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Knockout of Highly Conserved Seminal Fluid Protein Ablates Male Fertility in Drosophila melanogaster

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ABSTRACT

In *Drosophila,* seminal fluid proteins (SFPs) are key components of the male ejaculate and are essential determiners of reproductive fitness. SFPs are required for inducing a range of post-mating physiological responses in females. To date nearly 300 SFPs have been identified, however the vast majority of these remain uncharacterized and their role in reproduction and/or postcopulatory sexual selection is not clear. SFPs are functionally diverse and contain proteins from a variety of biochemical processes, most notably proteases and protease inhibitors that are thought to be critically important in biochemical interactions with female proteins. Another class of proteins that is enriched among SFPs are carbohydrate bonding proteins, presumably involved in glycolytic reactions within the accessory glands and in mated females. However, the molecular mechanisms and fertility roles of these ejaculate glycolysis modulators have yet to be explored. One of these uncharacterized glycolysis SFPs is the highly conserved *beta*-glucoronidase (*CG15117*), which catalyzes the breakdown of complex carbohydrates. CG15117—which I will refer to as BGLUC—is (1) predominately expressed in the male accessory gland—but maintains low level expression in other tissues, (2) is transferred to the female during mating, and (3) is one of the most conserved SFPs in *Drosophila.* To identify BGLUC's role in reproduction, we created a CRISPR/Cas-9 knockout mutant by ablating 5 bp from the coding sequence and introducing a premature stop codon. We found that BGLUC is required for male fertility: females that are mated to knockout males do not produce progeny, fail to store sperm and will readily remate, suggesting that an ensemble of key post-maing processes is disrupted. To further examine the molecular basis of this male sterility we performed label-free quantitative proteomic analysis and bulk RNA sequencing on mutant males as well as females mated to mutant and control males and find systematic abnormal abundance of several

proteins—including proteins of ACP36DE and ACP62F— in the accessory glands as well as ACP36DE and other proteins in the transferred male ejaculate. ACP36DE and ACP62F have major roles in modulating sperm storage in the female and their reduced abundances in mutant male accessory glands likely contributes to sperm storage defects observed in females mated by mutant males. Together, our results show that the often-ignored carbohydrate metabolism proteins that are part of the seminal plasma are essential for fertility in *Drosophila*.

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Introduction

Chapter 1.1Background

Drosophila melanogaster is an excellent model for studying the evolution of sexual reproduction. Female Drosophila, like many other insects, can mate with multiple males (polyandry) and store sperm for extended periods of time, resulting in an overlap of ejaculates from several males in the female reproductive tract. The overlap of ejaculates creates the potential for post-copulatory sexual selection (PCSS) because sperm from different males can compete for fertilization (Parker, 1970). In addition to sperm competition, females can engage in cryptic female choice, where females can bias sperm usage from the stored set from different males following mating, thus selecting for ejaculate traits favored by the female (Eberhard, 1996). This intense selection can result in the rapid diversification and/or optimization of traits that aid in sperm competition and ultimately lead to higher male reproductive fitness. The bulk of PCSS research has focused on sperm traits and sperm competition. However, the non-sperm component of the ejaculate, seminal fluid proteins (SFPs), also play a prominent role in molecular reproductive interactions and fertilization success.

SFPs are products of the male reproductive tract secretory tissues, particularly the accessory glands, but also include components that are derived from the seminal vesicles, ejaculatory duct, ejaculatory bulb and testes, and are transferred to female as part of the ejaculate during mating (Avila et al., 2011, Wigby et al., 2020). SFPs are a highly diverse set of proteins composed of various functional classes. The prominent functional classes among SFPs include proteases, protease inhibitors, redox-related proteins, immunity-related proteins, and lipid metabolism-related proteins (Findlay et al., 2008a; Walker et al., 2006).

SFPs are critical in providing sperm the support required for successful fertilization through complex interactions within the female reproductive tract, so much so that the absence of SFPs results in adverse effects in reproductive success for both sexes (Avila et al., 2011; Hopkins et al., 2017; Sirot & Wolfner, 2015). Critically, SFPs provide the signals for females to regulate the storage and release of sperm and are key drivers of the stimulus for ovulation and egg production following mating (reviewed in Wigby et al., 2020). These processes are at the core of reproductive success, and thus understanding the molecular functions of SFPs and their role in fertilization success is essential for understanding the evolutionary consequences of PCSS postcopulatory sexual selection on reproductive protein evolution. In this chapter I will review some of the known functions of SFPs and our current understanding of their molecular function.

Identifying SFPs in *Drosophila* is a challenging task and has been subject to debate among researchers (Hurtado et al., 2021). Transfer from males to females must be demonstrated, which is experimentally challenging due to SFP modification and degradation in the female shortly following mating. Early identification efforts of SFPs focused on mRNA's predominately expressed in male accessory glands and that possessed a predicted signal secretion sequence (K. B. Chapman & Wolfner, 1988; Monsma & Wolfner, 1988; Ravi Ram & Wolfner, 2007; Swanson et al., 2001; Walker et al., 2006; Wolfner et al., 1997). Following these studies higher throughput proteomic studies were performed. Proteomic analysis was performed on female reproductive tracts following mating to isotopically labeled spermless males (Findlay et al., 2008a, 2009; McCullough et al., 2022). This method identified transferred seminal proteins while excluding sperm proteins and proteins native to the female reproductive tract. In a similar experiment, quantitative proteomics was performed on male accessory glands, and female

reproductive tracts post mating to identify proteins that are depleted in the male and increased in the female (Sepil et al., 2019) which result in the identification of additional SFPs.

The most utilized list of *D. melanogaster* SFPs comes from a combination of past studies on the seminal proteome, with 292 proteins being considered 'high-confidence' based on proteomic and transcriptomic analysis (Wigby et al., 2020). In addition to the high-confidence SFP list, a second 'candidate' list of 321 proteins was generated. The candidate list was established with the criteria that they (1) are likely SFPs based on expression data, but fail to find evidence of transfer to the female, (2) are included in predicted intracellular housekeeping functions, (3) were first defined as SFPs, but are also included in the sperm proteome (Wigby et al., 2020). More recently, a full set of SFPs was identified using a combination of semiquantitative proteomics and sex-specific isotopic labeling to identify sperm proteins and SFPs at the major stages following posttesticular maturation (McCullough et al., 2022). This approach demonstrated significant compositional changes of both sperm proteins and SFPs as they move through the male reproductive tract into the FRT where they interact with female-derived proteins. Together this approach generated the most complete set of SFPs having empirical evidence for classification as a SFP.

Many methods are available to characterize SFPs' functional roles in *D. melanogaster*. *D. melanogaster* has a complete and well-annotated genome with a wealth of genetic and molecular tools available. These tools include collections of mutants with knock-downs, knock-outs, and overexpression for specific genes (Zirin et al., 2020). If the desired mutant collection is not available, there are several methods one can utilize to manipulate gene expression to create a mutant collection (Bassett & Liu, 2014; Gratz et al., 2015; Heigwer et al., 2018; Prelich, 2012). A particular SFP's function can be characterized by mating males with

altered SFP expression to wild type females and observing for deviations in the typical female post-mating behavior and reproductive output. *Drosophila* reproductive processes are observable and well characterized (Hall, 1994), therefore observed deviations in the reproductive prosses suggests that the male's altered SFP expression is responsible for producing the deviation. Another way SFPs have been functionally characterized by being injected with a purified SFP (Cirera & Aguadé, 1997; Yamane et al., 2008). If the female displays a behavior(s) typically seen in mating, then this SFP likely has a role in this behavior.

A SFP's role in competitive paternity can also be determined in the laboratory. Competitive paternity success can be assayed in *Drosophila* by mating a female to multiple males that differ in genotype and/or carry a visible marker (Gilbert & Richmond, 1981; Prout & Bundgaard, 1977). Setting up reciprocal matings allow for the determination of sperm 'defense' (P1) and sperm 'offense' (P2) (Clark et al., 1995). P1 measures the first male's paternity success rate, while P2 measures the second male's paternity success rate. After mating, the number of progeny produced from the singular female is counted, and paternity is assigned by the visible marker(s). Lastly, direct quantification of rival males' sperm storage and utilization in the female reproductive tract can be observed using RFP and/or GFP-labeled sperm (Laturney et al., 2018; Manier et al., 2010). Applying this method to males having either a knockdown or knockout for an SFP of interest allows for the identification of a SFP's role in competitive paternity. Using a wild type male as the rival male, lower P1 and or P2 for the mutant male compared to the wild type male can be attributed to the SFP mutation. Thus, suggesting the SFP of interest has a role in competitive paternity.

