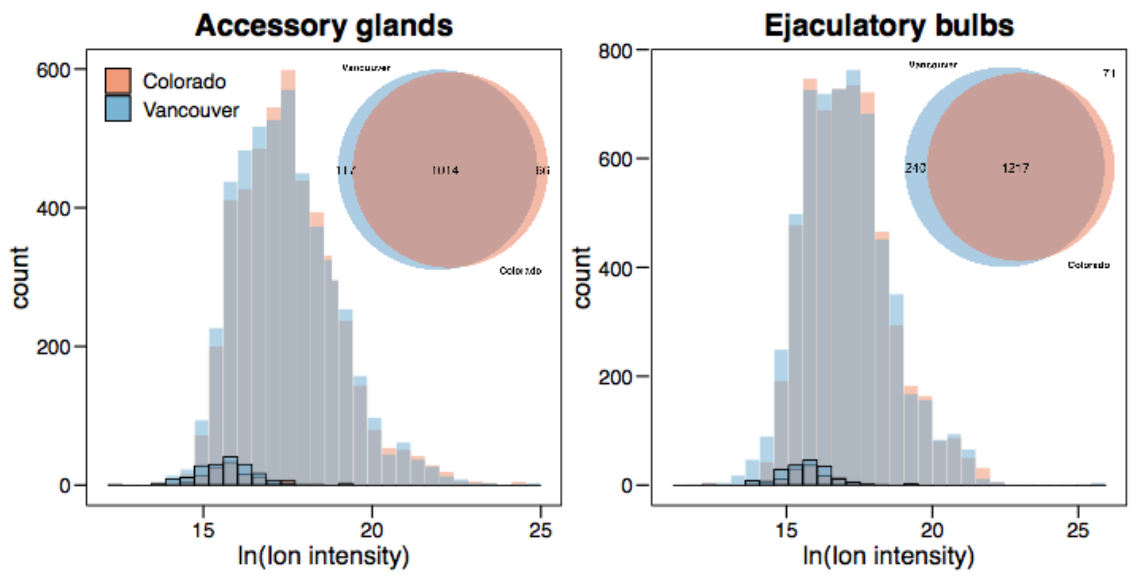

Figure S4.7. Shared proteins between populations in the AgP (left) and EbP (right) using all available data. Distribution of unique proteins shown in black outline. Inset: Venn diagrams showing shared and unique proteins.

