Proteomic Analysis of Remating Inhibition in Queensland Fruitfly

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Abstract

The Queensland fruit fly (Qfly) *Bactrocera tryoni* is a major pest of Australian horticulture. A Sterile Insect Technique is being developed to control Qfly but remating of females with wild males could undermine its success. In Qfly, the transfer of seminal fluid during mating influences the remating propensity of females. However, the protein and peptide composition of Qfly seminal fluid has not been characterised. This thesis presents a proteomic analysis of Qfly seminal fluid to identify candidate peptides for sexual inhibition. It characterises the reproductive gland proteomes of sexually mature virgin males, which possess full gland contents, just-mated males of the same age whose gland contents have been transferred to their mates, and males dissected 13–16.5 hours after mating whose gland contents should be substantially replenished. I find 63 candidate seminal fluid proteins, 21 of which could be sufficiently annotated. I do not find homologues of the Sex Peptide responsible for remating inhibition in *Drosophila melanogaster*, but I do find matches to other peptide hormones. I also find several proteases, other post-translational protein modifying enzymes, and ligand-binding proteins, some number of which could be responsible for activation and transport of prohormones to their receptors in the female.

Keywords: Queensland Fruit fly, Remating Inhibition, Seminal Fluid Proteins, Proteomics

1. Introduction

1.1 Polyandry and its significance in nature and pest management

Diverse insect species are known to utilise polyandrous mating systems, in which females mate with multiple males (Birkhead and Moller, 1998). Even though multiple mating can be costly to females, resulting in reduced longevity (Arnqvist and Nilsson, 2000) and increased risk of sexually transmitted diseases (Hurst et al., 1995), it increases their Darwinian fitness and, by increasing effective population size and reducing the risk of inbreeding depression, the viability of the population (Tregenza and Wedell, 2002; Zeh and Zeh, 2001).

Multiple mating by females can cause sperm competition and consequently sexual selection among their different suitors (Rowe and Arnqvist, 2012). The reproductive success of the males depends not only on their success in courtship behaviour, copulation and insemination, but also on the success of their sperm in fertilisation (Simmons, 2001). Males' reproductive fitness is enhanced if they can monopolise the fertilisation of their partners' eggs. However, if once-mated females accept additional males, then the latters' sperm can displace or otherwise impede the first mates' sperm. In some insects, the ejaculates of subsequent male partners show allospermicidal functions in the females' reproductive tracts, attacking the sperm from previous matings (see for example Tracey Chapman, 2001 for *Drosophila melanogaster* and Fry and Wilkinson, 2004 for *Cyrtodiopsis whitei*).

Apart from its importance to Darwinian fitness in natural populations, female remating propensity is also an important issue for insect pest control straegies based on the Sterile Insect Technique (SIT). SIT is a birth control method for pests which involves mass rearing of the species under laboratory conditions, sterilising the males using ionic or chemo-radiation and finally area-wide mass release of those sterile males (Knipling, 1955, 1960). The treated males act as biological control agents against their untreated, wild partners by nullifying their potential to produce viable offspring (Klassen and Curtis, 2005). Critically therefore, SIT depends on mated females not remating to any significant extent; otherwise wild females initially mated to sterile SIT males can still produce fertile eggs with any wild male with which they subsequently remate. Given minimal remating, the size of the pest population will be reduced drastically in the next generation.

Efficacy and off-target safety concerns with the use of traditional chemical insecticides (Dominiak and Ekman, 2013) have seen growing interest in the development of SIT programs around the globe to suppress or eradicate a diverse range of insect pests. In particular, SIT programs have been widely applied against diverse tephritid fruitflies, such as the mediterranean fruit fly *Ceratitis capitata* (Hendrichs et al., 1983), the melon fly *Bactrocera cucurbitae* (Kuba et al., 1996) and the Queensland fruit fly (Qfly) *Bactrocera tryoni* (Fisher, 1994).

Qfly is Australia's most economically damaging pest of fruit and vegetable crops, causing losses of approximately $160 million per year (Clarke et al., 2011). So far, more than 250 species of horticultural crops have been recorded as potential hosts for this pest (Yonow, 2014). Endemic to eastern Australia, Qfly has been reported to disperse into southern parts of mainland Australia, Tasmania, New Caledonia, French Polynesia, the Cook Islands, Pitcairn Island and New Zealand (Clarke et al., 2011; Dominiak and Daniels, 2012; Kean et al., 2018). Recurrent outbreaks of Qfly in the southern parts of Australia have increased the threats to various horticultural crops in these regions (Sutherst et al., 2000). Although earlier attempts at Qfly control with SIT were not successful (Klassen and Curtis, 2005), a major new SITPlus initiative is underway to improve the pest control outcomes in southern Australia (https://horticulture.com.au/what-we-do/sitplus/), and one important component of this initiative is to gain a deeper understanding of the molecular mechanism of remating inhibition in Qfly.

1.2 The protein contents of seminal fluid and male reproductive tissues

Females of many dipteran species become sexually unreceptive to later suitors for varying lengths of time after their first mating (See Chen, 1984; Gillott, 1996, 2003; Ringo, 1996 for reviews). Most of the evidence to date indicates that certain small seminal fluid proteins play a major role in inhibiting females from remating (Avila et al., 2011, 2016). Male seminal fluid is a complex mixture of sperm, seminal fluid proteins, salts, sugars, lipids, hormones, water, immune regulators and vesicles from male reproductive tissues (Perry et al., 2013). However in the relatively few systems which have been analysed in any depth it is the proteins which are generally found to modulate the post-mating behaviours of females (Avila et al., 2011, 2016).

Seminal fluid proteins (Sfps), which are generally synthesised in the accessory glands and are consequently often also known as accessory gland proteins (Acps), are mainly comprised of members of a relatively small number of protein classes, specifically proteases, protease inhibitors, lectins, prohormones, lipases of various categories and antioxidant protective proteins (Avila et al., 2016). Although these few classes recur across species (Mueller et al., 2004), the primary sequences of Sfps diverge between species much more rapidly than do most other proteins (Findlay et al., 2014; Mueller et al., 2005; Thomas and Singh, 1992).

Our knowledge of Sfps has expanded substantially over the last two decades as transcriptomic (initially mainly expressed sequence tag (EST) screening and microarrays) and proteomic (mass spectrometry) techniques have become available (Avila et al., 2011). Before the turn of the century just 18 proteins had been reported from the accessory glands of the model organism *Drosophila melanogaster* (Chen et al., 1988; Chen and Balmer, 1989; Wolfner et al., 1997). However Swanson et al. (2001) identified 57 new candidate Acps from the sibling species *D. simulans* by EST screening and Mueller et al. (2005) then identified homologues of 34 of these in the newly published *D. melanogaster* genome. The major functional classes were serpins and other proteases, protease inhibitors, C-type lectins, acid lipases, cysteine-rich secretory proteins (CRISPs), and alkaline phosphatases.

Dorus et al. (2006) also used an early form of proteomics based on two dimensional electrophoresis and mass spectrometry to identify 381 proteins from mature *D. melanogaster* sperm isolated directly from seminal vesicles. Gene Ontology (GO) analysis found many of these proteins were involved in energy and cellular metabolism (21%) and catalysis (17%), with significant numbers of cytoskeletal proteins (9%) proteases and peptidases (3%) and DNA/RNA binding proteins (6%). About half of the proteases/peptidases were leucyl aminopeptidases (Laps). A subsequent comparison with homologues in the *D. simulans* genome (Swanson et al., 2001) showed that the sperm proteins were much more highly conserved between the two species than were the Acps. Subsequently Findlay et al. (2008) used a combination of whole-organism isotopic labelling and mass spectrometry to identify a total of 82 *D. melanogaster* Sfps which they could show were transferred to females during mating. Many of these were proteases, protease inhibitors and proteins involved in immunity and lipid metabolism as above but they also included carbohydrate and chitin

binding proteins plus several odorant binding proteins (Obp22a, Obp51a, Obp56e, Obp56f, Obp56g, Obp56i, Or82a).

Another early proteomics study of male *D. melanogaster* reproductive tissues by Takemori and Yamamoto (2009) found 440 proteins from five different male reproductive organs, 232, 168, 129, 113 and 124 from testis, seminal vesicle, accessory gland, ejaculatory duct and ejaculatory bulb respectively. About a quarter of the proteins were involved in cellular metabolism, with others often associated with protein folding/modification (9%), gene regulation (9%) and proteolysis (11%). One hundred and ninety six proteins were found to be specific to the testis and seminal vesicle, many of them involved in the production of sperm, specifically protein folding, cytoskeletal organisation (with Tektin A and C particularly abundant), axoneme assembly and other microtubule functions (e.g. the tubulins αTub84B and βTub85D), and sperm protection (e.g. the 'heat shock' proteins Hsp60B and Hsp60C, the glutathione s-transferase GstS1 and the odorant-binding proteins Obp44a and Obp99a), plus various enzymes involved in primary metabolism. On the other hand, 121 proteins were found to be specific to one or more of the other reproductive organs, 40 of them being previously reported Acps. Proteases, protease inhibitors, CRISPs, lipases, lectins and odorant binding proteins were relatively common. Two other peptides/proteins however are considered in more detail in the following section because functional studies have linked them directly to the post-mating reproductive behaviours of females. These are the so-called Sex Peptide (SP, or Acp70A) produced in the accessory glands and an esterase produced by the ejaculatory duct (Esterase 6, or Est-6).

1.3 Remating inhibition systems in insects

1.3.1 Drosophila: Remating inhibition has been most extensively studied in *D. melanogaster*, where the Sex Peptide (SP) is the primary male factor that regulates remating inhibition and other post-mating responses (PMRs) in mated females (Aigaki et al., 1991; Chapman et al., 2003; Chen et al., 1988; Liu and Kubli, 2003). The SP is encoded by an autosomal gene which is expressed exclusively in the male accessory glands (Chen et al., 1988). The 36-amino-acid SP is derived from a 55-amino-acid precursor whose first 19 residues constitute a signal sequence for secretion into the gland lumen, and thence the seminal fluid. The N-terminal region of the mature SP can bind to sperm whereas the C-terminal region is critical for receptor interaction and downstream PMRs (Ottiger et al., 2000). During mating, both free

SP and sperm-bound SP are transferred into the female to induce respectively short- and long-term PMRs (Peng et al., 2005a). Specific binding between the SP and the Sex Peptide Receptor (SPR) in *fruitless*-expressing neurons abolishes sexual receptivity in mated females (Yapici et al., 2008). Following the identification of the SPR by Yapici et al. (2008), Häsemeyer et al., (2009) and Yang et al., (2009) have independently demonstrated that the SP-SPR interaction in a subset of *fruitless* neurons that co-express *pickpocket* is sufficient to trigger remating inhibition. Subsequent functional mapping has refined the early steps of SP-signalling to six uterine neurons that express *fruitless*, *pickpocket* and *doublesex* (Rezával et al., 2012). The transmission of these uterine SP-signals to the brain requires the *ap* interneurons in the ventral nerve cord (Soller et al., 2006) and neurons that express myoinhibitory peptides (MIPs) in the abdominal ganglion (Jang et al., 2017). Knowledge of SP-mediated sexual inhibition in *D. melanogaster*, afforded by its sophisticated genetic tools, serves as a model not only for testing specific hypotheses on gene function, but it is also useful for appreciating the complexity of the phenotype, including the mechanism of long-term sexual inhibition and collateral hormonal regulations.

In *D. melanogaster*, apart from inducing acute inhibition, SP-signally also drives long-term sexual inhibition and other PMRs, which involve additional factors and pathways. For example, cessation of remating inhibition is associated with the depletion of stored sperm in mated females (Manning, 1962, 1967). Injection experiments have also shown that the inhibitory effects of injected accessory gland extract or synthetic SP into the haemolymph of virgin females were relatively short-lived compared to that of normal mating (Chen et al., 1988; Chen and Bühler, 1970). To attenuate remating inhibition, sperm-bound SPs have to be enzymatically cleaved and progressively released (Liu and Kubli, 2003; Peng et al., 2005a). In addition to the direct interaction with SPR and sperm, biochemical evidence (affinity blotting) suggests that SP is able to bind to two distinct protein targets, one in the nervous system and the other in the genital tract (Ding et al., 2003; Kubli, 2003). Even in a genetic background that lacks a functional SPR, ectopic expression of SP can still cause a significant reduction in sexual receptivity, indicating the existence of a pathway independent of SP/SPR interaction (Haussmann et al., 2013). Artificial introduction of SP into the haemolymph by injection of accessory gland extract (Chen and Bühler, 1970), injection of synthetic SP (Chen et al., 1988; Tsuda et al., 2015) or ectopic expression of SP (Aigaki et al., 1991; Nakayama et al., 1997) is still able to elicit full short-term PMRs, without

any obvious involvement of the reproductive tract. The broad binding profiles of SP in the female CNS and PNS (Ottiger et al., 2000) also lend support to the possible direct interaction between SP and its CNS/PNS targets(s) via the haemolymph, circumventing the reproductive tissues. Apart from its roles in regulating remating inhibition and oviposition, SP can induce many pleiotropic physiological and behavioural responses in the mated females (Avila et al., 2016; Domanitskaya et al., 2007; Gioti et al., 2012; Moshitzky et al., 1996; Peng et al., 2005b), some of which are not directly related to neurological activities, and are likely to involve non-SPR target(s) and pathway(s). It is also unclear if other seminal fluid co-factors are required for these diverse SP roles in PMR regulation.

Although the *D. melanogaster* SP is a useful paradigm for understanding the mechanism of sexual inhibition, it is probably not the only inhibitory pathway in insects. Tsuda et al. (2015) noted that SP is only found in the *Drosophila* genus and the SP/SPR-based post-mating response is highly variable within the genus *Drosophila*. Heterologous injection experiments have also demonstrated clear phylogenetic constraints on SP function (Tsuda et al., 2015; Tsuda and Aigaki, 2016). When synthetic SP of *D. melanogaster* was injected into females of different *Drosophila* species, remating inhibition was observed in species relatively closely related to *D. melanogaster* in the melanogaster group of the subgenus Sophophora (*D. simulans, D. sechellia, D. yakuba, D. erecta and D. ananassae*), but not in more distantly related species (*D. pseudoobscura, D. persimilis, D. willistoni and D. mojavensis)* (Tsuda et al., 2015). Consistent with the latter finding, the genomes of the distantly related *D. mojavensis* and *D. grimshawi* have not been found to contain genes that would encode SP orthologues (Tsuda et al., 2015).

However, while the SPs themselves may be phylogenetically restricted, even within the Drosophilidae, SPR genes have been found in diverse insect genomes (Kim et al., 2010; Tsuda et al., 2015; Tsuda and Aigaki, 2016; Yapici et al., 2008), which suggests that SPRs may have additional ligands and functions. Indeed, myoinhibitory peptides (MIPs) have also been found to be potent agonists for SPRs (Kim et al., 2010) but activation of specific MIP expressing neurons in the abdominal ganglion can override SP-signalling, in an SPR-independent fashion (Jang et al., 2017). Although SP homologues are not detectable in non-Drosophilid insects, MIPs are found in diverse species (Kim et al., 2010), and may contribute to the regulation of remating inhibition in the oriental fruit fly, *Bactrocera dorsalis* (Gui et al.,

2017). Nonetheless, given that sexual inhibition is common in insects, and that the SP-responsive species are restricted to the melanogaster group in Drosophila, additional remating inhibition mechanisms must exist. The well-characterised mode of action of *D. melanogaster* SP may represent one pathway that can lead to sexual inhibition; the search for inhibitory molecules in other taxa therefore need not be confined to specific proteins/peptides (e.g. SP homologues) or organ secretions (e.g. male accessory gland contents).