SFP levels in the male reproductive tract are not limitless. SFPs are energetically demanding to make, biologically constrained and resource limited (Perry et al., 2013). Males

that mate and remate in quick succession are depleted of SFPs and although they are transferring sperm, the SFP depletion may result in reduced fertility (Findlay et al., 2008). With SFP production being limited in males, males can specifically tailor their SFP abundances present in their ejaculate transferred during mating based on perceived competition from rival males. Quantitative proteomic analysis has shown that SFP production and transfer peaks in the presence of rival males (Hopkins et al., 2019). In addition to elevating production and transfer of SFPs in the presence of rival males, males can regulate abundances of specific SFPs transferred during mating based on if the female has recently mated with a rival male. If a SFP has a lasting effect in females, the second male can transfer less of this SFP and "piggyback" off the reproductive response initiated by the first male, thus saving SFP stores for future matings (Hodgson & Hosken, 2006; Sirot et al., 2011). SFPs that persist in the FRT and produce long lasting postmating responses in females have a reduced benefit to paternity success of males mating to females that have recently mated. Therefore, it is energetically more efficient to transfer less of these SFPs in their ejaculate while allocating more energy to the production and transfer of SFPs that persist for a shorter time period in the FRT.

Chapter 1.2 SFP Function

Drosophila SFPs are extremely diverse in their functional roles. Drosophila mating creates an opportunity for microbes to enter the female reproductive tract. During copulation males can physically damage the genitalia of the female, which can result in transfer of pathogens. SFPs have been implicated in upregulating antimicrobial genes in the female post-mating (McGraw et al., 2004). The SFP Sex Peptide (*SP*) is a primary factor driving transcription of antimicrobial genes such as *metchikowin*; their stimulation may act as a preventative step in preventing the infection pathogens following mating (Peng, Zipperlen, et al., 2005). In addition to invoking the upregulation of immune genes, there is evidence that SFPs have antimicrobial effects themselves. Females ectopically expressing SFPs CG6168*,* CG9334*, or* CG10284 could resist bacterial infection significantly better than their control counterparts (Mueller et al., 2007). These findings suggest that SFPs may have protective roles within the female reproductive tract, and their transfer may, directly or indirectly, aid in the female's ability to clear microbes that could lead to infection.

Drosophila SFPs also function in forming mating plugs in females. In *D. melanogaster,* a mating plug forms shortly after mating begins at the posterior end of the bursa. The mating plug is thought to decrease receptivity to remating by blocking subsequent copulations and maintaining sperm in the bursa. The composition of the mating plug consists of a posterior region containing ejaculatory bulb proteins PEBme, PEB-II, and PEB-III (Bretman et al., 2010; Lung & Wolfner, 2001) and an anterior region containing a collection of SFPs (Lung & Wolfner, 2001). PEB-II is an essential component of the mating plug, for PEB-II knockdown males form smaller mating plugs in the uterus of their mates. Consequently, females mated to these knockdown males are more receptive to remating in the first four hours after mating (Bretman et

al., 2010). The smaller mating plug does not function as well in physically blocking competitor males from inseminating the female. This study supports the idea that the mating plug functions to reduce the occurrence of female remating in the short-term before SFPs that mediate long-term reductions in mating receptivity take effect. In a similar study, knocking down PEBme expression by 25% failed to produce a mating plug and greatly affected female fertility (Avila et al., 2015). Females mated to PEBme knockdown males have coagulation failure at the posterior end of the mating plug, thus resulting in the inability of females to retain the ejaculate after mating. In these matings, as the male and female separated following copulation, the ejaculate was often pulled out of the female reproductive tract (Avila et al., 2015). Together, these studies show that SFPs have an essential role in mating plug formation and function in females, and that the mating plug is required for short-term reductions in female remating.

In Drosophila, SFPs play essential roles in mated females, including sperm viability, initiating and maintaining sperm storage, and mediating sperm release during fertilization. In *D. melanogaster*, there is evidence that SFPs aid in sperm survival in females following mating. Analysis of sperm within the reproductive tract of singularly mated (monogamy) and doubly mated (polyandry) females showed that the receipt of SFPs not only improved the survival of sperm from the same male but rival males too (Holman, 2009). This occurrence contrasts with other insects, such as honeybees and ants (den Boer, Baer, et al., 2009; den Boer, Boomsma, et al., 2009). In these insects, there are differences in the effects of SFPs on sperm viability between monogamous and polyandrous species. In polyandrous species, SFP secretions are detrimental to rival males' sperm, while in monogamous species SFPs aid in the survival of rival males' sperm and their own (den Boer et al., 2010).

Once sperm enters storage, its viability must be maintained for extended periods for fertilization. Based on association studies, it has been hypothesized that Acp29AB in *D. melanogaster* affects sperm competitive ability (Fiumera et al., 2005) and the Acp29AB locus is undergoing positive selection (Žurovcová et al., 2006). In addition, functional analysis was performed on Acp29AB loss-of-function males: males without Acp29AB in their ejaculate were able to induce sperm to enter female sperm storage organs following mating. However, the sperm viability declined in the storage organs over time (Wong et al., 2008). This observed sperm retention defect suggests that Acp29AB plays a role in mediating sperm retention in female sperm storage organs. The retention of sperm in the female sperm storage organs provides a competitive paternity benefit while also maintaining a supply of sperm to be utilized for fertilization.

Some SFPs mediate sperm storage and the subsequent release from storage required for fertilization. As previously mentioned, Acp36DE aids in the conformational changes in the bursa that are required for sperm storage (Avila & Wolfner, 2009). Sperm stored in the female in the absence of several SFPs fails to be utilized in the fertilization of eggs. Knockdown of five SFPs present in the male ejaculate leads to sperm retention in sperm storage organs without fertilization (Ram & Wolfner, 2007). Four of these SFPs (CG9997, CG1652, CG1656, and CG17575) are required for the localization of the SFP SP to sperm tails. These SFPs work together in a functional pathway that transports *SP* through physically binding SP to sperm, thus maintaining its presence in the female reproductive tract for as long as sperm is present (Peng, Chen, et al., 2005). While being bound to sperm tails, the female post-mating response is maintained; however, only after SP being cleaved from sperm tails can sperm be utilized for fertilization (Peng, Chen, et al., 2005). The binding of SP to sperm tails ensures SP's stability in

the female reproductive tract, and may protect it from premature degradation by proteases in the hemolymph (Peng, Chen, et al., 2005). SP also benefits the paternity of the first male in a competitive mating environment through decreasing mating receptivity of the female, maintaining sperm in storage, and only allowing for fertilization as SP it is gradually cleaved from sperm tails (Liu & Kubli, 2003; Misra & Wolfner, 2020; Peng, Zipperlen, et al., 2005)

Chapter 1.3 SFP Female Interactions

The reproductive tract of an unmated female Drosophila is an obstacle that must be overcome for successful sperm storage and fertilization. The reproductive tract of unmated females is tightly closed with flaps of tissue covering the openings to sperm storage organs (Adams & Wolfner, 2007). The ejaculatory components of the male initiate the conformational changes required for sperm storage and fertilization. SFPs, rather than sperm, are the molecules that mediate uterine conformational changes (Adams & Wolfner, 2007). Females mated to males with a full complement of SFPs but lacking sperm experience the uteri conformational changes required for sperm storage (Adams & Wolfner, 2007). Conversely, females mated to males lacking SFPs but having sperm only experience the first few stages of uterine conformational changes and store a reduced amount of sperm (Adams & Wolfner, 2007). Together, these data show that conformational changes in the female reproductive tract mediated by SFPs are essential for proper sperm storage.

The full complement of SFPs required for mediating conformational changes in the female reproductive tract is unknown. However, Acp36DE is a prerequisite for the progression of conformational changes required for sperm storage. *D. melanogaster* males Acp36DE knock-down males cannot initiate the progression of female reproductive tract conformational changes during mating experiments (Avila & Wolfner, 2009, 2017; Chapman et al., 2000). Knockdown of Acp36DE in males results in sperm lagging in the mid-bursa instead of forming dense masses near the sperm storage organ entrances (Adams & Wolfner, 2007). In females mated to Acp36DE knock-down males, the uterus fails to expand fully, thus inhibiting sperm movement toward the openings of sperm storage organs. Additionally, Acp36DE protein localizes to the anterior mating plug and the common oviduct in mated females (Bertram et al., 1996). Together,

these data suggest Acp36DE functions in mediating conformational changes in the female reproductive tract that orient sperm near the openings of sperm storage organs where it can subsequently be stored.

Reductions in sexual receptivity after mating occur in *Drosophila* and various other insects. In *D.melanogaster*, actively rejecting rival male suitors is a component of the induced post-mating behaviors. These behaviors benefit the first male in a competitive mating environment. Without a rival male(s) sperm in the female reproductive tract, the first male's paternity share is more significant and can fertilize more eggs before the female remates. Males with traits capable of producing substantial, long-lasting reductions in mating receptivity in females sire more offspring possessing these traits. Therefore, these traits are selected for over time.