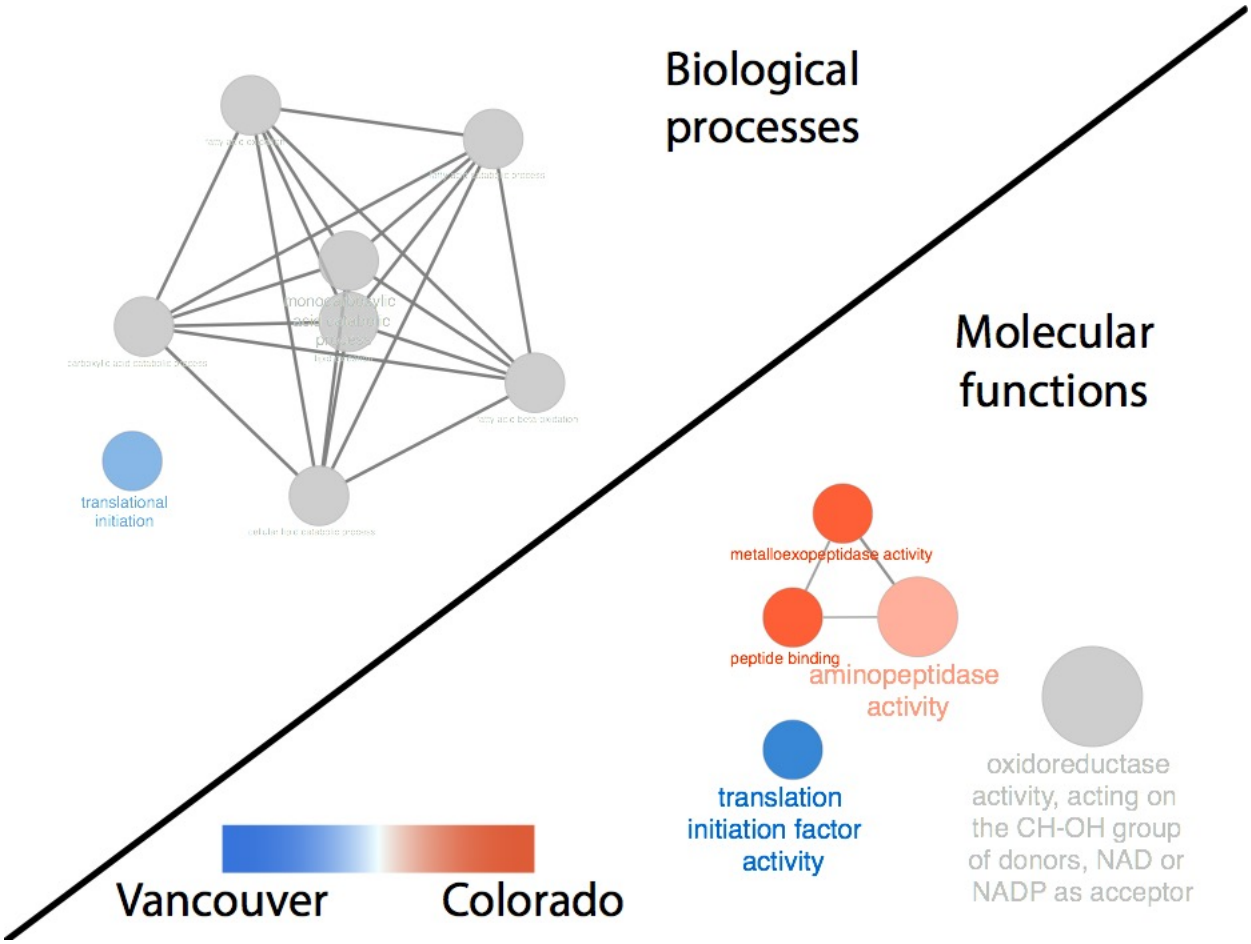

Figure S4.8. ClueGO network view of differentially abundant proteins found in the accessory gland proteome (AgP). GO terms in the network enriched in Colorado (red), Vancouver (blue), or showing no difference (grey).