Interestingly however, while the SP itself might be naturally restricted to certain *Drosophila*, injected SP still shows some bioactivity in a few other non-drosophilid species. For example, injection of the SP from *D. melanogaster* into adult females of the moth *Helicoverpa armigera* suppresses their calling behaviour and oviposition activity (Hanin et al., 2012). Injection of SP into adult female medflies decreases their juvenile hormone levels (Moshitzky et al., 2003), although the opposite effect has been observed in *D. melanogaster* (Moshitzky et al., 1996). Thus, although Qfly is phylogenetically quite distant from *Drosophila*, components of the sexual inhibitory pathways might still be conserved and even functional.

As a further complication to the story, *D. melanogaster* also secretes an ejaculatory duct-derived 31 residue paralogue of SP named DUP99B, which is also transferred during mating with the seminal fluid and induces some minor short-term PMRs in mated females, independent of SP and sperm (Liu and Kubli, 2003). DUP99B is produced from a 54 amino acid precursor with a signal peptide of 21 amino acids and shows high sequence similarity in both its N- and C-terminal regions to the mature SP peptide (Saudan et al., 2002). Males with intact ejaculatory duct but no accessory glands only induce a weak post-mating response in their partners (Rexhepaj et al., 2003) whereas males lacking this DUP99B gene but retaining the SP gene still induce as strong a refractory response in their partners as do wild-type males, suggesting that DUP99B might only have some limited inhibitory role. Consistent with this, whereas SP is apparently conserved within the subgenus Sophophora, DUP99B in not seen in *D. erecta* and may be limited to *D. melanogaster*, *D. simulans, D. sechellia* and *D. yakuba* (Kim et al., 2010).

Three other seminal fluid proteins have also been implicated in remating inhibition in *D. melanogaster*. Two of them which are produced in the ejaculatory bulb (PEBme and PEBII) are known to influence the size of the female's 'mating plug' (Ludwig et al., 1991; Lung and

Wolfner, 2001), which contributes to the short term reduction of receptivity (Bretman et al., 2010). The other seminal fluid protein is the enzyme esterase 6 (EST 6), which is produced in the ejaculatory bulb of the male, rapidly translocated from the female's reproductive tract to her haemolymph following mating and then inhibits remating and stimulates egg laying for up to a week afterwards (Gilbert et al., 1981; Scott, 1986). The mechanism by which EST 6 acts is as yet unknown, although interestingly it has also been shown to be strongly expressed in the antennae of both sexes, where it acts as a general odorant degrading enzyme (ODE) (Younus et al., 2017).

1.3.2 Mosquitoes: Acp-induced sexual inhibition has been reported from several mosquito species (Craig, 1967; Shutt et al., 2010; Young and Downe, 1983). Remating inhibition studies in *Anopheles gambie* reported a male accessory gland derived steroid hormone, 20-hydroxyecdysone (20E) which is transferred to female during mating and confers long term sexual refractiveness after 1-2 days post mating – similar to SP of *D. melanogaster* (Chapman et al., 2003; Gabrieli et al., 2014; Pondeville et al., 2008). Mates of sperm-less males do not remate further, suggesting a sperm-independent inhibitory role of 20E in *A. gambie* (Thailayil et al., 2011).

1.3.3 Other insects: As yet uncharacterised accessory gland secretions have also been shown to have remating inhibitory activity in females of several dipteran insect species and other arthropods (Davies and Chapman, 2006; Radhakrishnan and Taylor, 2007 and references therein). Seminal fluid induced sexual refractoriness has also been described in seed beetles (Yamane et al., 2008; Yamane and Miyatake, 2010) and several families of ticks and spiders (see Mendez et al., 2017 for a list). When injected with male accessory gland substances, remating refractiveness has been observed in ground beetles (*Leptocarabus procerulus)*, West Indian sweet potato weevil (*Euscepes postfasciatus*), Tobacco cutworm (*Spodoptera litura),* Corn earworm (*Helicoverpa zea)*, Western tarnished plant bug (*Lygus Hesperus*) and bumblebees (*Bombus terrestris)* (Baer et al., 2001; Brent and Hull, 2014; Hartmann and Loher, 1999; Hayashi and Takami, 2014; Himuro et al., 2017; Kingan et al., 1995; Yu et al., 2014). However, none of these studies of other insect Orders identified specific proteins responsible for the remating inhibition effects.

1.3.4 Tephritids: The molecules in male ejaculates responsible for remating inhibition and stimulation of egg laying in females have not yet been identified in tephritids but several lines of study provide useful information on their characteristics.

A bimodal pattern of sexual inhibition is observed in the Mediterranean fruit fly *C. capitata*, with an initial and short term refractiveness induced by sperm storage and a longer term inhibition induced by accessory gland products (Mossinson and Yuval, 2003). In contrast to the sperm effect in *C. ceratitis*, propensity of female remating does not seem to be influenced by the number of stored sperm in several species of *Bactrocera* and *Anastrepha* (see Abraham et al., 2011, 2016 for *A. fraterculus* and *A. ludens*; Harmer et al., 2006 for *B. tryoni*; Kuba and Itô, 1993 for *B. dorsalis*; Landeta-Escamilla et al., 2016 for *A. serpentina*). However, Harmer et al. (2006) detected a significant negative association between sperm storage and remating probability of females mated with non-irradiated males. Such a quantitative relationship could be explained by the proportionality of sperm number and titre of seminal fluid, which might exert remating inhibition in mated females in a concentration–dependent manner (Harmer et al., 2006). This finding in *B. tryoni* is analogous to early observations in *D. melanogaster,* where the magnitude of short-term PMRs (measured as number of eggs laid and remating rate of once mated females) declined as the male accessory gland secretion progressively reduced in serially mated males, whose fertility (measured the proportion of adults emerging from egg laid) remained stable (Hihara, 1981). On the other hand, irradiation of male *C. ceratitis* and *A. fraterculus* can reduce the remating inhibitory effect (Abraham et al., 2012; Kraaijeveld and Chapman, 2004), but no such effect was observed in bactroceran flies (Haq et al., 2013; Harmer et al., 2006; Radhakrishnan et al., 2009b). However, the seminal fluid of Qflies at least is apparently not affected by the irradiation treatment (Harmer et al., 2006), which might suggest that the inhibiting factors are in the seminal fluid.

Injection of male accessory gland extracts into virgin females has been shown to induce sexual refractiveness in recipient females of *C. ceratitis* (Jang, 1995) and *A. fraerculus* (Abraham et al., 2011), but not *A. suspensa* and *A. ludens* (Abraham et al., 2014; Lentz et al., 2009). Radhakrishnan, and Taylor (2007) injected a mixture of Qfly accessory gland, ejaculatory apodeme (=bulb) and ejaculatory duct extracts into virgin females and observed increased remating inhibition. While this latter result confirms the existence of molecules

with some analogous effects to *Drosophila* SPs in Qflies, it leaves unanswered the question of the glands of origin.

To investigate this issue further Radhakrishnan and Taylor (2008) compared the sizes of various reproductive organs of Qfly males before and after mating. The dimensions of the accessory glands but not the testis or ejaculatory apodeme were found to be reduced after mating. These results suggest accessory gland secretions may be involved in the post-mating effects on female behaviour but the involvement of the ejaculatory duct and apodeme has not been ruled out owing to difficulty in measure relative size change in those organs, which are both thick muscular tissues lined by a thick cuticle and may have not contracted even if they did secrete significant material into the seminal fluid (Radhakrishnan et al., 2009a).

However Radhakrishnan et al. (2008) also performed a radio-isotopic bioassay, where mature males were provided with a radiolabelled diet, finding that the radioactivity levels of the accessory glands were reduced after mating. Further, they found that the radioactivity was transferred to females during mating, moving from their reproductive tracts to other somatic tissues (haemolymph and thorax and brain) immediately after mating, and finally accumulating in the thorax and the head (Radhakrishnan et al., 2008). These results are consistent with, but not conclusive evidence, that the male donated products responsible for remating inhibition and stimulation of egg laying in the females may be of accessory gland origin and suggest that binding sites for those products may be located in the female CNS and Peripheral nervous system (PNS). However the identity of the male-derived products, their binding partners, their modes of action in inhibiting remating remain unknown.

Several transcriptomic studies of male reproductive tissues have identified possible components of the seminal fluid of different tephritids. The first organ-specific study, by Davies and Chapman (2006), reported 13 ESTs with Open Reading Frames (ORFs) encoding proteins in a size range of 73-262 amino acids from accessory glands of *C. capitata*. Six of them included putative signal peptides, which would be necessary for secretion into the lumen of the gland, and thence the seminal fluid. Nine of them showed strong sequence identity with *D. melanogaster* Acps and four did not show significant similarity to any known proteins, indicating considerable evolutionary lability at the sequence, if not functional

level. Putative functions of the annotated peptides included a juvenile hormone binding protein (JHBP), cysteine-rich secretory protein (CRISP), trypsin-like serine protease, lipase, γ-aminobutyric acid transaminase and thioredoxin peroxidase. However, expression of these annotated proteins is not restricted to reproductive tissues in *D. melanogaster,* based on anatomical expression data available in FlyAtlas (Robinson et al., 2013).

In the oriental fruit fly, *B. dorsalis*, proteomic and/or transcriptomic profiling has been directed at specific reproductive tissues (Tian et al., 2017; Wei et al., 2015a, 2015b, 2016). Ninety putative accessory gland proteins (Acps), i.e., proteins found in accessory glands that carry a signal peptide, have been identified (Wei et al., 2015a). The 26 putative Acps with functional prediction included proteases, Obps, metalloproteinase, ribosomal protein, serine protease inhibitor and proteins associated with immunity. Amongst the 90 Acps detected, the most abundant one was a putative JHBP, believed to facilitate transportation and protection of juvenile hormone (Wei et al., 2015a). Tian et al. (2017) re-assembled existing RNAseq datasets from multiple tissue types and identified unigenes specific to the accessory gland or ejaculatory duct. These included a C-type lectin and antigen 5 involved in immunity; perlucin, glucose dehydrogenase and lipase-1 believed to be involved in sperm function; and pheromone-binding protein-related protein-5 putatively involved in mating. These specific genes may be involved directly in the functioning of the seminal fluid. Targeted annotation of the *B. dorsalis* genome and transcriptome datasets from six tissue types by Gui et al. (2017) identified 39 candidate neuropeptides, including the *B. dorsalis* homologue of allatostatin B (or MIP), which has been implicated in modulating SP-signalling in specific MIP-expressing neurons and, as a consequence, sexual recptivity in *D. melanogaser* (Jang et al., 2017).

Besides seminal fluid proteins, Zingerone, a plant derived fruit fly lure, can also modulate *B. tryoni* male behaviour and physiology, which can indirectly affect the post-mating reproductive activity of their mates (Kumaran et al., 2013; Kumaran and Clarke, 2014). No organ-specific transcriptomic studies has been conducted so far regarding the effect of the lures but some whole body transcriptomic comparisons between lure-fed versus control males (Kumaran et al., 2014) showed 3198 differentially expressed genes (DEGs), the vast majority of which were enriched in the lure-fed group. Interestingly several of the upregulated genes had functional annotations related to mating behaviours, including some

encoding accessory gland proteins. Kumaran et al. (2018) then compared whole body transcriptomes of *B. tryoni* virgin females, females mated with normal males and females mated with lure-fed males. Three hundred and thirty one DEGs were identified in the control-mated females, and several of these DEGs were immune responsive or chorion genes. In the lure-mated females, an additional 80 DEGs were found, including sperm and testes specific genes. None of the genes identified in these studies, whether DEG or not, were homologues of genes with known remating inhibition porterties in other species.

1.5 My project

The goal of my study is to identify proteins/peptides of Qfly seminal fluid which may have roles in remating inhibition and stimulation of egg laying in females, and to identify their glands of origin.

My study uses modern proteomics rather than transcriptomics as the screening tool, for three reasons. Firstly, proteins are generally the bioactive products of the genome whereas the transcripts are generally the intermediaries between the genome-encoded information and those bioactive products. Secondly, the transcriptomes are often not reliable indicators of the relative abundances, or even sometimes the presence or absence of particular proteins; wide variation in mRNA stabilities and the extracellular transport of the proteins often make the transcripts poor indicators of protein amounts in particular samples (Belle et al., 2006; Gygi et al., 1999; Rogers et al., 2008). Thirdly, proteins are often subjected to post-translational modifications which cannot often be inferred from mRNA sequences (Dhingra et al., 2005). As noted, previously proteomics was a relatively cumbersome and insensitive technology combining two-dimensional gel electrophoresis and mass spectrometry (Figeys et al., 1996; O'Farrell, 1975), but the latest LC-MS/MS shotgun strategies as performed at CSIRO permit detection and quantitation of several thousand proteins per sample.

My study also includes three other novel aspects of experimental design compared to the previous work on other tephritids. Firstly, it not only compares the proteomes which are specific to different male reproductive tissues but it also compares them between virgin and just-mated males. Secondly, it also looks at the proteomes of sexually mature virgin versus mated males 13-16.5 hours after mating, to see what molecules are being replenished during their recovery periods. Finally an extensive bioinformatic analysis is used in combination with four different databases which are specifically adapted to include insect neuropeptides

and proteins related to reproduction and small peptides in the size ranges similar to SP and other candidate inhibitory molecules reported in other insects.

2. Method and Materials

2.1 Experimental approach: This thesis is based on two complementary proteomic experiments on the reproductive tracts of sexually mature Qfly males. The ultimate aim of the experiments is to identify the proteinaceous components of the seminal fluid and in particular to identify the functional equivalent(s) of the Drosophila sex peptides (SP and DUP99B). The task is thus to identify proteins and peptides secreted into the lumens of the male reproductive glands after the seminal fluid has been generated but not yet transferred to the female during mating.

The Qfly strain used for these experiments, S06 (see below), is fully sexually mature at 6 days post-eclosion. All mating experiments were conducted using 8-day old flies. The first comparison was between virgin males and just-mated males (i.e., the 'depleted' males). Dissection of reproductive tissues (testes, accessory glands and ejaculatory apodeme) was done within 3 hours upon mating completion. The second comparison was between males that had mated at 8 days (i.e., the 'depleted' males) and males that had been aged for a further 13 – 16.5 hours after mating (i.e., the 'replenished' males). Compared to unmated males, the area and length of mesodermal accessory glands of the mated males are known to diminish immediately after mating, and gland size recovers steadily over a period from 5.5 to around 22 hours post-mating (Radhakrishnan and Taylor, 2008). The time point 13 - 16.5 hours was chosen because, based on the estimation by Radhakrishnan and Taylor (2008), the accessory glands are approximately 90% of their original size at that time. This time point allowed me to collect materials for subsequent transcriptomic analyses (beyond the scope of this thesis) while the relevant RNAs should still be present.