In *D. melanogaster*, the receipt of SFPs plays a crucial role in inducing reductions in mating receptivity in females (T. Chapman et al., 2003; Häsemeyer et al., 2009; Liu & Kubli, 2003; Ram & Wolfner, 2009; Yang et al., 2009; Yapici et al., 2008). Males that transfer sperm but not SFPs induce a short-term reduction in mating receptivity (approximately one day) in females. In contrast, males that transfer sperm and SFPs induced a ~1 week reduction in females' mating receptivity. Males with reduced expression of sex-peptide (SP*)* induce a weak reduction in short-term mating receptivity and no long-term reduction compared to females mated to wild-type males (Liu & Kubli, 2003). Since SP binds to the tails of sperm (Peng, Chen, et al., 2005), the binding of SP to sperm is presumably required to induce more substantial short-term reductions in mating receptivity and sustained long-term reductions in mating receptivity. Regarding reducing mating receptivity in females post-mating, sperm likely serves only as a carrier for SP and has little to no effect on mating receptivity itself.

SFPs also have major influences on ovulation and oviposition in females. Ovulation is the process by which a mature egg is released from the ovary, where it can be fertilized, and oviposition, the egg-laying process, are key indicators of reproductive fitness in females. Following mating, ovulation and oviposition are increased. For females, ovulation and oviposition are energetically costly. Activation of ovulation and oviposition following mating ensures that these processes are activated when sperm is available for fertilization. Activation of ovulation and oviposition in females following mating benefits the first male in a competitive mating environment. Activation of these processes ensures that the first male's sperm fertilizes the maximum number of eggs before the female remates.

In *Drosophila*, SFPs are the components of the male ejaculate that have been shown to stimulate the activation of ovulation and oviposition in females following mating (Gillott, 2003; Heifetz et al., 2000; Ravi Ram & Wolfner, 2007). In *D. melanogaster*, following mating SP and the four SFPs that localize it to sperm tails (Peng, Chen, et al., 2005; Ram & Wolfner, 2007, 2009) contribute to the long-term activation of ovulation and oviposition in the female as long as sperm is present in the reproductive tract. SP does not only benefit the male that produced it; SP received from one male can bind to the sperm of another male in the female sperm storage organs, thus stimulating the usage of this stored sperm for fertilization (Misra & Wolfner, 2020). Given that males can specifically tailor their SFP composition in their ejaculate (Hodgson & Hosken, 2006; Sirot et al., 2011), SP likely has complex interactions in a competitive mating environment where the activation of ovulation and oviposition may be from one or more males.

The SFP Acp26Aa has been shown to stimulate an initial increase in oviposition in females following mating (Herndon & Wolfner, 1995). Acp26Aa was first identified for its role in oviposition through genetic analysis and it has been shown that females mated to Acp26Aa

knockout males lay fewer eggs than females mated to wild-type males (Herndon & Wolfner, 1995). Another SFP, ovulin, has been shown to stimulate an increase in ovulation in females following mating (Heifetz et al., 2000, 2005). The ectopic expression of ovulin or either of its cleaved products could stimulate ovulation in unmated females (Heifetz et al., 2005). The exact methods of ovulin's interactions in the female reproductive tract to upregulate ovulation are unknown. However, they likely involve interactions with neuromuscular targets in the female reproductive tract's lateral oviduct or indirectly through altering the neuroendocrine system's activity (Heifetz et al., 2000; Heifetz & Wolfner, 2004). In the reproductive tract of mated females, ovulin is proteolytically broken down step-by-step in a process that depends on SFP CG11864 and possibly others (Ravi Ram et al., 2006). The proteolytic cleavage of ovulin following mating could serve as a way to increase the number of available molecules that can interact with their neuromuscular targets, thus resulting in increased ovulation.

The female molecules that interact with SFPs have been relatively unexplored. Currently, one molecule has been identified in *D. melanogaster*: the Sex peptide receptor (SPR)—which localizes in female sperm storage organs as well as the nervous system—interacts with SP in addition to in addition to the SFP DUP99B (Rexhepaj et al., 2003; Yapici et al., 2008). Through genetic experiments, it was shown that SPR's function is required in specific neurons for SP to stimulate post-mating responses in the female (Wolfner, 2009). SPR serves as a prime example of a female molecule that interacts with SFPs of the male ejaculate. Going forward, there is a need to further identify additional female molecules involved in the reproductive process to better understand the mechanisms females use to process and modify SFPs after mating.

A component of the post-mating response in Drosophila females induced by SFPs is an increase in the amount of nutrients they consume. This increase in feeding behavior is likely in response to the energetic demand resulting from the activation of ovulation that occurs in females following mating. Sex peptide (SP) has been shown to have a role in stimulating ovulation and oviposition in females following mating (Gillott, 2003; Heifetz et al., 2000; Ravi Ram & Wolfner, 2007). SP has also been associated with post-copulatory feeding in *D. melanogaster* females. Supporting this, males lacking SP in their ejaculate fail to stimulate an increase in post-copulatory feeding in their mates, and unmated females with ectopic expression of SP experience an increase in feeding behavior (Carvalho et al., 2006). However, there is no evidence that SP directly influences an increase in female post-copulatory feeding. Sterile females do not display increased feeding behavior following mating (Barnes et al., 2008). This suggests that the increase in female post-copulatory feeding behavior observed in Drosophila is a product of increased ovulation and oviposition rather than SP directly causing the behavior.

Chapter 1.4 Longevity of females and sexual conflict

SFPs benefit male reproduction by increasing sperm competitive ability, stimulating their mates to move sperm into storage, and upregulating ovulation and oviposition. Together, these processes aid in or are required for the production of offspring. Despite SFPs benefits, some SFPs have been implicated in reducing a female's lifespan and fitness (T. Chapman et al., 1995; Fricke et al., 2010, 2013; Lung et al., 2002; Mueller et al., 2007; Smith et al., 2017; Wigby & Chapman, 2005). One SFP that significantly contributes to SFP-mediated mating costs in females is sex peptide (SP). Compared to control females, females consistently exposed to SP-deficient males—who do not produce any detectable SP—had much higher fitness and lifetime reproductive success (Wigby & Chapman, 2005). SP reduces female remating receptivity and increases ovulation and oviposition in the initial matings of unmated females. The reduction in lifespan and fitness experienced by mated females is likely due in part to the SP-mediated upregulation of ovulation and oviposition.

Additionally, Drosophila females go through a sleep-like state known as "siesta" after mating. SP mediates siesta: when receiving it, there is a 70% reduction in siesta and an increase in feeding and egg-laying behavior (Isaac et al., 2010). The combination of reduced sleep, increased feeding behavior, and the upregulation of ovulation aids in producing progeny yet harms the female over time. Due to these fitness-reducing side effects that some SFPs have on females, females are expected to evolve resistance to SFPs that harm them. Therefore, this provides the potential for an evolutionary arms race between the sexes (Wigby et al., 2020). For instance, if females develop resistance to an SFP as a result of the of the SFP causing them harm, the males no longer receives fitness benefits from that SFP. If the SFP provides a reproductive fitness benefit to the male (such as paternity share) the males may be selected for higher levels of

this SFP over time to overcome female resistance, thus resulting in increased harm to females (Edward et al., 2015). Without female resistance to SFPs that harm them yet provide reproductive fitness benefits to males, it is expected that selection for these SFPs would be driven only by male-male competition.

Chapter 1.5 Rapid molecular evolution of SFPs

Drosophila SFPs are among the most evolutionarily divergent proteins between species, with many displaying rapid sequence changes. In Drosophila, SFPs' rapid evolution and turnover may be attributable to the combination of sperm competition, cryptic female choice (CFC), and the continuous arms race between the sexes (Haerty et al., 2007; Ramm, 2020; Swanson & Vacquier, 2002). As a result of these highly selective forces SFPs may be experiencing rapid sequence change as new sequence combinations provide a selective advantage. In contrast, the rapid change of SFP genetic sequences may not be due to rapid evolution of SFPs but, rather nonadaptive forces such as relaxed selection. Relaxed selection may be occurring in the absence of purifying selection on these genetic sequence allowing for changes to occur without functional consequences A comparison of SFP genetic sequence information within *D. melanogaster* (intraspecific) and within the *D. melanogaster* species group (interspecific), approximately 50-57% had signatures of relaxed selection, 35-37% showed signatures of being selectively constrained, while only 7-12% had signatures of positive selection (Patlar et al., 2021). Given SFPs' roles in PCSS, the prevalence of SFP genetic sequences having signatures of relaxed selection is counterintuitive. The prevalence of SFP genetic sequences having signatures of relaxed selection suggests that the rapid sequence change observed in SFP genes is not due to PCSS but rather by weakened selection in maintaining genetic sequence identity. The minority of SFP genes, the genetic sequences displaying signatures of positive selection, may still be rapidly changing sequences because of selective pressures from PCSS. However, the precise mechanisms responsible for this selection need to be better understood at the functional level. The second most prevalent SFP genetic signature was the signature of being selectively constrained. 35-37% of SFP genes displayed signatures of

being selectively constrained (Patlar et al., 2021). These SFP genes likely maintain sequence identity intra- and interspecifically, due to these genes' essential and conserved roles in reproduction.