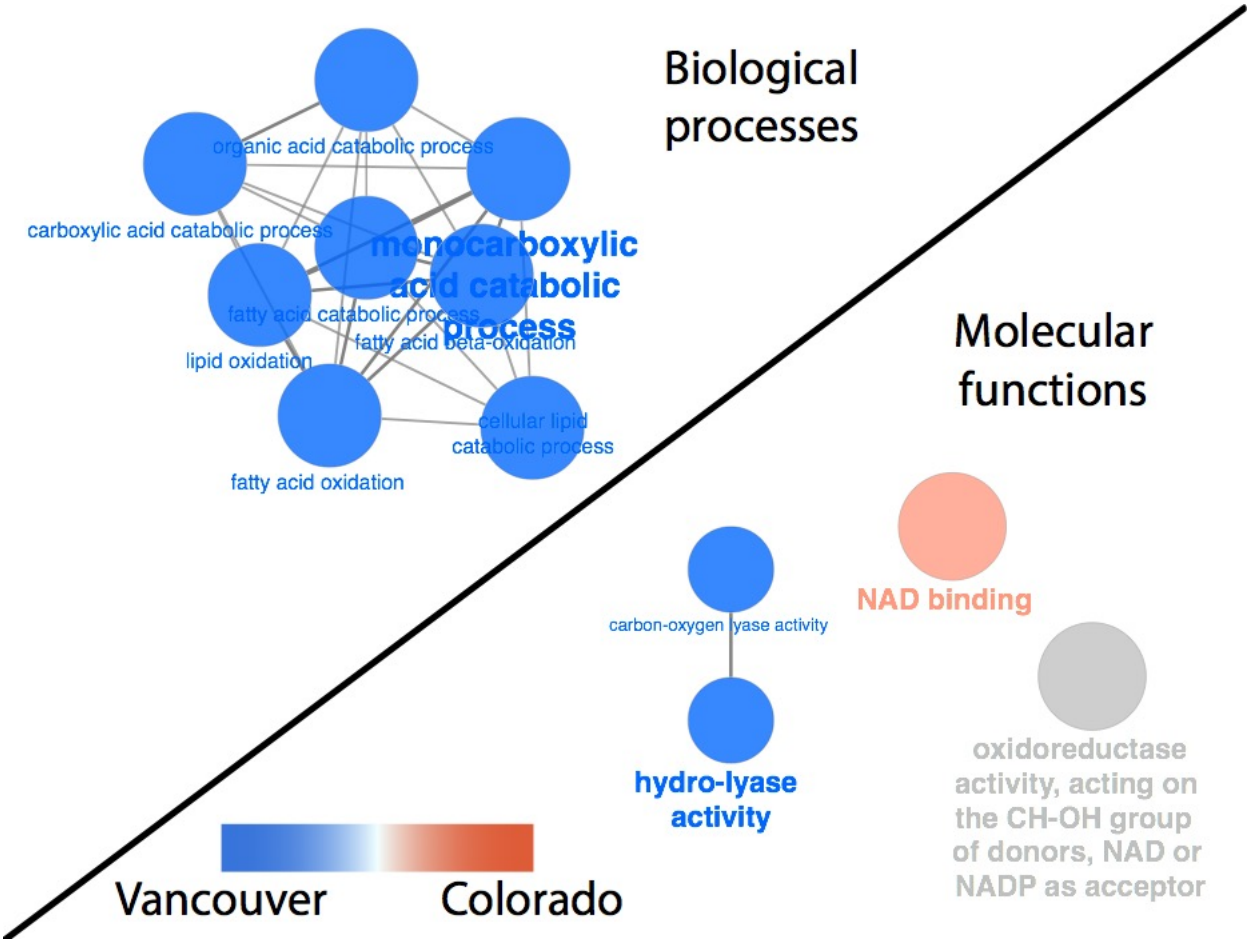

Figure S4.9. ClueGO network view of differentially abundant proteins found in the ejaculatory duct and bulb proteome (EbP). GO terms in the network enriched in Colorado (red), Vancouver (blue), or showing no difference (grey).

Table S5.1. Live weight (mg) of individuals used in metabolic rate assays.

Sex	Treatment	mean	s.e.	n
Female	M	1.19	0.05	12
	P	1.41	0.11	12
Male	M	0.812	0.07	12
	P	0.859	0.07	12

Table S5.2. Wing vein VI length (µm) measurements of individuals used in development time assays.

Sex	Treatment	mean	s.e.	n
Female	M	2324	4.80	152
	P	2335	5.76	118
Male	M	2099	4.93	154
	P	2114	5.96	127

Table S5.3. Multivariate analysis of variance (MANOVA) results of the effects on variation in relative metabolite composition across selection lines. Model based on mean values per line and sex.

	Wilks's λ	F value	df	Pr(>F)
Selection	0.286	3.99	5,8	0.041
Sex	0.253	4.72	5,8	0.026
Selection x Sex	0.475	1.77	5,8	0.226

Table S5.4. Summary table from MANOVA.

Metabolite	Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Lipids	Selection	1	1.849	1.849	4.612	0.053
	Sex	1	5.442	5.442	13.571	0.003
	Selection x sex	1	0.086	0.086	0.214	0.652
	Residuals	12	4.812	0.401		
Aqueous fraction	Selection	1	1.126	1.126	1.807	0.204
	Sex	1	0.656	0.656	1.052	0.325
	Selection x sex	1	0.305	0.305	0.489	0.498
	Residuals	12	7.478	0.623		
Protein	Selection	1	0.181	0.181	0.401	0.538
	Sex	1	1.296	1.296	2.868	0.116
	Selection x sex	1	0.835	0.835	1.849	0.199
	Residuals	12	5.421	0.452		
Glycogen	Selection	1	1.355	1.355	2.325	0.153
	Sex	1	1.708	1.708	2.932	0.113
	Selection x sex	1	0.331	0.331	0.567	0.466
	Residuals	12	6.993	0.583		
Chitin	Treatment	1	0.828	0.828	1.866	0.197
	Sex	1	5.711	5.711	12.867	0.004
	Treatment x Sex	1	0.125	0.125	0.281	0.606
	Residuals	12	5.326	0.444		