While the accessory glands are known to regain their size substantially in the above interval, there are no published data on the replenishment rate of seminal fluid components from the other male reproductive tissues. I therefore prepared separate proteomes for three tissue types: testes, accessory glands, ejaculatory apodeme. Attempts to extract sufficient proteins directly from the lumens of these tissues were not successful, so my inference about the secreted status of the proteins and peptides is based on the presence of a consensus N-terminal secretion peptide predicted using SignalP 4.1

(http://www.cbs.dtu.dk/services/SignalP/; Nielsen, 2017; Nordahl Petersen et al., 2011) in the reference sequences which produce tryptic peptide profiles that matched those recovered empirically from the tissue proteomes (see below).

2.2 Provenance of S06 and culture conditions

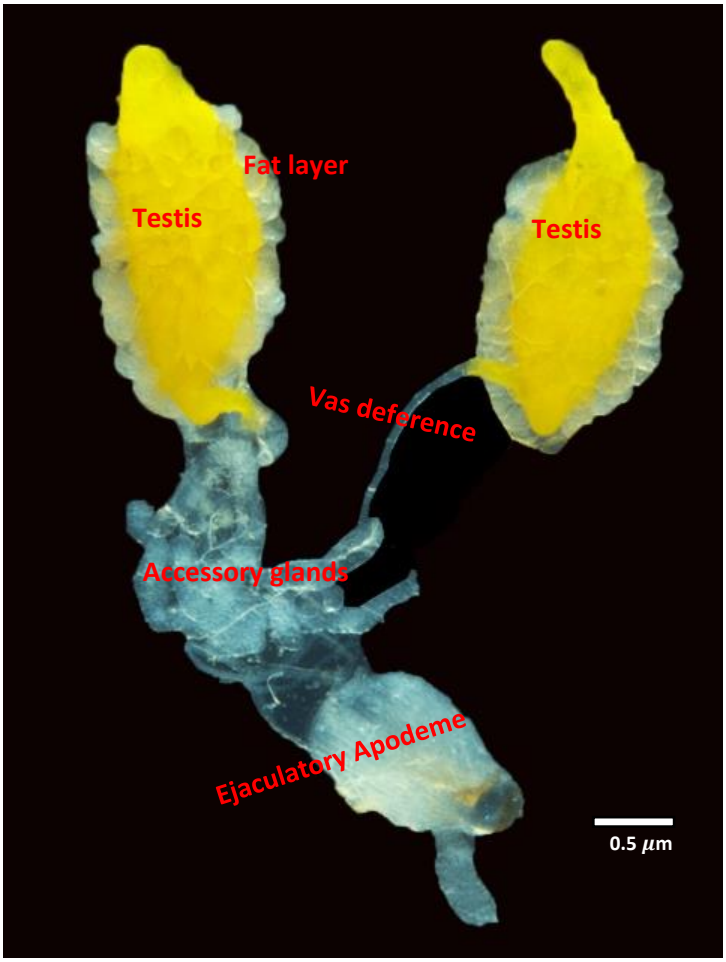
The S06 strain was originally established by Dr Stuart Gilchrist and colleagues from flies collected from loquats in Sydney, Australia in 2006. The strain was then maintained at the University of New South Wales, Sydney, Australia. A sub–strain was established in the CSIRO Black Mountain Laboratories in Canberra, Australia, in February 2015. The S06 sub-strain has since been maintained at CSIRO in a 6 week life cycle at 25-26℃ (±1℃), 65% (±5%) relative humidity, 11 hour complete dark and 11 hour full light cycle, with one hour each of dawn and dusk. At the time of my experiments, the S06 strain had been kept in laboratory conditions for at least 100 generations.

S06 eggs for the experiments were collected by placing a 250 ml plastic sauce dispenser (Décor) pierced with about 100 small pores on one side of the dispenser. A small piece of apple was suspended in the dispenser on the opposite side of the oviposition pores (to avoid females laying eggs into the apple) and this egging device was hung inside a standard 27 L insect rearing cage (BugDorm-4M3030) in the presence of about 200-300 adult SO6 flies. The pores on the dispenser were coated with apple juice to provide additional attractant for oviposition. The females are attracted to the apple and lay eggs through the pores. Females were allowed 2 hours to lay their eggs, the bottles were removed from the cage, and eggs were collected by rinsing the inside surface of the dispenser with water. Using a pipette, eggs collected at the bottom were dispensed into 30 ml plastic cups, each containing approximately 15 ml of Qfly gel diet prepared according to Moadeli et al. (2017). The cups were covered with lids to maintain moisture and placed in 1 L plastic takeaway food storage boxes, each containing ~1 cm thick of vermiculite into which the jumping larvae burrow. Each plastic box was covered with a nappy liner for ventilation. The lids of the cups were opened five days after egg collection so that the late stage third instar larvae could jump out into the vermiculite to pupate. About 10 days later the pupae were sieved from the vermiculite and placed into 27 L rearing cages (BugDorm-4M3030), at a density of approximately 250 per cage. Adult flies were provided with water, sugar and yeast hydrolysate.

2.3 Bio-assay 1: Virgin vs depleted 8-day old males

2.3.1 Set up of the experiment: Five days of daily virgin collection were used for this experiment, and each day's collection was considered a replicate. Virgin flies were collected within one day of eclosion and kept in separate cages until the mating experiment on day 8. On the eighth day after collection the flies in that collection were checked to confirm there was no contamination with the opposite sex and then sorted into three 'treatment' classes, namely mating pairs, virgin males and virgin females. Each mating pair was established by putting a single virgin female and male in a 425 ml transparent plastic cup (Cat. No. 72205; Dowlings of Canberra) together with a small moist sponge which was then covered with a transparent dome-shaped lid (Fig. 1). The virgin male class was set up in the same way except that no female was included. The virgin female class was set up in the same way except that no male was included. Each collection day/replicate consisted of 40-50 cups per treatment class (120-150 cups in total). The cups were set up about one hour before dusk to allow the flies time to settle before the onset of the conditions (dusk) conducive to mating. The cups were placed on cardboard cup holders/trays in sets of three; each set consisted of one cup from each of the three treatment classes. Female flies used in this experiment were kept for future proteome analysis.

a



b

Fig 1. Trio arrangement of mating cups (a) and reproductive organs of *B. tryoni* (b). (In Fig. 1.a VM denotes virgin male only, VF denotes virgin female only and M denotes mating pairs).

2.3.2 Mating observations: The cups containing potentially mating pairs on each replicate/day of the experiment were monitored continuously from the onset of dusk and the start and end times of the mating of each pair were recorded. Mating was considered to start when the male and female stably joined and to end when they subsequently disjoined. Males that sustained an uninterrupted copulation for 30 minutes or more were considered mated males, and were used for tissue dissection, along with the unmated males. The same 30 min copulation threshold was applied to males that mated more than once. Approximately 10-20% of the males in the mating pair treatment class were discarded either because they did not mate at all or their copula duration was less than 30 minutes. Once a completed mating had been recorded, the mated male and an unmated male were dissected simultaneously. In cases where dissection could not be done immediately after mating, cups were held in a light-sealed box to minimise light exposure prior to dissection.

2.3.3 Dissections: Mated and virgin males were dissected in ice cold phosphate-buffered saline (PBS, pH 7.4) to protect the protein and RNA contents of the glands from degradation. Their accessory glands, testes and ejaculatory apodemes were separated under a dissecting microscope and transferred to 2 ml microcentrifuge tubes (one tube per tissue type), which themselves were kept on dry ice. Glands of each type were pooled from at least 30 males of each treatment class and replicate, and each tube was then sealed and transferred to a -80 °C freezer. For tissue specific comparisons, carcasses (whole body without reproductive tissues) were also collected from both treatments and stored similarly. Dissections were completed within 3.5 hours.

2.4 Bio-assay 2: Replenished day-9 males

This experiment was carried out in the same way as Experiment 1, but with the following modifications. Firstly, a limitation on the number of flies available meant that only three collection days/replicates of virgins were collected. Secondly, after the mating observations, the mated males were transferred to separate rearing cages with provision of water, sugar and yeast hydrolysate as described above and kept under the standard laboratory conditions described in Section 2.2. About 13 hours after the mated males had mated, dissections were begun on these flies. The dissections were performed within 3.5 hrs. Reproductive tissues as well as carcass were dissected, pooled and frozen as described in Experiment 1. The flies were 9 days old at the time of dissection.

2.5 Sample preparation and analysis

2.5.1 Protein extraction: Three hundred microliter of ice cold 70% methanol (Sigma-Aldrich) and three 3mm stainless steel beads (Qiagen) were added to each tube of 30 glands from experiments 1 and 2 above and the glands homogenised using a TissueLyser II (Qiagen) for two minutes at 20 oscillations per second. Then 200 μl of extract was transferred into a separate tube and 140 μl chloroform (Sigma-Aldrich) was added and the mixture homogenised briefly for 2 minutes at 20 oscillations per second. Afterwards, 46 μl of 0.1M KCl (Sigma-Aldrich) was added and the mixture homogenised as above. Samples were then centrifuged at 16,110 g for five minutes, yielding three phases; an upper aqueous phase mainly containing metabolites and nucleic acids, an interface layer mainly containing proteins, and a lower phase containing mainly lipids. However to enhance the protein yield, it was ultimately necessary to retain all three phases. The samples were air dried in a 100 mBar vacuum for 2.5 hours.

2.5.2 Protein quantification: After the samples have been dried, 20 μl of 8 M urea (Sigma-Aldrich) was added to denature the proteins. The mixture was then sonicated (Model no: FXP4, Unisonics) for 10 minutes and left at -4 ℃ overnight to dissolve the denatured proteins. Protein concentrations were determined using a Quick Start Bradford protein assay kit (Bio-Rad) according to the manufacturer's instructions, with bovine serum albumin (BSA, BioRad; concentration range 125–2000 μg.mL^{-1}) as standard. Absorbance was measured at 595 nm using a spectrophotometer (SpectraMax Plus 384, Bio-strategy).

2.5.3 Protein digestion: Eight molar urea (Sigma-Aldrich) was added to each sample to bring the protein concentration to about 5 $\mu g/\mu l$. Two replicates from bioassay 1 were discarded because of lower protein concentrations. Then 1.5 μl of 15% dithiothreitol (DTT, Sigma-Aldrich) was added and the mixture incubated at room temperature for 30 minutes to break the disulfide bonds in the proteins. Following this 1 μl of 40% acrylamide (Sigma-Aldrich) solution was added and the mixture allowed to incubate at room temperature for 30 minutes to prevent the disulfide bonds from reforming. The denatured proteins were then digested in an overnight incubation in a trypsin solution (60 μl of 25 mM ammonium bicarbonate and 0.1 μg trypsin; Sigma-Aldrich) at 37 ℃. After this 1 μl of 10% formic acid was added to stop the reaction and the mixture filtered through a 0.2 μm syringe-driven filter unit (Millex-LG).

2.5.4 Bottom up MS/MS analysis: A MS/MS analysis was performed using an Orbitrap Fusion mass spectrometer (ThermoFisher Scientific) at the CSIRO Black Mountain site. The resolution of the machine was 120,000, with a scan range of m/z 400-1500. The automated gain control (AGC) target was set to $4.0e^5$, the radio frequency (RF) of the lens was 60% and the maximum injection time was 50 ms. The intensity threshold was $1.0e^4$ and the dynamic exclusion duration was set to 15 seconds. Data acquisition was performed at full speed for 120 minutes with a cycle time of 3 seconds. Ions with charge states from 2+ to 7+ were successively fragmented by Higher energy Collisional Dissociation (HCD) with a collision energy of 32%. For the HCD, the first mass was m/z 120, the AGC target was $4.0e^3$ and the maximum injection time was 300 ms. Other parameters were as per default settings.

2.5.5. Reference protein/peptide databases: A draft genome assembly of *B. tryoni* (Gilchrist et al., 2014) is available but the protein annotation is not yet complete. So, we constructed four databases, collected from NCBI and Uniprot protein resources. The first two databases comprised the inferred proteomes from the genomes of the phylogenically closely related *B. dorsalis* and *D. melanogaster* (~20,000 proteins and ~31,000 proteins respectively). The major inhibitory proteins which have been identified in *D. melanogaster* (SP, DUP99B) are small peptides (30-60 amino acids), so a third database was also used which includes all the male reproductive tissue- and neuro-peptides known from all insects (~64,000 proteins/peptides). Finally a fourth database of small peptides from all insects with a size range of 10-100 amino acids was constructed (~60,000 peptides). The search terms to query NCBI for the third database were -"Ejaculatory bulb"[All Fields] OR "Testis"[All Fields] OR "accessory gland"[All Fields] OR "vas deferens"[All Fields] OR "ejaculatory duct"[All Fields] OR "ejaculatory bulb"[All Fields] OR "apodeme"[All Fields] OR "odorant"[All Fields] OR "pheromone"[All Fields] AND "Insecta"[ORGN]. For the fourth database, Genbank database was searched for all insect short peptides 10-100 amino acids in length.

2.5.6 Analysis of MS/MS data: The SEQUEST HT search engine embedded in Proteome Discoverer 2.2 (Thermo Fisher Scientific) was used to match MS spectra against each of the four reference databases above, and the peptide spectral matches (PSM) were validated using the Percolator algorithm (strict and relaxed false discovery rate (FDR) set at 0.01 and 0.05 respectively). Peptides of 6-144 amino acids long were retained for analysis and no more than three missed cleavage sites were allowed. Default settings for precursor mass

tolerance (10 ppm) and fragment mass tolerance (0.6 Da) and peptide relevance factor (0.4) were used. The following dynamic modifications were selected: oxidation (amino acids H, M, W; delta mass +15.99492 Da), deamidation (amino acids N, Q; delta mass +0.98402 Da), amidated C-terminus (delta mass -0.98402 Da). Propionamide-cysteine (delta mass +71.03711 Da) was set as static modification. Samples from both bioassays were run simultaneously and normalised using "total peptide amount" parameters in the "precursor ion quantifier" module of Proteome Discoverer 2.2 software. Replicates of each tissue type and treatment were used to estimate average abundances (measured as summed signal intensities) and variability (e.g. all the replicates of accessory glands of virgin 8 day old flies were grouped into VA8) using the Label-Free Quantification method in Proteome Discoverer 2.2. Three tissue-specific abundance ratios (VT8/VC8, VA8/VC8 and VE8/VC8) and four depletion and subsequent replenishment abundance ratios (MA8/VA8, MA9/MA8, ME8/VE8, ME9/ME8) were then generated, where V and M denote Mating status (virgin and mated), A,C,E,T denote tissue type (accessory glands, carcass, ejaculatory apodeme and testes) and 8,9 denote age of the fly (Day8 and Day9). An ANOVA was then carried out in Proteome Discoverer 2.2 to compare average grouped abundances between treatment classes and generate associated adjusted P-values.

2.5.7. Data Filtering and analysis: The output files from the Proteome Discoverer 2.2 analyses above were analysed further in Microsoft Excel. Only proteins of high detection confidence (Exp. q-value: Combined < 0.01; minimum one unique peptide) were retained for downstream analyses. SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/; Nielsen, 2017; Nordahl Petersen et al., 2011) was used to predict the secretory proteins that might be transferred to females in the seminal fluid. *D. melanogaster* homologues were identified by blastp search against the *D. melanogaster* reference protein database on NCBI. Gene Ontology (GO) (Ashburner et al., 2000; Carbon et al., 2017) terms were obtained primarily from Flybase but in some cases also from Uniprot.