The drivers of SFP genetic sequence evolution warrant further examination. Studying what drives SFP genetic sequence evolution allows for (1) an understanding of how lineage specific SFPs may be involved in reproductive isolation. (2) An understanding of how rapid sequence changes of SFP genes between closely related species may provide a selective advantage. And lastly, (3) how highly conserved SFP genes may be essential for reproduction. Going forward, I will focus on the third point by functionally characterizing one of the most conserved Drosophila SFP genes in *D. melanogaster*.

Chapter 2

Chapter 2.1 Introduction

Drosophila SFPs are among the most evolutionarily divergent class of proteins, however the functional consequence of their rapid divergence remains unclear. Many characterized SFPs have known roles in reproductive success of males, and are thought to be targets of PCSS. Yet, relaxed constraints could also be causing their rapid divergence. Examining the molecular evolution of approximately 300 SFP genes, it was found that more genes are evolving under relaxed selection or were selectively constrained than genes that showed evidence of positive selection (Patlar et al., 2021). Based on this molecular evolutionary data, it is unclear if SFPs are rapidly evolving because of PCSS, as there is a large proportion of genes displaying signatures of nonadaptive forces. To better understand what forces may predominantly be shaping SFPs rapid divergence we need to achieve a better understanding of the functional roles uncharacterized SFP genes have that fall into the categories of positive selection, relaxed selection, and selectively constrained. By developing an understanding of functional roles or lack thereof in genes in each of these three categories, we will be able to make a better connection between a gene's functional role and its molecular evolutionary pattern. Additionally, there is a lack of knowledge of the precise molecular functions of SFP's. By characterizing uncharacterized SFPs functional roles we will add to the short list of characterized SFPs.

Genes with molecular signatures of positive selection indicate that these genes are recent advantageous genetic variants that swept the population. PCSS can result from forms of directional forms of selection such as positive selection giving some SFP variants advantages in ejaculate function, or forms of sexual conflict that contribute to an escalating coevolutionary chase between sexes (Rowe et al., 2020; Sirot & Wolfner, 2015; Wigby et al., 2020). Therefor I

hypothesize that uncharacterized SFP genes that display signatures of positive selection will mainly have roles in reproduction that affect male paternity and progeny output in females. Genes with molecular signatures of relaxed selection indicate that these genes under weakened selective strength. SFP gene expression is male-limited, which means that selection is limited to males (Ellegren & Parsch, 2007; Ranz et al., 2003). SFP gene copies in males, which are approximately half of all copies for an autosomal diploid gene, experience no selection each generation (Dapper & Wade, 2020). Thus, the selective coefficient acting on SFP genes in males must be twice as strong as that acting on genes expressed in both sexes to experience positive selection. SFP genes that exhibit signatures of relaxed selection are not experiencing strong enough selection in males to overcome their sex-limited expression. Therefore, I hypothesize that uncharacterized SFP genes that display signatures of relaxed selection will mainly have no observable functional roles in reproduction. Genes with molecular signatures of being selectively constrained may have once been adaptive variants, but recently are limited in the production of new variants. Constrained SFPs genes are overrepresented in older genes, present in ancestors to the genus *Drosophil*a and may be essential for housekeeping maintenance of reproduction (Patlar et al., 2021). I hypothesize that uncharacterized SFP genes that display signatures of being selectively constrained will mainly have roles that are essential to reproduction. Moving forward, I will start by characterizing a selectively constrained SFP gene and hope to characterize additional genes in the future.

One of the most evolutionarily conserved SFP genes is *CG15117*. *CG15117* is an uncharacterized *D. melanogaster* SFP that is predicted to enable beta-glucuronidase activity and carbohydrate-binding activity (Baycin-Hizal et al., 2011; Findlay et al., 2008b; Wigby et al., 2020) Because of this, I will refer to CG15117 as BGLUC. BGLUC is highly conserved, with

orthologs present in *Homo sapiens*, *Mus muscus*, *D. obscura*, and *D. yakuba* to name a few (Supplementary Table 2).

D. melanogaster BGLUC was identified as a high-confidence SFP (Wigby et al., 2020). BGLUC is transferred as a component of the male ejaculate to the female during mating (Findlay et al., 2008a, 2009; McCullough et al., 2022). BGLUC also has expression bias towards the male accessory gland, is moderately expressed in the testes and has reduced expression in other tissues (Li et al., 2022). Single-cell data from (Li et al., 2022) of the male reproductive tract shows BGLUC expression is predominant in male accessory gland main cells, male accessory gland secondary cells, anterior ejaculatory duct, seminal vesicle, and the secretory cells of the male reproductive tract. Expression in the testes is limited to gonadal associated epithelium. Thus, BGLUC is a *bona fide D. melanogaster* SFP.

BGLUC is predicted to enable beta-glucuronidase and carbohydrate-binding activity (Baycin-Hizal et al., 2011). Beta-glucuronidases are members of the glycosidase family of enzymes that function in the catalytic breakdown of complex carbohydrates (*Comprehensive Biological Catalysis. 4*, 1998). In humans beta-glucuronidase is a lysosomal enzyme that catalyzes the hydrolysis of beta-glucuronide residues as part of a sequential degradation of glycosaminoglycans and several sulfates (Morel & Levin, 2008). The autosomal recessive disorder known as Sly syndrome is due to a deficiency of beta-glucuronidase which leads to accumulation of incomplete degraded glycosaminoglycans in secondary lysosomes in many tissues (Morel & Levin, 2008). There is a knowledge gap in how beta-glucuronidase proteins are involved in sexual reproduction. Currently there are no available studies on beta-glucuronidase activity in sexual reproduction in insects, and the studies that do exist are based on its activity in bull seminal plasma and reproductive organs (Jauhiainen & Vanha-Perttula, 1986). Therefore,

functionally characterizing beta-glucuronidase in *D. melanogaster* reproduction will provide insight into its role in insect reproduction and other organisms due to its evolutionary conservation.

In an effort to functionally characterize the role of BGLUC in *D. melanogaster* reproduction, I generated a CRISPR knockout mutation for this gene. By generating a knockout mutation of this gene and analyzing the consequences on reproduction deviations from wild-type reproductive behavior were characterized. A line containing sgRNA for the coding sequence of BGLUC was obtained to generate this mutation (Zirin et al., 2020). Using an sgRNA-expressing strain allows many flies to be mated with a line expressing Cas9 and then screened for the knockout mutation. This method of generating CRISPR knockout mutations has a higher throughput than methods where gRNAs are microinjected into fly embryos expressing Cas9 and then screened for the mutation. Additionally, in my experience, using lines already expressing a sgRNA was more effective at generating knockout mutations. Here I analyze *D. melanogaster* BGLUC mutants to identify phenotypes, and thus elucidate the genes' role in sexual reproduction.

Chapter 2.2 Methods

Fly stocks and husbandry

Flies were kept at consistent population densities and maintained at a constant temperature of 24° in a ∼12-hour day/night cycle on standard cornmeal medium. I collected flies to be utilized for all experiments every six hours using CO2 anesthesia to ensure they were unmated. Collected flies were sexed, divided based on phenotype and placed into standard cornmeal vials, and aged for five days. Twenty-four hours before planned mating experiments I placed male flies into their own individual fresh vial. Additionally, 24 hours before all planned mating experiments, I placed females in groups of ten female flies into fresh vials with a dried yeast mixture (4g yeast/7 ml H2O) to increase mating receptivity in females.

The following lines were used in this study: BL#77081 y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TKO.GS00872}attP40, BL#3628 y[1] w[*]; nub[2] b[1] sna[Sco] pr[1] cn[1]/CyO, BL#54591 y[1] M{w[+mC]=nanos-Cas9.P}ZH-2A w[*], BL#25211 Oregon-R-modENCODE, and pBac{Ub nls-EGFP, ProtamineB-EGFP} (J. Belote, personal communication).

BGLUC null stock generation

To generate CRISPR knockouts for BGLUC, I utilized a TRiP-KO line that expresses an sgRNA targeting the coding sequence of BGLUC. First, I mated TRiP-KO females with males carrying a germline source of Cas9 (*nos*-Cas9) to induce a double-strand break in the coding sequence of BGLUC. (Non-homologous end-joining repair occurs, generating a knockout mutation for BGLUC if frame-shift mutation occurs.) Next, I collected fifteen male F1 progeny containing nos-Cas9 and sgRNA transgenes were. I then mated these males *en masse* to second

chromosome balancer females. By crossing in a balancer chromosome line, recombination is suppressed in the second chromosome as a result of inverted chromosome segments that prevent inheritance of products of recombination events and suppress recombination near inversion breakpoints (Crown et al., 2018). Thus, allowing for mutations that may be homozygous lethal or result in sterility to be maintained in a stock. I then collected thirty F2 male progeny with wild-type cuticle color (mutations in yellow gene resulting in yellow cuticle color reduce male mating success), RFP eyes (to eliminate sgRNA transgene from stock on the third chromosome), and curly wings (balanced). I then individually paired F2 males with a balancer female. Next, I collected the resulting F3 progeny with wild-type cuticle color and vermilion non-RFP eyes (to eliminate nos-Cas9 from stock). After I confirmed the knockout mutation of BGLUC by sequencing (see below), I maintained these stocks by mating collected F3 progeny *en masse*. The resulting F4 progeny with wild-type wings are then homozygous for knockout of BGLUC. F4 progeny with curly wings are heterozygous for BGLUC knockout and balanced.