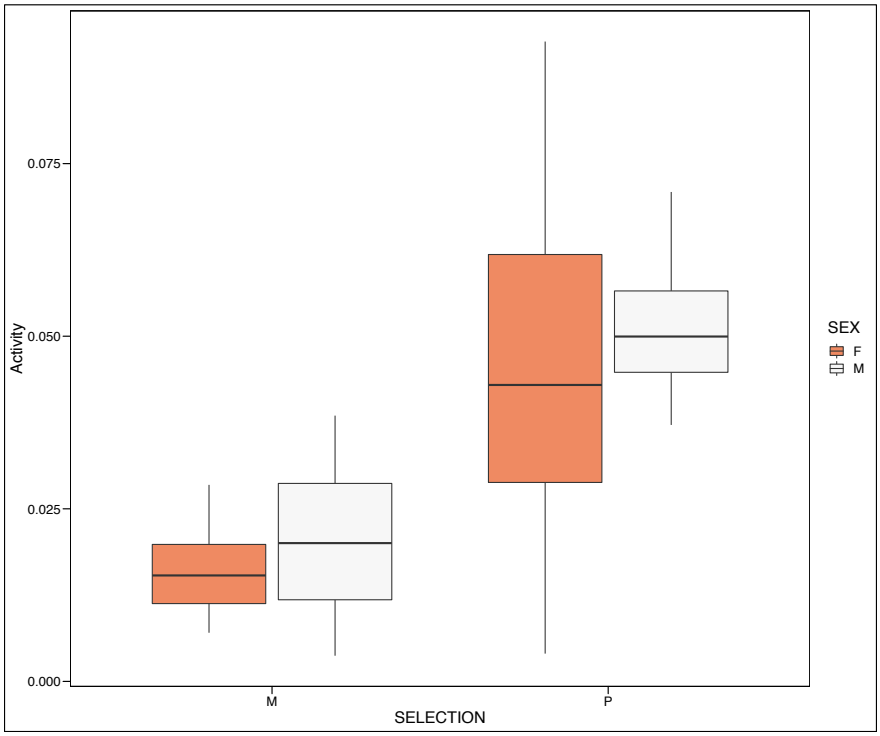

Figure S5.1. Activity measured from flies used in metabolic rate experiments.

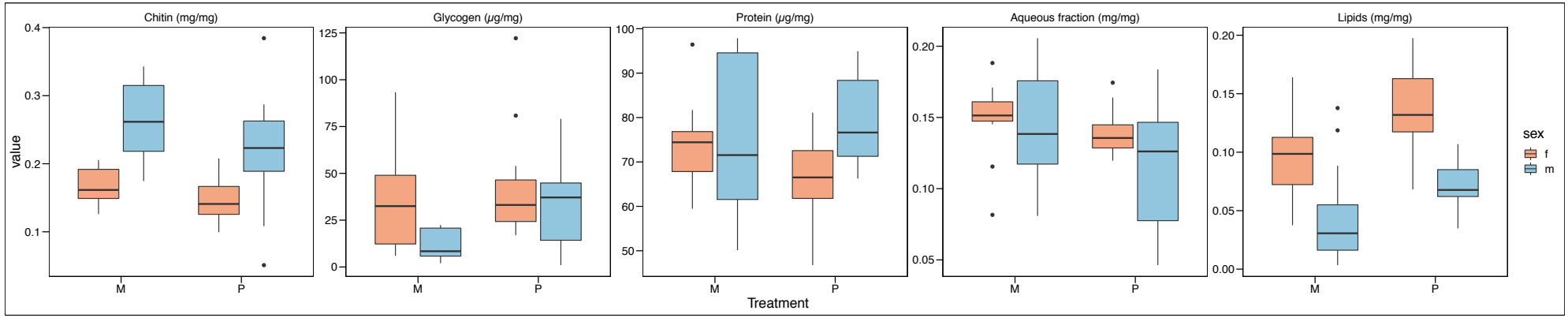

Figure S5.2. Metabolite raw values (µg or mg per milligram dry weight). Note y-axis scale varies in each panel.