3. Results

This project aimed to identify seminal fluid proteins (Sfps) that contribute to remating inhibition in *B. tryoni*. Literature in *D. melanogaster* (particularly around the mode of action of the Sex Peptide) and tephritid fruit flies (particularly in Qfly) has created the expectations that the inhibitory molecule(s) would be expressed in the male reproductive tissues (accessory glands for SP and ejaculatory duct for DUP99B), reduced in abundance in those tissues immediately after mating, and possibly replenished 16.5 hours postmating. The experiments in the current study therefore aligned with these expectations.

3.1 Detection of proteins in tissues of males of different mating histories

The proteomic analyses covered four male tissue types, including three male reproductive organs and the carcass, with three mating histories. As noted above, four protein/peptide reference databases (DBs) were used to detect tryptic fragments from *B. tryoni* samples. Matching the MS/MS profiles to these four databases yielded thousands of high confidence hits (FDR q ≤ 0.01; minimum 1 unique peptide match). Table 1 provides an overview of the numbers of protein/peptide matches. For a given database, the number of hits appeared stable across tissue types and experiments. Amongst the 63,037 proteins detected across the dataset, 5,324 (~8.4%) were predicted to possess a signal peptide. Of the two species-specific databases, the *B. dorsalis* reference database (DB1; ~20,000 sequences) produced more hits (mean ± stdev: 2,879 ± 137; N=10; range: 2,668 – 3,054) and higher proportion (mean ± stdev: 10.7 ± 0.7%; N=10; range: 9.5% - 11.7%) of signal peptide positive proteins, whereas the *D. melanogaster* reference database (DB2; ~31,000 sequences) yielded 1617 ± 66 hits and 6.5 ± 0.5% (N=10; range: 5.8% - 7.2%) proteins with a putative signal peptide. The proportions of the signal peptide positive proteins/peptides were atypically low in the ejaculatory apodeme (3.2%) and testes (2.4%) of day 9 mated males, compared to the remaining eight samples for DB4 (insect small peptides 10-100 a.a.), whose proportions ranged from 4.9% to 5.9%. In contrast, DB3 (~64,000 sequences male reproductive tissues and neuropeptides from all insects) produced only 317 ± 36 hits (N=10; range: 280 – 381), but with the highest average proportion of proteins with a signal peptide (16.1 ± 3.3%; N=10; range: 9.3% - 19.7%) amongst the four databases used. Despite the variation in protein detection rates in the four non-*B. tryoni* databases, the results demonstrated that the proteomic workflow was able to produce abundant, high-confidence, potentially secreted protein matches for three

downstream analyses to evaluate (1) tissue specificity expression, (2) protein depletion immediately after mating and (3) protein replenishment 13-16.5 hours after mating.

Table 1: Number of proteins detected in four male tissue types with three mating histories using four reference protein databases.

	Virgin male day 8 (V8)			
Database	**Accessory glands**	**Ejaculatory apodeme**	**Testes**	**Carcass**
DB1	2950 (328 or 11%)	2754 (311 or 11.3%)	2996 (315 or 10.5%)	2678 (313 or 11.7%)
DB2	1719 (119 or 6.9%)	1581 (114 or 7.2%)	1648 (113 or 6.9%)	1544 (110 or 7.1%)
DB3	289 (57 or 19.7%)	291 (57 or 19.6%)	381 (54 or 14.2%)	280 (54 or 19.3%)
DB4	1599 (81 or 5.1%)	1535 (80 or 5.2%)	1544 (75 or 4.9%)	1314 (77 or 5.9%)

	Mated male day 8 (M8)			
Database	**Accessory glands**	**Ejaculatory apodeme**	**Testes**	**Carcass**
DB1	2961 (318 or 10.7%)	2877 (314 or 10.9%)	3054 (302 or 9.9%)	ND
DB2	1682 (106 or 6.3%)	1607 (105 or 6.5%)	1645 (95 or 5.8%)	ND
DB3	304 (52 or 17.1%)	293 (50 or 17.1%)	361 (47 or 13.0%)	ND
DB4	1558 (78 or 5.0%)	1490 (73 or 4.9%)	1516 (76 or 5.0%)	ND

	Mated male day 9 (M9)			
Database	**Accessory glands**	**Ejaculatory apodeme**	**Testes**	**Carcass**
DB1	2868 (297 or 10.4%)	2668 (293 or 11.0%)	2984 (284 or 9.5%)	ND
DB2	1660 (100 or 6.0%)	1500 (96 or 6.4%)	1584 (95 or 6.0%)	ND
DB3	340 (52 or 15.3%)	285 (46 or 16.1%)	343 (32 or 9.3%)	ND
DB4	1537 (76 or 4.9%)	1346 (43 or 3.2%)	1471 (36 or 2.4%)	ND

Database abbreviations: DB1 = *B. dorsalis* reference proteins, DB2 = *D. melanogaster* reference proteins, DB3 = male reproductive tissue proteins and neuropeptides from all insects and DB4 = small peptides (10-100 a.a.) from all insects. Number and percentage of proteins with signal peptide are in brackets. ND = not determined.

3.2 Tissue-specific analysis to map potential sites of production of putative Sfps

Proteomes of individual reproductive tissues were examined to establish the baseline spatial expression patterns of the putative secreted proteins. The carcass (i.e., the rest of the male body less the three reproductive organs) was used as a control to screen for proteins that were exclusively expressed in the male reproductive tract. Results of the tissue-specific analysis are summarised in Table 2 and details of individual proteins that possess a putative signal peptide are presented in Table S1. In both species-specific analyses (i.e., DB1 and DB2) the majority of signal peptide positive proteins originated from all three reproductive organs (i.e., A+E+T), followed by accessory glands and testes (i.e., A+T) and testes only (i.e., T only). In addition to the consistency in rank order of tissue(s) of origin, the general lack of ejaculatory apodeme-specific proteins was also noted.

Apart from the tissue-specific analysis, two experiments were conducted to compare the proteins found in a) virgin males with just-mated males of at 8 days old and b) just-mated males with males 13-16.5 hours after mating. The analyses focused on proteins expressed in the accessory gland and ejaculatory apodeme in DB1 and DB2. A total of 67 proteins with a putative signal peptide (49 in DB1; 18 in DB2) showed significant reduction in abundance in accessory glands and/or ejaculatory apodeme (regardless of protein expression in the testes) immediately after mating (adj. $P \leq 0.05$). Ten of these 67 (7 in DB1; 3 in DB2) also showed significant increase in abundance 13-16.5 hours after mating (adj. $P \leq 0.05$). Accession numbers of these 67 proteins are listed in Table S2.

Table 2: Spatial expression patterns of proteins/peptides in day-8 unmated males.

		DB1	**DB2**	**DB3**	**DB4**
Not detected in carcass	A/E/T	470 (37)	241 (14)	24 (2)	318 (5)
	A+E+T	189 (15)	90 (11)	12 (1)	184 (2)
	A+T only	116 (9)	70 (2)	3 (0)	45 (1)
	A+E only	14 (2)	18 (0)	1 (1)	64 (2)
	E+T only	16 (2)	5 (0)	1 (0)	2 (0)
	A only	21 (3)	18 (0)	0	2 (0)
	E only	0	1 (0)	0	0
	T only	114 (6)	39 (1)	7 (0)	21 (0)
	C only	16 (9)	11 (3)	0	4 (1)

Database abbreviations: DB1 = *B. dorsalis* reference proteins, DB2 = *D. melanogaster* reference proteins, DB3 = male reproductive tissue proteins and neuropeptides from all insects and DB4 = small peptides (10-100 a.a.) from all insects. Tissue type abbreviations: A: Accessory glands, E: Ejaculatory apodeme and T: Testes C: Carcass. A/E/T denotes proteins detected in one or more reproductive tissues, A+E+T denotes proteins detected from all three reproductive organs. Number of proteins with signal peptide are shown in brackets.

3.3 Identification of the soluble fraction of male reproductive tissue proteomes

Identification of proteins that possess a signal peptide provided an entry point to predict their solubility, an essential characteristic of SPFs. However, the level of redundancy at the gene level was unknown at this stage. The uncertainties stemmed from: (1) multiple accession numbers mapped to the same gene in a given species, (2) proteins from different databases derived from a homologue pair, and (3) the same accession number(s) were included twice because they reached statistical significance threshold in both tissue types. These issues prompted the conversion of protein IDs to *D. melanogaster* gene accession numbers ("Fbgn" notation used in Flybase), to facilitate redundancy reduction as well as solubility assessment. The analysis examined three input datasets. Dataset 1 included the 58 proteins with a putative signal sequence (Table 2; DB1: 37; DB2: 14; DB3: 2; DB4: 5); dataset 2 included the 67 signal peptide-positive proteins that showed significant depletion after mating (Table S2; 49 in DB1; 18 in DB2); dataset 3 included the 10 proteins (Table S2; 7 in DB1; 3 in DB2) that showed significant depletion immediately after mating followed by a significant increase in abundance 13-16.5 hours after mating. *D. melanogaster* homologues were identified through BLASTp searches against the Genbank *D. melanogaster* reference protein database at E-value cutoff of 1e-20. Gene accession numbers (Fbgn numbers), their official protein names and their associated gene ontology (GO) terms were retrieved from Flybase and in some cases using GO terms from Uniprot. Protein accessions that shared the same *D. melanogaster* gene were then combined and treated as one gene in terms of GO assessments. The "Cellular Component" of the GO terms was examined to differentiate between putative soluble proteins (defined by the possession of GO terms "extracellular space" or "extracellular region") and the membrane associated ones (defined by possession of GO terms "membrane" or "extracellular matrix"). The re-classification of soluble and membrane-associated proteins are summarised in Table 3. This analysis identified 49 putative soluble proteins (21 from tissue-specific analysis and 28 from V8>M8 analysis), whose *D. melanogaster* homologue information and GO terms lent themselves to redundancy reduction and overlap analysis.

Table 3: Identification of soluble fraction of proteins with signal peptide in male reproductive tissues.

Detected in A or E or T but not C			
***D. melanogaster* homologue +ve at blastp e value < 1e-20**	**Annotated**	**Soluble**	21 (10, 9, 1, 1)
		Membrane-associated	10 (5, 4, 0, 1)
		Others	4 (2, 1, 0, 1)
	Unannotated	**Uncharacterised**	13 (12, 0, 1, 0)
		***D. melanogaster* homologue -ve**	10 (8, 0, 0, 2)

V8>M8 in A or E at P≤0.05			
***D. melanogaster* homologue +ve at blastp e value < 1e-20**	**Annotated**	**Soluble**	28 (14, 14, 0, 0)
		Membrane-associated	16 (12, 4, 0, 0)
		Others	0
	Unannotated	**Uncharacterised**	9 (9, 0, 0, 0)
		***D. melanogaster* homologue -ve**	14 (14, 0, 0, 0)

V8>M8 & M9>M8 in A or E at P≤0.05			
***D. melanogaster* homologue +ve at blastp e value < 1e-20**	**Annotated**	**Soluble**	3 (2, 1, 0, 0)
		Membrane-associated	5 (3, 2, 0, 0)
		Others	2 (2, 0, 0, 0)
	Unannotated	**Uncharacterised**	0
		***D. melanogaster* homologue -ve**	0

Numbers in brackets represent a numerical breakdown of the number of contributing proteins from DB1 (*B. dorsalis*), DB2 (*D. melanogaster*), DB3 (reproductive tissues + neuropeptides) and DB4 (small peptides) respectively. Tissue type abbreviations: A: Accessory glands, E: Ejaculatory apodeme and T: Testes C: Carcass. Mating status abbreviations: V8: day 8 virgin (or unmated) male; M8: day 8 mated male; M9: day 9 (or 13-16.5 hours after mating) mated male.

3.4 Evidence-based categories of annotated candidates

Annotated proteins from Table 3 that passed through the filters requiring secretion and solubility were then classified on the basis of the following three lines of evidence:

1) Their tissue specificity; specifically whether they were found in the accessory glands or apodeme (regardless of testes) but not the carcass,
2) Their depletion immediately-post-mating; specifically whether they were significantly less abundant in the accessory glands or apodeme in mated than virgin Day 8 males, and
3) Their replenishment the day after mating; specifically whether they were significantly more abundant in the accessory glands or apodeme in the mated males harvested at Day 9 than Day 8.

Combining the three lines of empirical evidence listed above and taking into account the nomenclature redundancy yielded a list of 21 Sfps (Table 4). These putative Sfps were classified into diverse protein classes (nomenclature system in accordance to Findlay et al., 2008) and placed into five groups based on the overlaps between three experiments.

Primary candidates were those that satisfied all three of the above criteria (i.e., Group 1) and secondary candidates were those that satisfied one or two of them. The secondary candidates were then divided into four subclasses as follows:

Group 2: those that satisfied criteria 1) and 2) but not 3) above, i.e., they were not found outside the accessory glands or apodeme (regardless of testes) and they were depleted but not replenished after mating,

Group 3: those that satisfied criteria 2) and 3) but not 1) above, i.e., they were depleted and then replenished after mating but were not confined to reproductive tissue,

Group 4: those that satisfied criterion 1) but not 2) and 3) above, i.e., they were not found outside the accessory glands or apodeme (regardless of testes) but they were not depleted nor replenished after mating, and

Group 5: those that satisfied criteria 2) but not 1) and 3) above, i.e., they were depleted but not replenished after mating nor confined to reproductive tissue.

Table 4: Candidate proteins/genes for sexual inhibition in Qfly.