Verifying knockout

After pairing with a balancer female for three days, I collected individual F3 males and sequenced to check for the presence of a deletion in BGLUC at the target site. I flash froze the males in liquid nitrogen, and obtained genomic DNA from each fly using a proteinase-K extraction protocol (Gloor & Engels, 1992). I PCR amplified genomic DNA using primers ~100 base pairs upstream and downstream of the sgRNA. I PCR purified samples and then sent the samples to Azenta for Sanger sequencing. I then aligned to *D. melanosater* reference genome to the sequence data and visualized with Geneious Prime. I maintained stocks corresponding to samples that were heterozygous for base pair deletions near the sgRNA target site that caused a

frame shift mutation. I also maintained one stock corresponding to a sample without deletions near the sgRNA target sequence as a control in the following experiments. This stock has the same genetic background as the BGLUC null stock; however, it has no alterations to the BGLUC coding sequence. Lastly, I collected F4 males, extracted their genomic DNA, and submitted it for sequencing to confirm the presence of a knockout mutation in BGLUC. I identified the mutant stock contained a five-base pair deletion in the sgRNA target sequence, followed shortly by a premature stop codon (Supplementary Figure 1).

ProtamineB-eGFP BGLUC null stock generation

To generate a homozygous null BGLUC stock expressing protamineB-eGFP, I first mated heterozygous males with females carrying pBac{Ubnls-eGFP, protamineB-eGFP}. I then sorted the resulting F1 progeny for the presence of curly wings (balanced) and ubiquitous expression of GFP (marking the presents of protamineB-eGFP). Next, I mated these F1 males to homozygous null BGLUC females. I sorted F2 progeny were by ubiquitous expression of GFP, curly wings, and the presence of wild-type bristles (contained one copy of BGLUC knockout). Flies with scutoid bristles indicated the presence of the unbalanced second chromosome rather than the chromosome containing the BGLUC knockout mutation. I then mated the sorted male and female flies from the F2 generation to generate F3 homozygous null males for BGLUC while also expressing protamineB-eGFP. To maintain this stock, I selected for ubiquitous expression of GFP in the preceding generations to maintain its presence in future stocks. I used the same previously mentioned mating scheme to generate a control line. I used the previously generated BGLUC control flies in place of BGLUC null flies to generate a protamineB-eGFP stock having the same genetic background without alterations to the BGLUC coding sequence.

Fecundity assay

To determine fecundity levels in females following mating to BGLUC homozygous null, BGLUC heterozygous null and BGLUC control, I maintained and sorted fly stocks as previously mentioned. After aging, I individually paired male flies with an Oregon R female. I observed pairs for a maximum of three hours for the occurrence of mating. I discarded pairs that did not mate within three hours. Immediately following separation, I removed the male. I also discared pairs that mated for less than ten minutes. Mated females were left to oviposit for a total of six days. On the third day following mating, I switched mated females into a fresh vial. I discarded the females after three days in the fresh vial (six days total). I discarded females that died prior to the six days of ovipositing were excluded in the analysis. As the resulting progeny eclosed, I counted progeny and discarded them until no more progeny eclosed. I calculated progeny count averages for all three groups and calculated significant difference in means using Tukey's HSD (honest significant difference) test.

Mating latency, mating rate and copulation duration

To determine mating latency, mating rate and copulation duration in females mated to BGLUC homozygous null, BGLUC heterozygous null, and BGLUC control males, I maintained fly stocks sorted them as previously mentioned. After aging, male flies I individually paired male flies with a *D. melanogaster* Oregon R female. I observed mating following the same protocol used in the fecundity assay. I measured mating latency starting at the time from introducing the male into the vial until the start of copulation. I measured copulation duration starting at the time the male mounted the female and ending at the time the male dismounted the female. I

calculated mating rate (percent) by totaling the number of pairs that mated for each group and dividing that total by the total number of pairs that were observed. I calculated mating latency and copulation duration means for each of the three groups and calculated significant differences in means using Tukey's HSD test.

Remating assay

To determine the proportion of females that remate following mating to BGLUC homozygous null, BGLUC heterozygous null, and BGLUC control flies independently, I maintained and sorted them as previously mentioned. I individually paired male flies from each group with an Oregon-R female and observed for mating. After separation, I removed the male fly, and left the mated female in the mating vial for three days. I discarded mating pairs that mated for less than ten minutes and females who died three days after mating. On the fourth day following mating, I transferred mated females into a fresh vial and aspirated an Oregon R male into the vial. I observed the mating pairs for the occurrence of mating for 90 minutes. I calculated the percentage of females that remated and statistically compared the percentages that remated to the percentages that did not remate using a pairwise Fisher's exact test to account for differing sample sizes.

Post-mating female reproductive tract imaging

To assess sperm storage dynamics in females following mating to protamineB-eGFP BGLUC homozygous null and protamineB-eGFP BGLUC control flies, I maintained stocks and sorted them as previously mentioned. For each genotype, I individually paired males with a *D. melanogaster* female and observed for the occurrence of mating. After dismounting, I

discarded the male a left the female in the vial for 1hr. After 1hr, I flash-froze the female in liquid nitrogen. I then removed the lower female reproductive tract using fine forceps and mounted it on a glass slide using a coverslip and 1x PBS solution. Using fluorescent light microscopy, I examined the lower FRT. I then recorded sperm presence or absence, and took pictures and videos of the samples.

Chapter 2.3 Results and Discussion

Using CRISPR to disrupt the protein-coding sequence of BGLUC and stabilizing the knock-out mutation in *D. melanogaster* allowed me to functionally analyze the role of BGLUC in reproductive success. Since *Drosophila* has reproducibly observed mating and post-mating behaviors, using BGLUC homozygous null, BGLUC heterozygous null, and BGLUC control males in mating assays allowed me to identify deviations from the typical mating and or post-mating behaviors.

BGLUC homozygous null males have a protein coding sequence disruption near the 5' end of the coding sequence shortly followed by a stop codon, thus prematurely terminating the translation of this gene's transcript (see Supplementary Figure 1). As an intermediate genotype I used BGLUC heterozygous null males. These males have one functional copy of BGLUC and one mutated BGLUC copy. As a control sample, I used BGLUC control males; these control males have the same genetic background as BGLUC homozygous null males but do not have BGLUC protein coding alterations. Using male samples with identical genetic backgrounds except for the alterations to the BGLUC coding sequence (homozygous null versus homozygous control), I can confidently attribute observed phenotypic effects directly to the BGLUC mutation. Additionally, by using BGLUC heterozygous null males, which is often used as the control sample for some SFP functional analysis, I was able to test if there are phenotypic differences between these male samples and BGLUC control males. The singular functional copy of BGLUC and the inverted 2nd chromosome segments in the heterozygous null males may contribute to altered phenotypes in these males.

Even though BGLUC is expressed at low levels in other male tissues, BGLUC homozygous null males appear healthy and survive long enough to become sexually mature and

mate with females. I observed several significant differences in mating assays of BGLUC male samples (Figure 1). In total mating latency was recorded from observations of 40 females mated to homozygous null BGLUC, 43 females mated to heterozygous BGLUC males, and 28 females mated to BGLUC control males were observed (Figure 1A). BGLUC homozygous null males have a significantly delayed copulation onset compared to heterozygous null males (p-value = 0.042) and BGLUC control males (p-value = 0.022). This was unexpected because BGLUC homozygous null males did not show any observable defects in locomotion or courting behavior. The observed increase in mating latency in BGLUC homozygous null males may be due to subtle behavior defects that are difficult to observe.

The same samples I observed in (Figure 1A) were also observed for copulation duration (Figure 1B). In terms of copulation duration, I observed only one significant difference between BGLUC heterozygous null males and BGLUC homozygous null males, with copulation duration being longer in BGLUC heterozygous null males (p-value = 0.029). This difference can likely be attributed to the curly wing phenotype that marks the 2nd chromosome balancer, as it may alter their ability to latch onto and inseminate the female, thus extending copulation duration.

I observed the most extreme differences in progeny production in wild-type females following mating to either BGLUC homozygous null, BGLUC heterozygous null, or BGLUC control males after six days of egg laying (Figure 1C). In total progeny counts were taken from, 20 females mated to BGLUC homozygous null males, 20 females mated to heterozygous BGLUC males, and 26 females mated to BGLUC control males. Progeny count differences were most significant between BGLUC homozygous null and BGLUC control mates (p-value = 2.4e-9), where BGLUC control males sired an average of 52 offspring, while BGLUC homozygous null males did not sire any progeny. Progeny count differences were the second most significant

between BGLUC homozygous null and BGLUC heterozygous null mates (p-value = 1.1e-8), where BGLUC heterozygous null mates produced an average of 42 offspring. Lastly, there was a significant difference between progeny produced from BGLUC heterozygous null mates and BGLUC control mates (p-value = 0.0064). Although progeny counts were low compared to typical *D. melanogaster* reproduction for all wild type females (likely due to the male genetic background), the lack of progeny produced from BGLUC homozygous null mates shows that complete disruption of the BGLUC coding sequence results in male sterility and that BGLUC is an essential male fertility gene. Additionally, the significant reduction in progeny produced from BGLUC heterozygous null males compared to BGLUC control mates suggests that having one functional copy of BGLUC results in an intermediate phenotype.

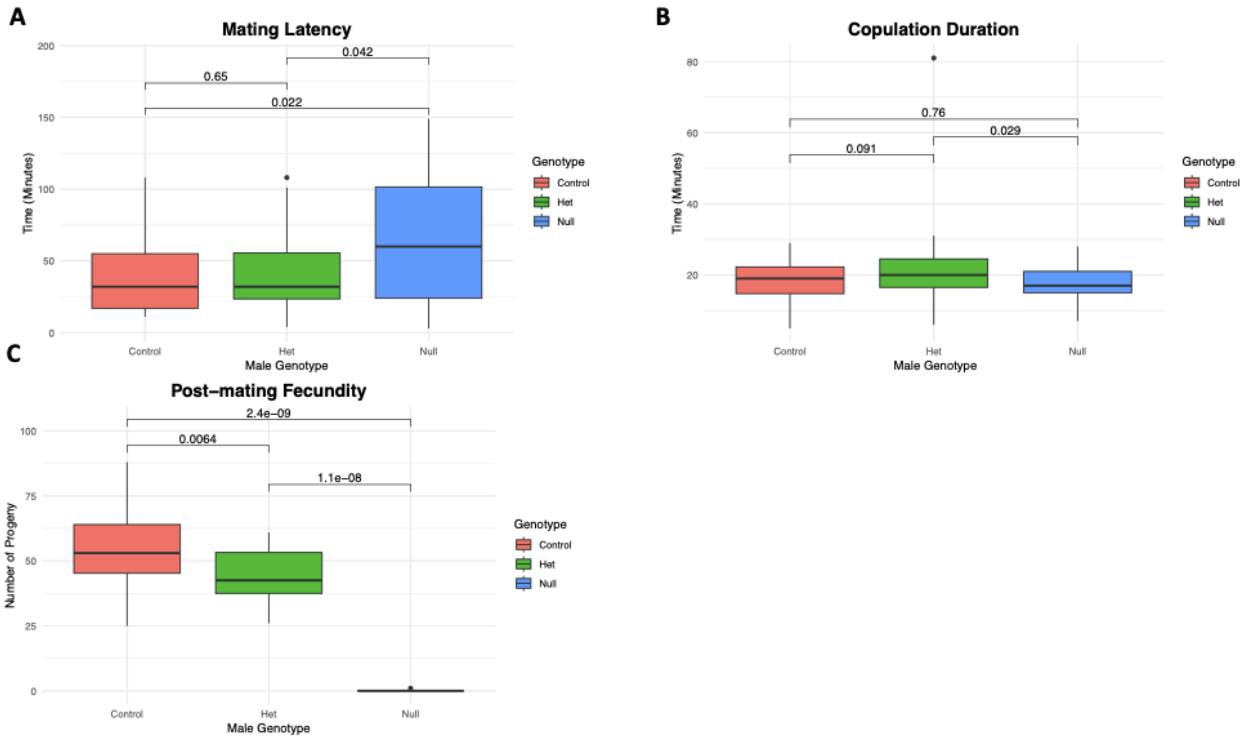


Figure 1. Mating assays of *D. melanogaster* BGLUC homozygous null (blue), BGLUC heterozygous null (green), and BGLUC wild-type control (red). (A) Time in minutes until the start of copulation in observed male-female pairs (mating latency). Significant differences in mating latency were observed in BGLUC homozygous null and BGLUC heterozygous null mating pairs compared to BGLUC control mating pairs as well as between BGLUC homozygous null and BGLUC heterozygous null mating pairs. (B) Time in minutes of mating duration in observed male-female pairs (copulation duration). A significant difference was observed in copulation duration between BGLUC homozygous null and BGLUC heterozygous null mating pairs. (C) Total counts of flies produced from a single mating in observed male-female pairs followed by six days of egg laying. Significant differences in progeny production were observed in BGLUC homozygous null and BGLUC heterozygous null mating pairs compared to BGLUC control mating pairs as well as between BGLUC homozygous null and BGLUC heterozygous null mating pairs. Homozygous null BGLUC mating pairs did not produce any progeny.

After observing a significant difference in mating latency between BGLUC homozygous null males and BGLUC control males, I calculated the mating rate for each male genotype (Figure 2 A). There were no significant differences in mating rate, supporting the idea that BGLUC homozygous null males have no phenotypic effects that result in the inability to mate. To determine if BGLUC homozygous null males trigger refractoriness to remating in females, BGLUC homozygous null, BGLUC heterozygous null, and BGLUC control males I individually paired males with Oregon-R females that were allowed to remate with a wild-type male four days following the initial mating. I observed for the occurrence of remating and recorded results in (Figure 2 B). I observed a significant difference in the percent remating in females who mated to BGLUC homozygous null males compared to females who mated to BGLUC control males (p-value = 2.78e-13) and between females mated to BGLUC homozygous null males and females mated to BGLUC heterozygous null males (p-value = 4.24e-11). 37.5% of females remated four days following an initial mating to BGLUC homozygous null males, while no females remated four days after an initial mating to BGLUC control males. 1.7% of females remated four days after initial mating to BGLUC heterozygous null males. However, this was not significant compared to the homozygous control. The reduction in refractory to remate suggests that females mated to BGLUC homozygous null males are experiencing an altered post-mating response, thus influencing their willingness to remate.

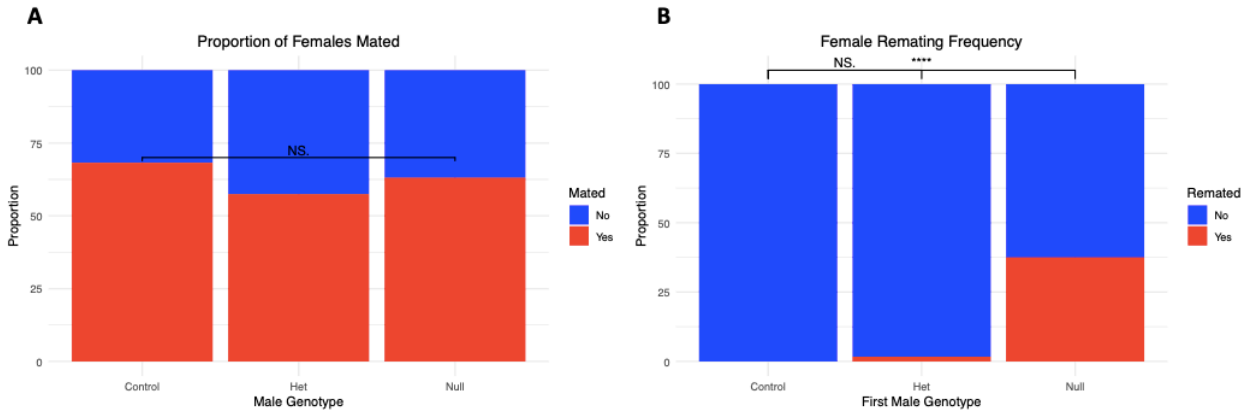


Figure 2. Mating assays of *D. melanogaster* BGLUC homozygous null, BGLUC heterozygous null, and BGLUC control. (A) Proportion of females mated in a three-hour observation of male-female pairs. No significant differences were observed. (B) The proportion of females that remated to a wild-type male four days after an initial mating to either D. *melanogaster* BGLUC homozygous null, BGLUC heterozygous null, and BGLUC control males during a 90-minute observation. Significant differences in progeny production were observed between BGLUC homozygous null and BGLUC control mating pairs, as well as between homozygous null BGLUC mating pairs and BGLUC heterozygous null mating pairs.

Following observation of the sterility defect in BGLUC homozygous null males, I examined sperm presence/absence in the male reproductive tract and in the female reproductive tract after mating. To examine this I introduced protamineB-eGFP, which labels sperm heads with a green fluorescent protein tag, to the BGLUC homozygous null stock and BGLUC control stock following the mating scheme outlined in Ch 2.2. I performed fluorescence imaging on protamineB-eGFP BGLUC homozygous null males as well as females mated to protamineB-eGFP BGLUC homozygous null males 1hr after mating. I performed the same imaging procedure was followed in protamineB-eGFP BGLUC control males and their mates. Imaging of protamineB-eGFP BGLUC homozygous null mates (Figure 3 D & C) showed a substantially reduced amount of sperm in the bursa compared to protamineB-eGFP BGLUC control mates' lower reproductive tract (LRT) (Figure 3 A & B). Additionally, among the few sperm in the female LRT, none of the sperm is observed in the sperm storage organs (spermatheca or seminal

receptacle). In contrast, sperm is present in protamineB-eGFP BGLUC control mates' sperm storage organs. An intact mating plug was formed in the mates of both male samples.