Group	A/E/T and not C	V8 > M8 in A or E at P≤0.05	V8>M8 & M9>M8 in A or E at P≤0.05	Found in	V8>M8 found in	M9>M8 found in	Accession number	Dm homologue	Protein name [Flybase]	Molecular functions	Protein class
1	✓	✓	✓	T,A,E	E	E	Q9VY87	FBgn0030521	Cathepsin B1	Cysteine-type endopeptidase activity	Protease
2	✓	✓		T,A,E	A,E	-	A0A034VK88	FBgn0030925	Hayan	Serine-type endopeptidase activity	Protease
	✓	✓		T,A,E	E	-	A0A034VRJ9	FBgn0035023	Ion transport peptide	Hormone activity; neuropeptide hormone activity	Neuropeptide signaling
	✓	✓		T,A,E	E	-	A0A034VSE0				
	✓	✓		A,E	E	-	A0A034WJT9	FBgn0041629	Hexosaminidase 2	Beta-N-acetylglucosaminidase activity; N-acetyl-beta-D-galactosaminidase activity	Carbohydrate metabolism
3		✓	✓	T,A,E,C	E	E	A0A034VM35	FBgn0004047	Yolk protein 3	Carboxylic ester hydrolase activity	Protein storage
		✓	✓	T,A,E,C	E	E	Q8T7E2				
4	✓			T,A,E	E	-	A0A034VT70	FBgn0035023	Ion transport peptide	Hormone activity; neuropeptide hormone activity	Neuropeptide signaling
	✓			T,A,E	E	-	E1JGW3				
	✓			T,A,E	E	-	Q0E8W5				
	✓			T,A,E	E	-	Q9W151				
	✓			T,A,E	-	-	XP_017462463.1				
	✓			A,E	-	-	ALS40432.1	FBgn0011294	Antennal protein 5		Odorant binding
	✓			T,A,E	-	-	O96690	FBgn0023178	Pigment-dispersing factor	Neuropeptide hormone activity; receptor binding	Neuropeptide signaling
	✓			A,E	-	-	A0A034VSS2	FBgn0028990	Serine protease inhibitor 27A	Enzyme inhibitor activity; serine-type endopeptidase inhibitor activity	Protease inhibitor
	✓			A,E	-	-	A0A034VQW8				
	✓			T,A	-	-	Q9VTZ5	FBgn0036299	Transferrin 2	Iron ion binding	Septate junction assembly
	✓			T,A,E	-	-	A0A075BK85				
	✓			T,A,E	-	-	A0A034VIL8				
	✓			T,A,E	-	-	A0A034WC42	FBgn0028936	Nimrod B5		Defense/ Immunity
	✓			T,A,E	-	-	Q7Z1E6.1	FBgn0005585	Calreticulin	Calcium ion binding; unfolded protein binding	Molecular chaperone
5		✓		T,A,E,C	A	-	A0A034VYQ4	FBgn0011280	Odorant-binding protein 19d	Odorant binding; pheromone binding	Odorant binding
		✓		T,A,E,C	A	-	A0A034VRE0	FBgn0012037	Angiotensin-converting enzyme	Carboxypeptidase activity; metal ion binding; metallopeptidase activity; peptidyl-dipeptidase activity	Protease
		✓		T,A,E,C	A	-	Q10714				
		✓		T,A,E,C	A,E	-	X2J8C3				
		✓		A,C	A	-	A0A034WKF4	FBgn0033873	CG6337	Cysteine-type endopeptidase activity	Protease
		✓		T,A,E,C	A,E	-	A0A034W5T7	FBgn0035781	CG8560	Metallocarboxypeptidase activity; zinc ion binding	Protease
		✓		T,A,E,C	E	-	Q9W227	FBgn0034753	CG2852	Peptidyl-prolyl cis-trans isomerase activity	Protein modification
		✓		T,A,E,C	A	-	A0A034WNT8	FBgn0037756	CG8507	Heparin binding; low-density lipoprotein particle receptor binding; receptor antagonist activity	Protein modification
		✓		A,E,C	A,E	-	A0A034VT37	FBgn0030828	CG5162	Carboxylic ester hydrolase activity; lipase activity	Lipid metabolism
		✓		A,E,C	A,E	-	A0A034WTR1	FBgn0022770	Peritrophin A	Chitin binding	Chitin binding
		✓		T,A,E,C	A	-	Q9W303	FBgn0026415	Imaginal disc growth factor 4	Chitinase activity; chitin binding; imaginal disc growth factor receptor binding	Chitin binding
		✓		T,A,E,C	A	-	X2JEB6				

Tissue type abbreviations: A: Accessory glands, E: Ejaculatory apodeme and T: Testes C: Carcass. Mating status abbreviations: V8: day 8 virgin (or unmated) male; M8: day 8 mated male; M9: day 9 (or 13-16.5 hours after mating) mated male. Additional details of the protein hits are presented in Tables S1 and S2.

Notably, the classification into primary and secondary candidates only reflects the number of the three criteria that are satisfied and does not carry with it any implication that the secondary candidates are necessarily less likely to be seminal fluid components that are passed to the female during mating. This is because of some uncertainties around the interpretation of each of the three criteria, as set out below.

In respect of criterion 1), the uncertainty lies in whether a protein or peptide contributing to the seminal fluid would necessarily be only produced in the accessory glands or apodeme (and possibly testis); at least some molecules contributing to some characteristics of the seminal fluid, for example antimicrobial functions, might well be expressed in other tissues. In respect of criterion 2), one major uncertainty concerns the timing of replenishment, the possibility being that the molecule in question has already been replenished in the 3.5 hours before all the flies were dissected. In this case, too, the requirement that the molecule only be only produced in the accessory glands or apodeme (and possibly testis) has been dropped so expression in other tissues might obscure any depletion in those reproductive tissues. In respect of criterion 3), the major uncertainty again concerns the timing of the dissections, the 13-16.5 hours used being based on the only evidence yet available on the time course of replenishment, namely that the reflation of the accessory glands was nearing completion at that time (Radhakrishnan and Taylor, 2008). No data has been published on how this physical reflation relates to the production of seminal fluid components in this or the males' other reproductive organs. And as with criterion 2), expression in other tissues could obscure the change in the reproductive tissue anyway.

For all these reasons, the three criteria and the classification scheme based on them are used in order not to exclude possible candidates rather than to prioritise the likelihoods around them.

3.5 Candidate proteins

3.5.1 Primary candidates: We found a match to the Cathepsin B1 protein in the soluble fractions of the male accesory gland and/or ejaculatory apodeme (and in all three cases also testis) secretomes which we classify as a prime candidate for involvement in remating inhibition on the basis that it was not found in the carcass proteomes and showed both a significant drop in abundance in the mated day 8 males (depleted males) and a significant

rise in the mated day 9 males (replenished males). These changes in the depleted and replenished males occurred in ejaculatory apodeme only.

Cathepsin B1 is a protease and, as noted in the Introduction, proteases are some of the most abundant components of the seminal fluid in diverse taxa ranging from mammals through to insects (Gillott, 2003; Mueller et al., 2004). Insects in which they have been identified as Sfps include *Heliconius* butterflies, Medfly, the Red flour beetle, Silkworms (Davies and Chapman, 2006; Walters and Harrison, 2010; Xu et al., 2013; Dong et al., 2016) and *D. melanogaster* (Findlay et al., 2008; Takemori and Yamamoto, 2009; Wasbrough et al., 2010). Certain seminal fluid proteases in *D. melanogaster* have been shown to cleave inactive SP and Ovulin prohormones into active forms, thus modulating their post-mating behaviour (Chapman, 2001). Others have been found to contribute to microbial immune response and the breakdown of mating plugs in the females, although little is known of the molecular mechanisms underlying these effects (Lung and Wolfner, 2001; Ram and Wolfner, 2007; Wolfner, 2009).

Another papain-like cysteine protease in the accessory gland secretome of *D. melanogaster*, CG4847 (Walker et al., 2006), which has 27.6% amino acid identity with Cathepsin B1, appears to be a potential orthologue of the mammalian cathepsin L which processes the prohormone proenkephalin (Hook et al., 2004). Tests of CG4847 (Peng et al., 2005) have shown it does not cleave SP at the sites required to activate the prohormone but it remains possible that it activates other male derived prohormones such as Ovulin in females.

A Cathepsin F-like protease similar to Cathepsin B1 and CG4847 has also been identified in the accessory gland proteome of *B. dorsalis* (Wei et al., 2015a) but no functional information on this protein has yet been published.

3.5.2. Secondary candidates

3.5.2.1 Candidates not specific to male reproductive tissue (Group 3 in Table 4): We found two matches to the Yolk protein 3 of *D. melanogaster* (FBgn0004047) in the soluble fraction of all three male reproductive tract secretomes and those in the ejaculatory apodeme showed a significant drop in abundance in the depleted males and a significant rise in the replenished males. This candidate was not classified as primary only because it was also found in the carcass secretome.

Yolk protein 3, otherwise referred to as vitellogenin 3, is a catalytically inactive member of the triacylglycerol lipase family in *D. melanogaster*. Yolk protein 3 shows very high expression in adult heads, fat bodies, carcasses and female reproductive organs but no expression in male reproductive tissues (Robinson et al., 2013; Sian Gramates et al., 2017). Three yolk proteins/vitellogenins have been reported as abundant components of adult female fat bodies and reproductive organs of *D. melanogaster* and *B. dorsalis*, accumulating in the oocytes during sexual maturation where they serve as a nutrient source during embryogenesis (Chen et al., 2012; Liddell and Bownes, 1991; Wei et al., 2017; Zuo and Chen, 2014).

Wei et al. (2017) have also observed some vitellogenin expression in male reproductive tissues of *B. dorsalis*, consistent with similar observations by others in male *D. melanogaster*, *Spodoptera littoralis* and *B. dorsalis* (Bebas et al., 2008; Majewska et al., 2014; Tian et al., 2017). The expression of one studied in *B. dorsalis* is relatively high in male accessory glands and ejaculatory ducts (Tian et al., 2017) and expression of the one studied in *S. littoralis* is high in the testes and seminal fluid, and is reported to form an external coating around the spermatozoa (Bebas et al., 2008). It is therefore possible that the yolk protein/vitellogenin match I find also has some sperm-associated function.

3.5.2.2. Candidates not restored in replenished males (Group 2 in Table 4): Matches to three different proteins were found in the soluble secretomes of the accessory glands and/or ejacultaory apodemes (but not necessarily testes and not carcasses) which also showed significant drops in abundance in the depleted males, but did not rise in abundance in the replenished males. These were proteins which matched two enzymes, namely the protease Hayan and Hexoaminidase 2, and two isoforms of ion transport (neuro)peptides.

The match for the *D. melanogaster* Hayan protease was found in the testes, accessory gland and ejaculatory apodeme but not carcass secretomes. Hayan belongs to the chymotrypsin subfamily of serine proteases. Similar to the cysteine proteases above, certain serine proteases have already been found among Sfps. In particular, CG9997, an accessory gland-specific serine protease of *D. melanogaster* has been found to be essential for the binding of SP to sperm and the maintenance of long-term post-mating responses of females (Swanson et al., 2001; Mueller et al., 2004; Ram and Wolfner, 2007; Findlay et al., 2008). Also possibly relevant here, serine proteases have been heavily implicated in immune functions in various

tissues in *D. melanogaster* (Murugasu-Oei et al., 1995; Ross et al., 2003), horseshoe crabs and *Anopheles* mosquitoes (Kawabata et al., 1996; Dimopoulos et al., 1997) and in cell adhesion in a crayfish (Huang et al., 2000).

The Hexosaminidase 2 was only found in the accessory gland secretome. This enzyme is a member of the beta-hexosaminidase protein family and plays a role in carbohydrate metabolism in other organisms (Finn et al., 2017; Jones et al., 2014). In *D. melanogaster,* it showed very high expression in accessory glands of adult males and may play a role in reproduction (Findlay et al., 2008; Robinson et al., 2013).

The matches to two small ion-transport neuropeptides were found in the three male reproductive tissue but not carcass secretomes. These ion transport peptides show depletion in the ejaculatory apodeme secretome only. These peptides are closely related but distinct isoforms produced by the *D. melanogaster* FBgn0035023 gene or its orthologue in the other species. While mainly expressed in the central nervous system (CNS), the three *B. dorsalis* peptides (ITS1, -2 and -3) have also been found at lower levels in male accessory glands and female reproductive tissues (Gui et al., 2017), as have some of the *D. melanogaster* peptides (Sian Gramates et al., 2017). The CNS-expressed peptides in *D. melanogaster* affect several behaviours, including some linked to circadian rhythms which might be of relevance here (Vanden Broek, 2001; Johard et al., 2009; Hermann-Luibl et al., 2014). Otherwise nothing is known of the specific functions of the reproductive tract ITSs.

3.5.2.3. Candidates found in reproductive tissues but not diminished in depleted males (Group 4 in Table 4): We found matches to eight proteins in the soluble secretomes of the accesory gland and/or ejaculatory apodeme (and possibly testes) but not the carcass proteomes which did not drop in abundance in the mated day 8 males. These proteins matched ion transport peptide (*D. melanogaster* homologue = FBgn0035023), two isoforms of the serine protease inhibitor 27A, the putative odorant binding protein (obp) A5, one Pigment Dispersing Factor (Pdf), three isoforms of a metal ion binding protein (Transferrin 2), one calcium ion binding protein (Calreticulin) and a protein implicated in defence or immunity named Nimrod B5 in *D. melanogaster* (Table 4).

Among the five isoforms of Ion transport peptide, three of them were found from *D. melanogaster* and one found from *B. dorsalis* peptides and the snowberry fruit fly *Rhagoletis*

zephyria each. All of them show homology with the *D. melanogaster* FBgn0035023 gene, which has been discussed in the earlier section.

Serine protease inhibitor 27A is a member of the functionally diverse but structurally conserved Serpin (SERine Proteinase INhibitors) family. Both serpin isoforms occurred in the accessory gland and ejaculatory apodeme secretomes. Even though most of the serpin family members are serine protease inhibitors, some also inhibit cysteine proteases such as caspases and cathepsins (Finn et al., 2017; Jones et al., 2014). Serpins occur commonly among the Sfps of several insect species (Dottorini et al., 2007; Findlay et al., 2008; Sirot et al., 2008; Walker et al., 2006; Wolfner et al., 1997), as do other families of protease inhibitors (Gillott, 2003; Mueller et al., 2004). However little is known of their functions in seminal fluid. Serpins have been implicated in a wide range of processes in other tissues, with hormone transport, immune responses, and signalling (De Gregorio et al., 2002; Hashimoto et al., 2003; Kong et al., 2010; Ligoxygakis et al., 2003) being possibly most relevant here.

The match with obp A5 was found in our accessory gland and ejaculatory apodeme, but not testes and carcass, secretomes. Very little is known about the function of obp A5-2 in *D. melanogaster* other than that it has only previously been reported in adult head tissue (Robinson et al., 2013), specifically olfactory hairs (Pikielny et al., 1994). While obps are principally known for binding pheromones and other bioactive odorants and transporting them to olfactory receptors in sensory tissues (Pelosi et al., 2006; Pelosi et al., 2014), several recent studies of *D. melanogaster* have shown some also occur, and in a few cases are largely confined to, reproductive tissues, suggesting they may bind and transport other ligands in these tissues (Galindo and Smith, 2001; Kim et al., 1998; McKenna et al., 1994; Pikielny et al., 1994; Takemori and Yamamoto, 2009). Specifically it has been suggested that some act as carriers for hormones which are transferred from male to female during copulation and modulate post-mating behaviour and oviposition in females (Arya et al., 2010). One obp, namely obp 10, which is expressed in both antennae and male reproductive organs of the moths *Helicoverpa armigera* and *H. assulta*, was found to be transferred to females during mating and subsequently associated with egg surfaces, suggested possible roles in oviposition behaviours (Sun et al., 2012). Five obps have been identified from male accessory gland and ejaculatory apodeme proteome of *B. dorsalis*, but their functions are yet to be explored (Wei et al., 2015a).

One match to the neuropeptide Pigment-dispersing factor (pdf), one isoform from each of *B. dorsalis* and *D. melanogaster*, were found in all three male reproductive tract secretomes. This neuropeptide acts via a G-protein coupled receptor to trigger intracellular signalling and has been found in several insects, including *B. dorsalis* (Gui et al., 2017). The *D. melanogaster* pdf is only highly expressed in brain tissues and has been linked to circadian rhythms and the production of male sex pheromones (Broeck, 2001; Johard et al., 2009; Krupp et al., 2013).

Matches to three isoforms, of an iron-ion binding protein from the transferrin family, two from *B. dorsalis* and one from *D. melanogaster*, were also found in the sectretomes of all three male reproductive organs. Transferrins have been associated with immunity, development and a variety of other biological functions in both invertebrates and vertebrates (Gomme et al., 2005 for a review). The *D. melanogaster* protein is highly expressed in ovaries and moderately-highly expressed in testes and accessory glands (Robinson et al., 2013). Yoshiga et al. (1997) reported up-regulation of transferrin proteins after bacterial infection in both *A. aegypti* and *A. albopictus* and their up-regulation has also been reported in ovaries of *A. aegypti* and *A. gambiae* 24 hours after mating (Alfonso-Parra et al., 2016; Gabrieli et al., 2014). Paralogous transferrins differ significantly in their expression patterns, suggesting different roles in *A. aegypti*; high expression of transferrin 2 after 72 hours of blood feeding suggests its involvement in egg development while upregulation of transferrin 1 upon bacterial infection suggests a role in immune response (Zhou et al., 2009).

A protein homologous to *D. melanogaster* Nimrod B5 was detected in the tissue-specific analysis against the *B. dorsalis* reference proteins (DB1). Based on the *D. melanogaster* annotation in Flybase, Nimrod B5 is involved in defense response and immunity. Nimrod B5 is present in the accessory gland and ejaculatory apodeme secretomes but was not identified in the carcass secretomes. This protein is a member of the Nimrod receptor family which has been implicated in microbial immunity via direct binding to microorganisms prior to their targetting for phagocytosis (Kurucz et al., 2007). Nimrod B5 in *D. melanogaster* is mainly expressed in adult head tissues, carcass, fat bodies and heart (Robinson et al., 2013) but it also shows upregulated expression in female spermatheca after mating (Robinson et al., 2013; Sian Gramates et al., 2017).

3.5.2.4. Candidates showing lower abundances after mating but not specific to male reproductive tissue and not restored in replenished males: Twelve matches were found among peptides/proteins which were found in the soluble secretomes of accessory glands and/or ejaculatory apodemes (and mostly also testes) as well as carcass that were also diminished in abundance after mating but not restored in the replenished males. Seven of the matches were to *B. dorsalis* proteins and five to *D. melanogaster* proteins but all the *B. dorsalis* matches also had *D. melanogaster* orthologues. Some of the proteins were isoforms, so only nine independent *D. melanogaster* proteins were implicated in the matches. Three of these were proteases, two had other functions in post-translational protein modification, one was an obp, one a lipase, and two were involved in chitin binding (Table 4).

One of the proteases was a metalloprotease homologous to mammalian Angiotensin-converting enzyme (Ance). Two isoforms of Ance (A0A034VRE0 and Q10714) showed depletion in accessory gland only, whereas the remaining one (X2J8C3) showed depletion in both accessory glands and ejaculatory apodeme. Orthologues of this protein have previously been reported in the male reproductive glands of diverse insects (Isaac et al., 1999; Leung and Sernia, 2003; Rylett et al., 2007; Sirot et al., 2008; Xu et al., 2013). In *D. melanogaster* at least, the accessory gland-derived enzyme has been shown to play a role in sperm individualization (Hurst et al., 2003) but its activity in accessory glands is significantly reduced after mating, suggesting some of it is also transferred in the seminal fluid to females, where it is assumed to have a different function (Rylett et al., 2007). Mammalian Ance is known to exist both as an integral membrane protein and a soluble secreted enzyme (Coates, 2003) and these properties of the insect enzyme suggest it too exists in both forms. Consistent with this, RNAi knockdown of Ance gene expression in male *Tribolium castaneum* causes abnormalities in their sperm, and reduces oviposition in their mates, which has been interpreted as indicating roles in sperm quality maintenance in both males and mated females (Xu et al., 2013). Similar effects on female fecundity have also been described in the mosquito *Anopheles stephensi* and the Cotton leafworm *Spodoptera littoralis* (Isaac et al., 1999; Vercruysse et al., 2005). Precedent work in mammals has led some to suggest that the Ance homologue in insect seminal fluid may also be involved in processing peptide prohormones (Fuchs et al., 2005; Isaac et al., 1999) but there is no direct evidence on this point as yet.

The other two proteases in this category of candidate were a cysteine protease (*D. melanogaster* orthologue FBgn0033873) and a metallo-carboxypeptidase (*D. melanogaster* orthologue FBgn0035781). The cysteine protease (FBgn0033873) occurred in the accessory gland and carcass, showing depletion in the accessory gland secretome, whereas the other one (FBgn0033873) was identified from all the reproductive glands as well as in carcass, and showed depletion in the accessory gland and ejaculatory apodeme only. Little is known about the function of either *D. melanogaster* orthologue but Flybase (Robinson et al., 2013; Sian Gramates et al., 2017) reports FBgn0033873 to be highly expressed in adult hindgut and FBgn0035781 to be highly expressed in adult digestive tract and testis, so their appearance in my accessory and ejaculatory secretomes represents a novel finding. The potential roles of seminal fluid cysteine proteases has already been discussed in the section above. Metallo-carboxypeptidases are generally involved in post-translational protein/peptide modifications (Finn et al., 2017; Jones et al., 2014), so it is possible that the FBgn0035781 orthologue recovered here has a role in activation of another protein, possibly a peptide hormone, either in the seminal fluid or endogenous in the reproductive tracts of the mated females.

Two other proteins with differing roles in post-translational protein modification were also identified in this category of candidates. One matched a *D. melanogaster* protein (FBgn0034753) annotated as a peptidyl-prolyl cis-trans isomerase, showing depletion in ejaculatory apodeme only. Proteins in this family, also known as cyclophilins and cyclophilin-like proteins, are found in all organisms, are usually located intracellularly and play roles in protein folding and the assembly of large protein complexes (Finn et al., 2017; Jones et al., 2014). FBgn0034753 is highly expressed in several male and female reproductive tissues, with highest expression in accessory glands (Robinson et al., 2013; Sian Gramates et al., 2017). Two proteins in this family have previously been identified in the accessory glands of *B. dorsalis,* and a role for them in antimicrobial defence was suggested (Wei et al., 2015a).

The other match to a protein involved in post-translational protein modification in this category of candidates was to an insect homologue of a mammalian heparin binding glycoprotein (FBgn0037756 in *D. melanogaster*). Even though it is found in all reproductive organ and carcass proteomes, but depletion is observed only in accessory glands. FBgn0037756 has previously been shown to be expressed in almost all life stage and tissues,

with highest levels reported in adult ovaries (Robinson et al., 2013). Although not well studied in insects, mammalian proteins in this family have been shown to bind with high affinity to a wide range of glycoproteins and other ligands and therefore to be involved in a wide range of processes (Finn et al., 2017; Jones et al., 2014).

A match to a *B. dorsalis* lipase orthologous to *D. melanogaster* FBgn0030828 was found in the accessory gland, ejaculatory apodeme and carcass secretomes which was also depleted in the accessory glands and ejaculatory apodeme secretomes after mating. Consistent with the behaviour of the *B. tryoni* enzymes in this study, FBgn0030828 is highly expressed in accessory glands of male *D. melanogaster* and transferred to females during mating (Findlay et al., 2008). Its specific function in the mated females is not known but catalytically active lipases have been reported previously in the ejaculates of many insect species (Davies and Chapman, 2006; Findlay et al., 2008; Mueller et al., 2005; Swanson et al., 2001; Takemori and Yamamoto, 2009; Tian et al., 2017; Walker et al., 2006) and it is speculated that they may affect the release of sperm from sperm storage organs or assist in fertilisation by modifying sperm membranes (Swanson et al., 2001).

A match to an odorant binding protein in *B. dorsalis* that was homologous to obp19d in *D. melanogaster* was also found in all reproductive organs and carcass secretomes and to be depleted in the accessory gland secretomes after mating. obp19d has previously only been associated with *D. melanogaster* head tissue (Robinson et al., 2013) but, as explained above, several obps have previously been found in the seminal fluids of various insects, and roles in the binding and transport of various small ligands proposed.

Matches to the chitin binding protein Peritrophin A of *B. dorsalis* and two isoforms of the Chitinase-like protein imaginal disc growth factor 4 (Idgf4) of *D. melanogaster* were found in this category of candidates. In my study, Peritrophin-A occurred in carcass and all reproductive organs except testes, and depletion was observed in both accessory glands and ejaculatory apodeme. Peritrophin-A has previously been found in various digestive tissues but has also been found to be secreted by *D. melanogaster* accessory glands (Findlay et al., 2008; Robinson et al., 2013; Sian Gramates et al., 2017).

Both isoforms of Idgf4 were identified from all reproductive organs and carcass secretomes, but depletion was observed only in the accessory glands. Idgf4 of *D. melanogaster* has been found in diverse tissues and been associated with processes ranging across wound healing,

ecdysis and imaginal disk development (Finn et al., 2017; Jones et al., 2014; Sian Gramates et al., 2017). Apart from its role in insects' cuticles and the peritrophic membranes of their guts, chitin is also a major component of the cell walls of fungi, so there may also be an antimicrobial function for these two components of the seminal fluid.

4. Discussion

4.1 Overview of results: I have found a total of 63 candidate seminal fluid proteins in the proteomes of the Qfly males analysed. Annotations (protein solubility and molecular functions) based on *D. melanogaster* homologues could be assigned to 21 of them, leaving 42 functionally anonymous or incomplete at this point (Table 5). As explained in the Section 4.2 below, there is good reason to believe that the 63 recovered significantly underestimate the actual number of seminal fluid proteins in Qfly.

Nevertheless it is instructive to compare the functional breakdown of the candidates recovered here with those of the previous studies of seminal fluid proteomes of *D. melanogaster* described in the Introduction. As explained in that section, most of these are inferred proteomes from transcriptomic analyses and they generally include significant numbers of proteins which may not be secreted and soluble. The one exception to this is the proteomic study of Findlay et al. (2008), who used an isotopically labelling technique to uncover transferred Sfps in the mated females. Table 5 compares my 63 candidate proteome of presumptively secreted, soluble proteins with that of Findlay et al. (2008), plus a summation of the results of that study and other prior *D. melanogaster* studies compiled by Findlay et al. (2008). The comparison is based on 13 functional groupings of the annotations made in each of the studies.

Table: 5. Comparison of the protein classes of putative Sfps detected in *D. melanogaster* and the current study.

Protein class	**Total number of Sfps detected in *D. melanogaster* by mass spectrometry (number of new Sfps detected by Findlay et al., 2008)**	**Number of putative Sfps detected in *B. tryoni***
Unknown function	47(23)	42*
Protease	15(5)	5
Protease inhibitor	14(6)	1
Defense/immunity	12(4)	1
Lipid metabolism	9(2)	1
Carbohydrate interactions	7(2)	1
Odorant binding	7(7)	2
Other functions	7(5)	3
Post-mating behaviour	5(0)	0
Sperm protein	5(0)	0
Chitin binding	4(4)	2
DNA interactions	3(3)	0
Protein modification	3(2)	2
Neuropeptide signalling	NA	3
TOTAL	138(63)	63

* In the current study, the "Unknown function" group contains proteins that 1) lacked detectable *D. melanogaster* homologues, 2) whose *D. melanogaster* homologues were insufficiently unannotated, 3) were annotated as membrane-associated and 4) had cellular locations other than extracellular space/region or membrane.

Findlay et al. (2008) identified 63 candidates in their own study, 40 of which they were able to annotate, and their compilation of those data with the other studies yielded a total of 138 candidates, 91 of which had annotations. My study thus has a higher proportion of candidates with poorly defined cellular locations/molecular functions (67%) than the others (37% in Findlay et al., 2008 alone and 34% in the compiled data). This is likely because of my reliance on homology with *D. melanogaster* for my annotations, given the current unavailability of a high quality annotated genome sequence for *B. tryoni*.

Notwithstanding the small sample size, the profile of my 21 annotated candidates across the 13 functional groupings is broadly similar to that in the proteome of Findlay et al. (2008), with both somewhat distinct from the cross-study compiled list generated by Findlay et al. (2008). Thus, their radiolabelled proteome included members of 11 functional groups and mine covered ten of these, the one exception being three proteins they classified as being involved in DNA interactions. One might expect such a function to be largely intracellular and may be related to leakage of material from damaged sperm. Findlay et al. (2008) identified two other functional groups among the other studies, namely post-mating behaviour and sperm proteins which did not appear in their own proteome and I do not find in mine either. The inclusion of these proteins in the compilation of other proteomes may reflect the more relaxed inclusion criteria for the candidates in some of those studies, as detailed in the Introduction and Section 4.2 below.

Notably, neither the radiolabelled proteome of Findlay et al. (2008) nor any of the other transcriptome-derived ones they compiled for *D. melanogaster* contained the Sex Peptide or the paralogous DUP99B. Indeed no short peptide hormones at all were recovered in these studies. The Sex Peptide was originally isolated by classical 'reverse-genetics', ie via the purification and characterisation of the peptide responsible for the phenotype in question, in this case remating inhibition (Chen et al., 1988), while DUP99B was originally detected by sequence homology (Chen et al., 1988; Saudan et al., 2002). Their absence from the proteome of Findlay et al. (2008) might reflect the difficulties of finding such small (36-37 residue) peptides in trypsin-processed extracts. This would not be a problem for the proteomes inferred from transcriptome data, but other problems such as transcript rarity could come into play there.

While I did not recover either the Sex Peptide or DUP99B, I was able to find three short, presumptive neuropeptide hormones. None of the other studies recovered such molecules. The peptides I found were somewhat longer (74-126 residues) than SP or DUP99B. My failure to recover SP or DUP99B is consistent with the absence of homologous sequences alignable to them in the *B. dorsalis* genome. Nevertheless, the successful detection of small neuropeptides in the current study suggests my workflow may at least be approaching the power and sensitivity required to find peptide hormones with similar characteristics if they exist in *B. tryoni* seminal fluid. The next section outlines some modifications to my workflow which might further enhance the power and sensitivity of my approach.

I do not suggest that any of the presumptive neuropeptide hormones I have recovered have a role in remating inhibition. While I cannot dismiss the possibility at this point either, I note that many other behavioural changes in females are triggered by mating (Avila et al., 2011; Chapman, 2001; Wolfner, 1997), so the seminal fluid could contain a variety of peptide hormones. However it is also relevant that I, like the precedent *D. melanogaster* studies, found a variety of protease and other post-translational protein modifying enzymes, and both Findlay et al. (2008) and I found ligand-binding proteins (in the form of proteins in the obp family). These findings suggest that *B. tryoni* seminal fluid contains the types of protein required to process prohormones like SP into their active forms and transport them to their cognate receptors. These findings are therefore at least consistent with the existence in the seminal fluid of a peptide hormone that is post-translationally activated and transported *in situ* to trigger remating inhibition in the female.

4.2 Plans for future work

4.2.1 Limitations of this study and plans to enhance the candidate list: While I have found several promising candidate proteins and peptides that might contribute to sexual inhibition or other important functions of *B. tryoni* seminal fluid, it is also likely that I have missed a significant number of candidates. However there are several reasons to think that some relatively straightforward modifications to my protocols could substantially improve the candidate list.

One readily addressed issue concerns the reference databases used. In the absence of a high quality genome assembly and annotation for *B. tryoni*, I was forced to utilise *B. dorsalis* and *D. melanogaster* databases. Seminal fluid proteins have been shown to evolve unusually

rapidly in a variety of organisms, including insects (Davies and Chapman, 2006; Findlay et al., 2008; Mueller et al., 2005; Thomas and Singh, 1992). Further, peptides as short as SP are particularly difficult to align across species. It is therefore likely that the necessity of using the *B. dorsalis* and *D. melanogaster* databases meant that several protein and particularly short peptide candidates have been undetectable in the analysis presented here.