To determine if there were errors in sperm production in protamineB-eGFP BGLUC homozygous null males, I performed fluorescence imaging on unmated protamineB-eGFP BGLUC homozygous null males' testes (Figure 4 A & B) and seminal vesicle (Figure 4 C & D). Errors in sperm production could result in decreased amount of sperm present in the female bursa post-mating (Figure 3A & 3B). Imaging of both tissues showed no defects in sperm production, individualization, or motility. Suggesting that the reduced amount of sperm present in mates of protamineB-eGFP BGLUC homozygous null males is due to defects in sperm transfer during mating, and likely a failure to induce sperm storage in mated females.

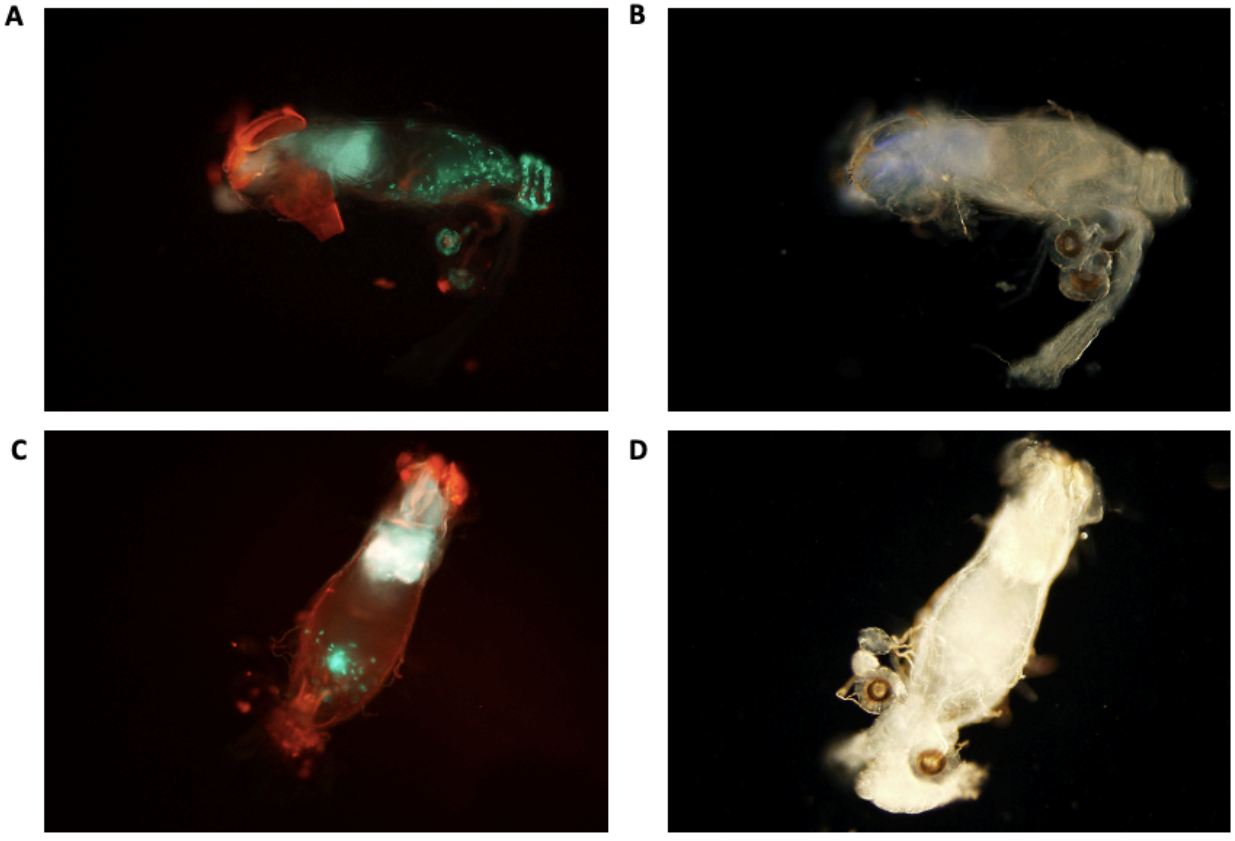


Figure 3. Lower female reproductive tract imaging 1hr post-mating. Green labels sperm. (A) Fluorescent imaging of the lower female reproductive tract after mating with a protamineB-eGFP BGLUC control male. (B) Darkfield image of the same lower female reproductive tract imaged in the image (A). (C) Fluorescent imaging of the lower female reproductive tract after mating with a protamineB-eGFP BGLUC homozygous null male. (D) A darkfield image of the same lower female reproductive tract is imaged in image (C). Notable reductions of sperm present in the lower female reproductive tract in females mated to a protamineB-eGFP BGLUC homozygous null male. Additionally, there is no sperm in the female sperm storage organs; the seminal receptacle and spermatheca in the female mated to protamineB-eGFP BGLUC homozygous null male.

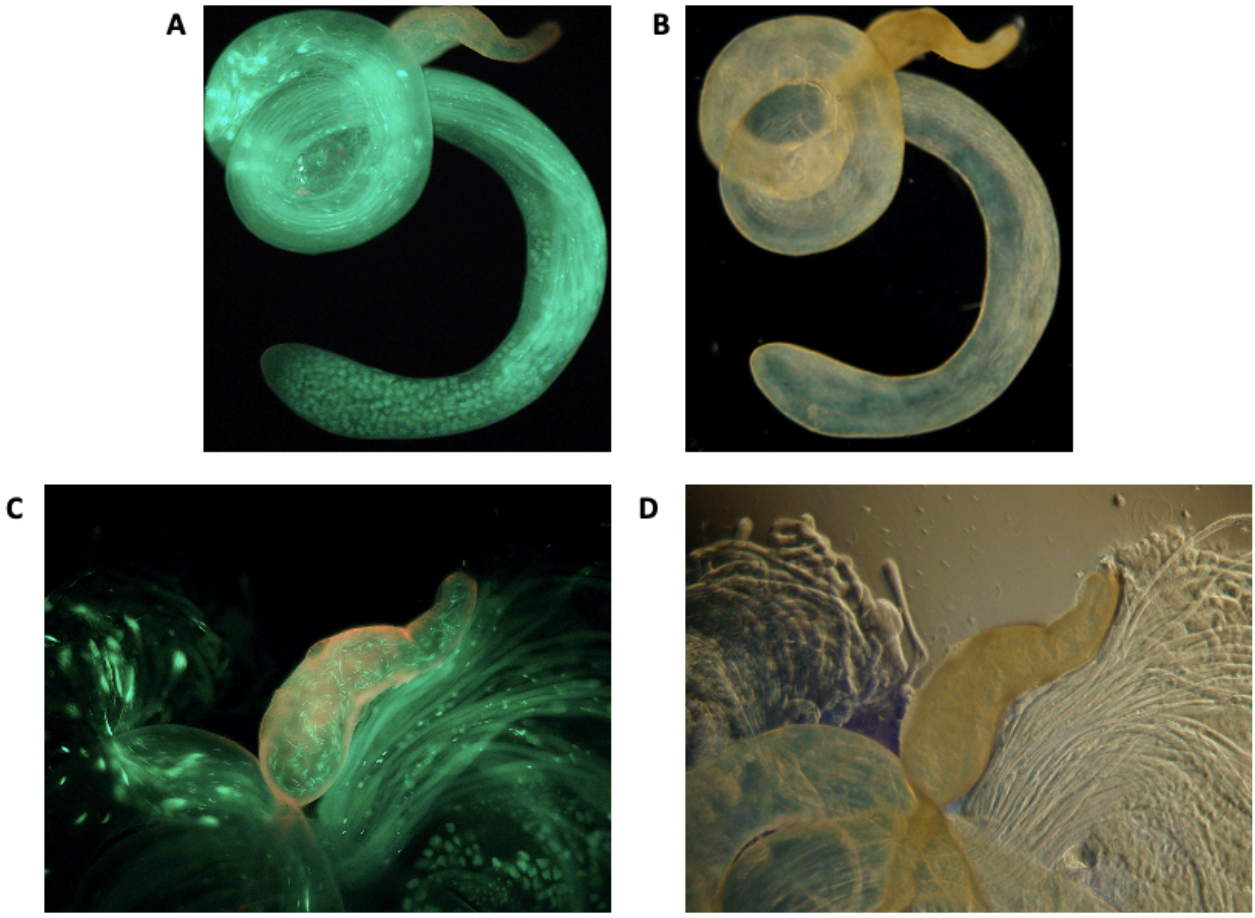


Figure 4. Unmated protamineB-eGFP homozygous null testis and seminal vesicle imaging from the same male reproductive tract. Green labels sperm. (A) Fluorescent image of the unmated protamineB-eGFP homozygous null testis. (B) Darkfield imaging of the same testis used in the image (A). (C) Fluorescent image of the unmated protamineB-eGFP homozygous null seminal vesicle. (D) Darkfield imaging of the same seminal vesicle used in the image (A). There were no observable defects in protamineB-eGFP homozygous null testes and seminal vesicle.