Fortunately, a much improved *B. tryoni* assembly and annotation is nearing completion. The CSIRO group has now generated over 100 fold coverage of Illumina data, significant long read Nanopore sequence, and over 50 stage- and tissue-specific transcriptomes. The new assembly and annotation including the additional data should be available shortly and the proteome predicted from those data can then be used as the primary reference database in a reanalysis of my data.

Additionally, I collected material for parallel transcriptomic analyses (using an extraction protocol optimised for small mRNA of less than 200 bases) of the various collections of males subjected to the proteomic analyses described in this thesis. I did not have time to process this material before preparing this thesis but will do so in the near future. As noted in my Introduction, there are some limitations in the use of transcriptomics for identifying peptides that contain post-translationally modified amino acids. However the method also has some compensating advantages, particularly for the recovery of peptides that are proteolytically cleaved into peptides that may lack sites for the trypsin-mediated cleavage carried out as part of the proteomic workflow. It might also recover matches for peptides or proteins which were for example too rare or too unstable to be evident in the proteomes. The combination of proteomic and transcriptomic data sets could provide a uniquely powerful approach to elucidating the protein contents of the seminal fluid.

As noted, there was also a technical issue (insufficient number of background elements) with my use of the peptide databases (i.e., databases 3 and 4 in the Results) which prevented me testing statistically whether the abundances of matches to these databases declined significantly in the just-mated depleted Day 8 males and then increased significantly in the subsequently 'replenished' Day 9 males. I did not have time to address this problem ahead of the thesis and therefore could not follow up matches to these databases seen in the tissue analyses in the analyses of the depleted and replenished males. At the least it should be

possible to apply a fold-change criterion in these latter analyses, even if a formal criterion of statistical significance cannot be deployed.

One issue which would require additional empirical work to address concerns the timeframes for the full restoration of the seminal fluid contents after mating. Timeframes of 1-3 and 13-16.5 hours post-mating respectively were selected for the collection of the 'depleted' and 'replenished' males. These timeframes were inferred from the results of Radhakrishnan and Taylor (2008), who showed that accessory gland size diminished immediately after mating but began to increase again from 5.5 hours post-mating, returning to pre-mating dimensions at 22 hours post-mating. However accessory gland size may not be a reliable indicator of the production of the proteins required to effect sexual inhibition in the males mates. Experiments are needed which directly measure the times it takes males to become competent, both in remating and in conferring sexual inhibition on their mates. Once these times are known it may be necessary to repeat the experiments I have carried out using more realistic collection times for the 'depleted' and 'replenished' males.

One issue which may be more problematic to overcome concerns the basis for my inference about the secreted status of the various matches. These inferences were based on agreement with consensus eukaroyte secretion signals. While the consensus sequences have proven to be excellent indicators there will still be occasional misclassifications, and possibilities like vesicle-mediated secretion will not be identified. Alternatively a different detection algorithm (e.g. DeepSig by Savojardo et al. 2017) could be used to identify signal peptides to minimise misclassification. I did try some empirical work to collect seminal fluid directly, but found it impractical to recover sufficient material for processing. However it may be worthwhile to pursue that possibility further in the future.

4.2.2 Future plans to validate and characterise key candidates: Implementation of the modified protocols outlined above should lead to a significantly larger list of candidate proteins and peptides than the 21 identified to this point. It is also important to note that another 42 matches which otherwise met one or more of our candidate criteria were not discussed further herein because they were difficult to annotate (either lacked a *D. melanogaster* homologue or their *D. melanogaster* homologue was also unannotated. Given the rapid sequence and functional divergence evident among seminal fluid proteins in other organisms, these unannotated proteins could nevertheless have important roles in sexual

inhibition. There will thus be more candidates for roles in sexual inhibition than it would be realistic to test empirically for function in the near term.

One additional filter that could be applied, albeit still requiring additional experimentation, would be to test which of the candidates can actually be recovered at higher titres in the proteomes of mated as opposed to virgin females. This might eliminate some classes of false positives among the candidate list. As noted earlier, I did in fact collect females for this purpose and aim to process their proteomes in the near future.

Beyond that, it may be necessary to work through a series of transient and stable genetic manipulations of the candidates and test them for mating inhibition abilities. Candidates meeting the most criteria, whether annotated or not, could be prioritised first, and the technically easier transient RNAi-based manipulations could be undertaken first. Effective RNAi-based 'knock-downs' of function have proven effective for other phenotypes mediated by testes-expressed genes in both *B. dorsalis* (Dong et al., 2016) and *B. tryoni* (S Whyard, University of Manitoba, pers. comm.), suggesting that phenotypes mediated by accessory gland- or ejaculatory apodeme-expressed genes should also be amenable to the technique. Candidates that show functional-knock down in these transient assays could then be validated in stable knock-downs generated by the recently developed CRISPR-Cas9 genome editing technology (Choo et al., 2018). However this latter technology is still practically problematic and candidates validated by RNAi-based knock-down experiments could be confidently considered important to sexual inhibition anyway.

Once key candidates have been substantively validated, strategic science questions concerning modes of actions and elucidating functional partnerships between eg peptides, binding proteins and receptors could be tackled with injections of synthetic candidate peptides, immuno- and radioactively-labelled reagents etc. From an applied perspective it would also be possible to test how various mass-rearing conditions affect the expression of the key genes and to screen natural Qfly populations for strains overexpressing those genes which might be more effective in remating inhibition in SIT control programs. Notwithstanding possible regulatory complications, it might ultimately be possible to use CRISPR-Cas9 'knock-in' technology to enhance the phenotype further in SIT strains. Finally, it would also be worthwhile to check the phylogenetic distribution of the key genes to see to what extent similar manipulations might be possible in other pest species of tephritids.

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Supplementary Table S1 part 1: Description of proteins with a signal peptide detected in reproductive tissue(s) and absent in carcass in unmated 8-day old males. Reference protein databases are denoted as DB1 (*B. dorsalis*), DB2 (*D. melanogaster*), DB3 (reproductive tissues + neuropeptides) and DB4 (small peptides).

Database	Genbank / Uniprot accession	Description
DB1	A0A034V6H9	CUGBP Elav-like family member 1 (Fragment) OS=Bactrocera dorsalis GN=CELF1 PE=4 SV=1
DB1	A0A034V8G8	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034V8H4	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034VAQ2	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034VBV4	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034VDD7	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034VDG3	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034VHY9	Uncharacterized protein (Fragment) OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034VIL8	Melanotransferrin OS=Bactrocera dorsalis GN=TRFM PE=4 SV=1
DB1	A0A034VIV5	Receptor-type tyrosine-protein phosphatase N2 (Fragment) OS=Bactrocera dorsalis GN=PTPR2 PE=4 SV=1
DB1	A0A034VK88	Serine protease persephone OS=Bactrocera dorsalis GN=PSH PE=3 SV=1
DB1	A0A034VM08	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034VQW8	Serpin-Z1C OS=Bactrocera dorsalis GN=SPZ1C PE=3 SV=1
DB1	A0A034VRJ9	Ion transport peptide OS=Bactrocera dorsalis GN=ITP PE=4 SV=1
DB1	A0A034VSE0	Ion transport peptide-like protein OS=Bactrocera dorsalis GN=ITPL PE=4 SV=1
DB1	A0A034VSS2	Serpin-Z1C OS=Bactrocera dorsalis GN=SPZ1C PE=3 SV=1
DB1	A0A034VSZ1	Venom allergen 3 OS=Bactrocera dorsalis GN=VA3 PE=3 SV=1
DB1	A0A034VT34	Angiopoietin-4 OS=Bactrocera dorsalis GN=ANGP4 PE=4 SV=1
DB1	A0A034VT70	Ion transport peptide OS=Bactrocera dorsalis GN=ITP PE=4 SV=1
DB1	A0A034VVW3	Serine protease easter (Fragment) OS=Bactrocera dorsalis GN=EAST PE=3 SV=1
DB1	A0A034VYW9	Contactin OS=Bactrocera dorsalis GN=CONT PE=4 SV=1
DB1	A0A034W119	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034W5F4	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034W6E4	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034W7E9	Fibrinogen C domain-containing protein 1 OS=Bactrocera dorsalis GN=FBCD1 PE=4 SV=1
DB1	A0A034W9B9	Uncharacterized protein (Fragment) OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034WAU7	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034WC42	von Willebrand factor D and EGF domain-containing protein OS=Bactrocera dorsalis GN=VWDE PE=4 SV=1
DB1	A0A034WCN9	Kielin/chordin-like protein (Fragment) OS=Bactrocera dorsalis GN=KCP PE=4 SV=1
DB1	A0A034WF84	Kielin/chordin-like protein (Fragment) OS=Bactrocera dorsalis GN=KCP PE=4 SV=1
DB1	A0A034WJT9	Chitooligosaccharidolytic beta-N-acetylglucosaminidase (Fragment) OS=Bactrocera dorsalis GN=HEXC PE=4 SV=1
DB1	A0A034WLW5	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034WNC7	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034WPR0	Endocuticle structural glycoprotein SgAbd-9 OS=Bactrocera dorsalis GN=CUD9 PE=4 SV=1
DB1	A0A034WQS1	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034WV88	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A075BK85	Transferrin 2 OS=Bactrocera dorsalis PE=2 SV=1
DB2	NP_001036569.2	Ion transport peptide, isoform C [Drosophila melanogaster]
DB2	NP_001163293.1	Ion transport peptide, isoform E [Drosophila melanogaster]
DB2	NP_001163294.1	Ion transport peptide, isoform F [Drosophila melanogaster]
DB2	NP_001247300.1	tenectin, isoform D [Drosophila melanogaster]
DB2	NP_001259536.2	cathepsin B1, isoform C [Drosophila melanogaster]
DB2	NP_001262163.1	golgi complex-localized glycoprotein 1, isoform B [Drosophila melanogaster]
DB2	NP_477392.1	supercoiling factor, isoform A [Drosophila melanogaster]
DB2	NP_524044.1	transferrin 2 [Drosophila melanogaster]
DB2	NP_524517.1	Pigment-dispersing factor [Drosophila melanogaster]
DB2	NP_524865.2	gelsolin, isoform B [Drosophila melanogaster]
DB2	NP_573131.1	calnexin 14D [Drosophila melanogaster]
DB2	NP_727948.1	CG9911, isoform D [Drosophila melanogaster]
DB2	NP_788563.1	golgi complex-localized glycoprotein 1, isoform A [Drosophila melanogaster]
DB2	NP_996148.2	gelsolin, isoform K [Drosophila melanogaster]
DB3	ALS40432.1	putative odorant-binding protein A5-2 [Zeugodacus tau]
Db3	Q7Z1E6.1	RecName: Full=Calreticulin; Flags: Precursor
DB4	XP_003694734.2	PREDICTED: PHD finger-like domain-containing protein 5A, partial [Apis florea]
DB4	XP_017305340.1	PREDICTED: protein ERGIC-53-like [Diaphorina citri]
DB4	XP_017462463.1	PREDICTED: ion transport peptide [Rhagoletis zephyria]
DB4	XP_018784790.1	PREDICTED: uncharacterized protein LOC108966385 [Bactrocera latifrons]
DB4	XP_018789908.1	PREDICTED: chymotrypsin inhibitor-like [Bactrocera latifrons]

Supplementary Table S1 part 2: Characteristics of proteins (Proteome Discoverer output) with a signal peptide detected in reproductive tissue(s) and absent in carcass in unmated 8-day old males.

Genbank / Uniprot accession	Coverage [%]	# Peptides	# PSMs	# Unique Peptides	# AAs	MW [kDa]
A0A034V6H9	11	1	17	1	159	16.6
A0A034V8G8	12	6	64	6	574	63.2
A0A034V8H4	16	5	61	5	382	41.9
A0A034VAQ2	15	5	61	5	402	44.3
A0A034VBV4	16	4	51	4	253	28.3
A0A034VDD7	18	3	21	3	237	28.4
A0A034VDG3	12	6	64	6	594	65.6
A0A034VHY9	7	5	12	5	1608	176.3
A0A034VIL8	9	4	19	4	847	96.4
A0A034VIV5	3	2	2	2	1176	130.9
A0A034VK88	9	2	15	2	375	41.3
A0A034VM08	7	3	11	3	556	62.8
A0A034VQW8	7	2	24	2	436	49
A0A034VRJ9	17	2	21	2	110	13.1
A0A034VSE0	15	2	21	2	126	14.7
A0A034VSS2	7	2	24	2	440	49.4
A0A034VSZ1	5	1	11	1	251	28.1
A0A034VT34	4	1	12	1	454	52.1
A0A034VT70	17	2	21	2	115	13.7
A0A034VVW3	11	4	11	4	401	43.9
A0A034VYW9	5	4	11	4	1393	158.4
A0A034W119	4	1	5	1	416	45.4
A0A034W5F4	13	2	27	2	246	28.4
A0A034W6E4	7	1	11	1	250	29.3
A0A034W7E9	4	1	1	1	406	47.3
A0A034W9B9	4	1	12	1	493	56
A0A034WAU7	33	4	12	3	277	29.9
A0A034WC42	3	1	13	1	349	39.4
A0A034WCN9	3	1	7	1	654	71
A0A034WF84	2	1	7	1	749	81.4
A0A034WJT9	4	1	8	1	451	51.2
A0A034WLW5	11	1	11	1	160	17.3
A0A034WNC7	10	2	2	2	265	29.3
A0A034WPR0	11	1	1	1	147	15.8
A0A034WQS1	12	2	11	2	151	16.3
A0A034WV88	7	1	1	1	201	21.8
A0A075BK85	9	4	19	4	841	95.6
NP_001036569.2	16	2	14	2	119	14
NP_001163293.1	18	2	14	2	108	12.9
NP_001163294.1	16	2	14	2	119	13.9
NP_001247300.1	1	1	21	1	2819	299.4
NP_001259536.2	6	1	6	1	340	37.4
NP_001262163.1	1	1	2	1	1107	125.4
NP_477392.1	5	1	4	1	329	38
NP_524044.1	2	1	4	1	819	92.3
NP_524517.1	13	1	2	1	102	11.5
NP_524865.2	2	1	6	1	798	88.3
NP_573131.1	2	1	1	1	639	72.6
NP_727948.1	2	1	1	1	412	47
NP_788563.1	1	1	2	1	1103	124.9
NP_996148.2	2	1	6	1	786	87.3
ALS40432.1	12	1	17	1	204	23.3
Q7Z1E6.1	2	1	10	1	398	45.8
XP_003694734.2	13	1	1	1	100	11.3
XP_017305340.1	13	1	4	1	84	9.6
XP_017462463.1	26	2	13	2	74	8.8
XP_018784790.1	35	1	2	1	71	8.3
XP_018789908.1	13	1	1	1	85	9.4

Supplementary Table S2 part 1: Description of signal peptide-positive proteins that showed a significant reduction in abundance in just-mated 8–day old males.

Database	Genbank / Uniprot accession	Description
DB1	A0A034V2A7	Renin receptor OS=Bactrocera dorsalis GN=RENR PE=4 SV=1
DB1	A0A034V7Q2	Protein yellow (Fragment) OS=Bactrocera dorsalis GN=YELL PE=4 SV=1
DB1	A0A034V9F1	Protein STRUBBELIG-RECEPTOR FAMILY 7 OS=Bactrocera dorsalis GN=SRF7 PE=4 SV=1
DB1	A0A034VCN2	Carboxypeptidase D OS=Bactrocera dorsalis GN=CBPD PE=4 SV=1
DB1	A0A034VGP4	UDP-glucuronosyltransferase OS=Bactrocera dorsalis GN=UDB31 PE=3 SV=1
DB1	A0A034VK88	Serine protease persephone OS=Bactrocera dorsalis GN=PSH PE=3 SV=1
DB1	A0A034VM35	Vitellogenin-1 OS=Bactrocera dorsalis GN=VIT1 PE=3 SV=1
DB1	A0A034VND1	Thrombospondin-4 OS=Bactrocera dorsalis GN=TSP4 PE=4 SV=1
DB1	A0A034VNS2	Angiotensin-converting enzyme-related protein OS=Bactrocera dorsalis GN=ACER PE=3 SV=1
DB1	A0A034VQT3	Thrombospondin-4 OS=Bactrocera dorsalis GN=TSP4 PE=4 SV=1
DB1	A0A034VRE0	Angiotensin-converting enzyme OS=Bactrocera dorsalis GN=ACE PE=3 SV=1
DB1	A0A034VRJ9	Ion transport peptide OS=Bactrocera dorsalis GN=ITP PE=4 SV=1
DB1	A0A034VRL7	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034VSA5	Cuticular protein 47Eg OS=Bactrocera dorsalis GN=LCP2A PE=4 SV=1
DB1	A0A034VSE0	Ion transport peptide-like protein OS=Bactrocera dorsalis GN=ITPL PE=4 SV=1
DB1	A0A034VT37	Vitellogenin-2 OS=Bactrocera dorsalis GN=VIT2 PE=3 SV=1
DB1	A0A034VT70	Ion transport peptide OS=Bactrocera dorsalis GN=ITP PE=4 SV=1
DB1	A0A034VUA4	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034VV73	UDP-glucuronosyltransferase OS=Bactrocera dorsalis GN=UDB13 PE=3 SV=1
DB1	A0A034VVD9	Uncharacterized protein (Fragment) OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034VXW9	Uncharacterized protein (Fragment) OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034VXX0	Maltase A3 OS=Bactrocera dorsalis GN=MAL3 PE=4 SV=1
DB1	A0A034VYQ4	Odorant binding protein 19d-2 OS=Bactrocera dorsalis GN=PBP2 PE=2 SV=1
DB1	A0A034VYV7	Endocuticle structural glycoprotein SgAbd-2 OS=Bactrocera dorsalis GN=CUD2 PE=4 SV=1
DB1	A0A034W5F4	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034W5T7	Zinc carboxypeptidase A 1 OS=Bactrocera dorsalis GN=CBPA1 PE=3 SV=1
DB1	A0A034W6E4	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034W9X5	Gamma-interferon-inducible lysosomal thiol reductase OS=Bactrocera dorsalis GN=GILT PE=4 SV=1
DB1	A0A034WAR3	Putative isoaspartyl peptidase/L-asparaginase GA20639 OS=Bactrocera dorsalis GN=ASGL1 PE=4 SV=1
DB1	A0A034WBF0	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034WEL3	Kunitz-type proteinase inhibitor kalicludin-3 OS=Bactrocera dorsalis GN=KC3 PE=4 SV=1
DB1	A0A034WFG5	Protease inhibitor OS=Bactrocera dorsalis GN=SBPI PE=4 SV=1
DB1	A0A034WG69	Uncharacterized protein (Fragment) OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034WGA7	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034WGV6	Pupal cuticle protein 20 OS=Bactrocera dorsalis GN=CU20 PE=4 SV=1
DB1	A0A034WHD4	Cuticle protein 1 OS=Bactrocera dorsalis GN=CU01 PE=4 SV=1
DB1	A0A034WJT9	Chitooligosaccharidolytic beta-N-acetylglucosaminidase (Fragment) OS=Bactrocera dorsalis GN=HEXC PE=4 SV=1
DB1	A0A034WKF4	Cathepsin L-like proteinase OS=Bactrocera dorsalis GN=CATLL PE=3 SV=1
DB1	A0A034WLZ0	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034WNT8	Alpha-2-macroglobulin receptor-associated protein OS=Bactrocera dorsalis GN=AMRP PE=4 SV=1
DB1	A0A034WP58	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034WPH2	Larval cuticle protein 5 OS=Bactrocera dorsalis GN=LCP5 PE=4 SV=1
DB1	A0A034WQS1	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034WSW5	Uncharacterized protein (Fragment) OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034WTR1	Peritrophin-1 OS=Bactrocera dorsalis GN=PE1 PE=4 SV=1
DB1	A0A034WUJ0	Uncharacterized protein OS=Bactrocera dorsalis PE=4 SV=1
DB1	A0A034WWI9	Chemosensory protein 2 OS=Bactrocera dorsalis GN=PEB3 PE=2 SV=1
DB2	NP_001285915.1	angiotensin converting enzyme, isoform C [Drosophila melanogaster]
DB2	NP_788655.1	midline fasciclin, isoform J [Drosophila melanogaster]
DB2	NP_788654.1	midline fasciclin, isoform M [Drosophila melanogaster]
DB2	NP_788650.1	midline fasciclin, isoform E [Drosophila melanogaster]
DB2	NP_788643.1	midline fasciclin, isoform H [Drosophila melanogaster]
DB1	Q8T7E2	Vitellogenin 2 OS=Bactrocera dorsalis PE=2 SV=1
DB2	NP_724578.1	boca [Drosophila melanogaster]
DB2	NP_727948.1	CG9911, isoform D [Drosophila melanogaster]
DB2	NP_788646.1	midline fasciclin, isoform L [Drosophila melanogaster]
DB2	NP_788649.1	midline fasciclin, isoform K [Drosophila melanogaster]
DB2	NP_573131.1	calnexin 14D [Drosophila melanogaster]
DB2	NP_001259536.2	cathepsin B1, isoform C [Drosophila melanogaster]
DB2	NP_477392.1	supercoiling factor, isoform A [Drosophila melanogaster]
DB2	NP_611695.1	CG2852, isoform A [Drosophila melanogaster]
DB2	NP_001285068.1	imaginal disc growth factor 4, isoform C [Drosophila melanogaster]
DB2	NP_001285915.1	angiotensin converting enzyme, isoform C [Drosophila melanogaster]
DB2	NP_001285068.1	imaginal disc growth factor 4, isoform C [Drosophila melanogaster]

Supplementary Table S2 part 2: Characteristics (Proteome Discoverer output) of signal peptide positive-proteins that showed a significant reduction in abundance in just-mated 8-day old males.

Database	Genbank / Uniprot accession	Coverage [%]	# Peptides	# Unique Peptides	# AAs	MW [kDa]
DB1	A0A034V2A7	10	2	2	277	30.6
DB1	A0A034V7Q2	22	4	4	261	29.7
DB1	A0A034V9F1	7	3	3	719	78.2
DB1	A0A034VCN2	9	3	3	446	50.6
DB1	A0A034VGP4	3	1	1	530	60.4
DB1	A0A034VK88	9	2	2	375	41.3
DB1	A0A034VM35	18	6	5	438	48
DB1	A0A034VND1	5	1	1	403	46.1
DB1	A0A034VNS2	13	6	6	637	73.8
DB1	A0A034VQT3	5	1	1	400	45.4
DB1	A0A034VRE0	15	7	7	617	71.4
DB1	A0A034VRJ9	17	2	2	110	13.1
DB1	A0A034VRL7	36	2	2	131	13.1
DB1	A0A034VSA5	23	2	2	117	12.6
DB1	A0A034VSE0	15	2	2	126	14.7
DB1	A0A034VT37	9	3	3	420	46
DB1	A0A034VT70	17	2	2	115	13.7
DB1	A0A034VUA4	38	2	2	123	12.4
DB1	A0A034VV73	13	4	4	524	60.6
DB1	A0A034VVD9	8	1	1	254	23.3
DB1	A0A034VXW9	8	1	1	248	22.9
DB1	A0A034VXX0	12	4	4	591	67.2
DB1	A0A034VYQ4	30	4	4	144	15.8
DB1	A0A034VYV7	38	3	3	135	14.6
DB1	A0A034W5F4	13	2	2	246	28.4
DB1	A0A034W5T7	18	5	5	445	50.2
DB1	A0A034W6E4	7	1	1	250	29.3
DB1	A0A034W9X5	32	5	5	212	23.3
DB1	A0A034WAR3	8	1	1	370	39.7
DB1	A0A034WBF0	8	1	1	210	22.7
DB1	A0A034WEL3	24	4	4	138	14.9
DB1	A0A034WFG5	15	2	2	164	17.1
DB1	A0A034WG69	16	1	1	166	17
DB1	A0A034WGA7	37	6	6	147	15.6
DB1	A0A034WGV6	23	3	3	185	21.3
DB1	A0A034WHD4	11	1	1	162	17.1
DB1	A0A034WJT9	11	3	3	451	51.2
DB1	A0A034WKF4	11	2	2	346	38.2
DB1	A0A034WLZ0	38	10	10	246	27.3
DB1	A0A034WNT8	6	2	2	393	46.9
DB1	A0A034WP58	5	1	1	393	44.1
DB1	A0A034WPH2	15	1	1	123	13.6
DB1	A0A034WQS1	33	6	6	151	16.3
DB1	A0A034WSW5	9	2	2	300	34.5
DB1	A0A034WTR1	20	2	2	231	25.6
DB1	A0A034WUJ0	7	1	1	138	15.1
DB1	A0A034WWI9	45	6	6	127	14.7
DB2	NP_001285915.1	2	1	1	615	70.9
DB2	NP_788655.1	3	1	1	905	100.2
DB2	NP_788654.1	3	1	1	840	93.1
DB2	NP_788650.1	3	1	1	839	93
DB2	NP_788643.1	3	1	1	815	90.5
DB1	Q8T7E2	18	6	5	438	48
DB2	NP_724578.1	8	1	1	180	21
DB2	NP_727948.1	2	1	1	412	47
DB2	NP_788646.1	3	1	1	816	90.6
DB2	NP_788649.1	3	1	1	881	97.7
DB2	NP_573131.1	2	1	1	639	72.6
DB2	NP_001259536.2	6	1	1	340	37.4
DB2	NP_477392.1	5	1	1	329	38
DB2	NP_611695.1	7	1	1	205	22.2
DB2	NP_001285068.1	3	1	1	442	48.6
DB2	NP_001285915.1	2	1	1	615	70.9
DB2	NP_001285068.1	3	1	1	442	48.6

Supplementary Table S2 part 3: *D. melanogaster* homologues and official (Flybase) protein names of signal peptide-positive proteins that showed a significant reduction in abundance in just-mated 8-day old males. Proteins that showed a significant increase in abundance 13-16.5 hours post mating are marked by an asterisk (*). Blank cells indicate proteins that lack a *D. melanogaster* homologue at blastp E-value 1e-20 threshold.

Database	Genbank / Uniprot accession	*D. mel* homologue	Protein name [Flybase]
DB1	A0A034V2A7	FBgn0037671	ATPase H+ transporting accessory protein 2
DB1	A0A034V7Q2	FBgn0041713	yellow-c
DB1	A0A034V9F1	FBgn0034718	windpipe
DB1	A0A034VCN2	FBgn0004648	silver
DB1	A0A034VGP4	FBgn0034605*	CG15661
DB1	A0A034VK88	FBgn0030925	Hayan
DB1	A0A034VM35	FBgn0004047*	Yolk protein 3
DB1	A0A034VND1	FBgn0031850	Thrombospondin
DB1	A0A034VNS2	FBgn0016122	Angiotensin-converting enzyme-related
DB1	A0A034VQT3		
DB1	A0A034VRE0	FBgn0012037	Angiotensin converting enzyme
DB1	A0A034VRJ9	FBgn0035023	Ion transport peptide
DB1	A0A034VRL7		
DB1	A0A034VSA5	FBgn0086519*	Cuticular protein 47Eg
DB1	A0A034VSE0	FBgn0035023	Ion transport peptide
DB1	A0A034VT37	FBgn0030828	CG5162
DB1	A0A034VT70		
DB1	A0A034VUA4	FBgn0259223	CG42323
DB1	A0A034VV73	FBgn0032684	CG10178
DB1	A0A034VVD9		
DB1	A0A034VXW9	FBgn0029170	TweedleT
DB1	A0A034VXX0	FBgn0050360	Maltase A6
DB1	A0A034VYQ4	FBgn0011280	Odorant-binding protein 19d
DB1	A0A034VYV7	FBgn0033730*	Cuticular protein 49Ag
DB1	A0A034W5F4		
DB1	A0A034W5T7	FBgn0035781	CG8560
DB1	A0A034W6E4		
DB1	A0A034W9X5	FBgn0037721	CG9427
DB1	A0A034WAR3	FBgn0030653*	CG7860
DB1	A0A034WBF0		
DB1	A0A034WEL3	FBgn0031562	CG3604
DB1	A0A034WFG5	FBgn0031563*	CG10031
DB1	A0A034WG69		
DB1	A0A034WGA7	FBgn0032897	CG9336
DB1	A0A034WGV6	FBgn0033726	Cuticular protein 49Ad
DB1	A0A034WHD4	FBgn0033308	CG8736
DB1	A0A034WJT9	FBgn0041629	Hexosaminidase 2
DB1	A0A034WKF4	FBgn0033873	CG6337
DB1	A0A034WLZ0		
DB1	A0A034WNT8	FBgn0037756	CG8507
DB1	A0A034WP58	FBgn0038405	CG8927
DB1	A0A034WPH2	FBgn0039719	CG15515
DB1	A0A034WQS1	FBgn0032021	CG7781
DB1	A0A034WSW5		
DB1	A0A034WTR1	FBgn0022770	Peritrophin A
DB1	A0A034WUJ0		
DB1	A0A034WWI9	FBgn0011695	Ejaculatory bulb protein III
DB2	NP_001285915.1	FBgn0012037	Angiotensin converting enzyme
DB2	NP_788655.1	FBgn0260745	midline fasciclin
DB2	NP_788654.1	FBgn0260745	midline fasciclin
DB2	NP_788650.1	FBgn0260745	midline fasciclin
DB2	NP_788643.1	FBgn0260745	midline fasciclin
DB1	Q8T7E2	FBgn0004047*	Yolk protein 3
DB2	NP_724578.1	FBgn0004132	boca
DB2	NP_727948.1	FBgn0030734*	CG9911
DB2	NP_788646.1	FBgn0260745	midline fasciclin
DB2	NP_788649.1	FBgn0260745	midline fasciclin
DB2	NP_573131.1	FBgn0264077	Calnexin 14D
DB2	NP_001259536.2	FBgn0030521*	Cathepsin B1
DB2	NP_477392.1	FBgn0025682*	supercoiling factor
DB2	NP_611695.1	FBgn0034753	CG2852
DB2	NP_001285068.1	FBgn0026415	Imaginal disc growth factor 4
DB2	NP_001285915.1	FBgn0012037	Angiotensin converting enzyme
DB2	NP_001285068.1	FBgn0026415	Imaginal disc growth factor 4