Chapter 2.4 Conclusions

Here I demonstrate that BGLUC is an essential gene for male fertility. BGLUC homozygous and heterozygous null males display identical mating behaviors, yet heterozygous null males sire progeny in their mates, unlike BGLUC homozygous null males. However, the number of progeny BGLUC heterozygous null males sire is significantly reduced in comparison to BGLUC control males (although this reduction is not dramatic). This indicates that having one functional copy of BGLUC is sufficient in maintaining male fertility yet decreases male reproductive fitness. Additionally, BGLUC homozygous null males fail to trigger post-mating responses in females, such as refractoriness to remating. This likely results from the lack of sperm present in the female sperm storage organs, or a failure of SFP transfer, which activates long-term refractoriness. The reduced amount of sperm present in the bursa and failure of sperm to enter the storage of females following mating to BGLUC homozygous null males strongly suggests defective accessory gland function that manifests as altered transfer of sperm and—possibly—failure to transfer key SFPs that are required for proper post-mating responses.

Chapter 3

Chapter 3.1 Introduction

The extreme sterility phenotype observed in BGLUC homozygous null mutant males in (Figure 1C, Figure 2B, and 3C), suggests a molecular disruption in accessory gland function. BGLUC is predicted to enable beta-glucuronidase and carbohydrate-binding activity (Baycin-Hizal et al., 2011). Beta-glucuronidases are members of the glycosidase family of enzymes that function to catalyze the breakdown of complex carbohydrates (*Comprehensive Biological Catalysis. 4*, 1998). Glycosylation is one of the most common post-translational modifications and plays important roles in in various biological processes (Bertozzi & Kiessling, 2001). BGLUC expression is also biased to the secondary cells of the male accessory glands (Li et al., 2022). A similar SFP, the Hox Gene *Abd-B,* which also has secondary cell expression bias, has been shown to glycosylate several SFPs in the male accessory gland, resulting in failure to transfer these SFPs to the female during mating and thus resulting in an altered female post-mating response (Gligorov et al., 2013)

Glycosylation of SFPs is not well understood; however three possible mechanisms for secondary cells to mediate glycosylation have been proposed: 1) by secreting glycosylation substrates that the main cells can absorb and utilize; 2) by secreting glycosylation regulators into the lumen, where they can alter SFPs from both accessory gland cell types; or 3) by re-uptake into vacuoles prior to secretion back into the lumen as mature, glycosylated proteins, e.g., ovulin (Gligorov et al., 2013). Studying BGLUC's role in mediating SFP production will provide insights into how glycolytic cleavage of proteins maintains SFP integrity.

I performed RNA sequencing to examine transcriptome dynamics in BGLUC homozygous null males and females mated to BGLUC homozygous null males. From here on I

will refer to BGLUC homozygous null males as BGLUC mutants. RNA sequencing was performed on BGLUC mutants and control male accessory glands and testes as well as female LRTs from females mated to either male genotype at one, three, and six hours post-mating. Furthermore, shotgun mass spectrometry with label-free quantification was performed on BGLUC mutant and control male accessory glands as well as female LRTs from females immediately after mating to either male genotype.

RNA sequencing in the male accessory gland and testes will allow for the identification of disruptions in transcriptome profiles that may indicate regulatory defects in SFP production. Furthermore, by performing post-mating time series RNA sequencing in the female LRT of females mated to either BGLUC mutant or control males, I will be able identify mRNA expression changes that are associated with disruptions in the female post-mating response. Shotgun mass spectrometry with label-free quantification of BGLUC mutant and control male accessory glands will allow me to identify altered protein abundances of additional proteins and thus likely suggest BGLUC has a role in post-translational modification of proteins in the male accessory glands. By performing shotgun mass spectrometry with label-free quantification of female LRTs, I was able to identify defects in the transfer of SFPs to the female during mating and/or altered protein abundances of female proteins in the LRT that are activated shortly after copulation.

Chapter 3.2 Methods

Fly stocks and maintenance

I maintained, aged, and sorted, fly stocks were as mentioned above. Notably, I excluded BGLUC heterozygous null males from these experiments. The unmated females I used in the following experiments are *D. melanogaster* Oregon R females.

Tissue collection

I paired flies in individual food vials and observed for the occurrence of matings for three hours. I discarded mating pairs that mated for less than ten minutes. For flies to be used in proteomics, I flash-froze pairs in liquid nitrogen within 15 minutes of separation and stored them at -80˚C until dissections. I processed unmated females to be used in RNA-seq using the same procedure, except they were never exposed to a male. For mating pairs to be used for RNA-seq analysis, I assigned the copulation pairs one of three flash freezing timepoints: 1hr, 3hr, and 6hr post-copulation. I discarded pairs that remated during this time frame. Due to the weak penetrance of the curly wing phenotype (balancer chromosome marker) and the subsequent difficulty in sorting out heterozygous males, BGLUC mutant males and their mates were pooled after flash freezing and placed in separate vials with corresponding labels. I stored samples at -80˚C until sequence data was obtained. Following, I genotyped each male pool as described previously. Female samples corresponding to male samples with heterozygosity near the sgRNA were discarded. Presence of heterozygosity for the BGLUC knockout mutation in pooled male samples indicated that one or more of the male(s) in the pool were BGLUC heterozygous null contaminates. Therefore, the corresponding female samples mated by these contaminate males would contaminate subsequent data analysis.

For male samples to be used in RNA-seq and proteomics, I flash-froze BGLUC mutant males in liquid nitrogen, pooled them in groups of five and vortexed them briefly to remove their heads. I placed the heads in a separate PCR tube from the carcass and labeled to correspond to each other. I stored carcasses at -80 ˚C until sequence data was obtained. I then extracted genomic DNA from the heads following the protocol mentioned above. I discarded carcass samples corresponding to head samples with heterozygosity near the sgRNA. Presence of heterozygosity for the BGLUC knockout mutation in pooled samples indicated that one or more of the heads came from a BGLUC heterozygous null male(s), Therefore the corresponding carcass samples had to be discarded to avoid contamination in subsequent data analysis.

For dissections, I thawed male and female samples at room for ~two minutes before dissecting. For females, I carefully removed the LRT using fine forceps (bursa, seminal receptacle, and spermathecae) and placed it in a drop of ice-cold 1x PBS and pooled in 1x PBS over ice. I pooled twenty female reproductive tracts per replicate and them stored at -80˚C. For males to be used in proteomic analysis, I removed the ejaculatory duct and accessory gland using the same technique as the female samples pooled the male samples at 20 male reproductive tracts per replicate. I used the same protocol and techniques as previously mentioned for male and female dissections to be used in RNA-seq analysis, except I removed and stored the testes in separate vials from the ejaculatory ducts and accessory glands. I then pooled the samples at 20 tissues per replicate and then stored them in TRIzol on ice before storing them at -80 ˚C.

RNA extraction and library prep

To extract RNA, I thawed replicates at room temperature for ~five minutes, and followed the protocol described in (Delbare et al., 2020). I then quantified purified RNA on a Qubit

fluorometer and standardized samples to 20 ng/μL and then stored them at -80° till library preparation. RNA-seq libraries were made using the Lexogen 3‘ FWD kit following the manufacturer’s protocol (Lexogen, NH). I quantified libraries on a Qubit fluorometer and checked peaks on an Agilent TapeStation 4200. Libraries were then pooled and sequenced at SUNY Upstate Medical Center on an Illumina NextSeq 2000 platform.

Protein extraction and shotgun mass spectrometry

After dissections, I removed excess 1xPBS tissue samples then homogenized the samples with a sterile pestle. I then extracted protein following the protocol utilized in (Garlovsky & Ahmed-Braimah, 2023) and delivered samples on dry ice to the BRC Proteomics and Metabolomics Facility at Cornell University for shotgun mass spectrometry.

Protein identification and quantification

All raw data files for gel slices from each sample were set as fractions in order and combined. The precursor abundance intensity for each peptide identified by MS/MS in each raw data files were automatically determined and their unique plus razor peptides in each fraction for each protein were summed and used for calculating the protein abundance by PD 2.4/2.5 software. Either “total peptide amount” or “none” will be used for Introduction of PD2.4/2.5 Reports for Protein ID/LFQ at Cornell Proteomics & Metabolomics Facility normalization of gel-based LFQ samples, while “specific protein: Enolase” will be used for normalization of in-solution non-yeast samples (if spiked). Protein ratios are calculated based on pairwise ratio as median of all possible pairwise between replicates of all connected peptides.

The raw files were searched against *D. melanogaster* database (dm6) that contains 22279 protein sequences. Spectral data from all replicates were run together with the following search parameters: mass tolerance of 10 ppm, for parent ions and 0.6 Da for fragment ions. Oxidation of M deamidation of N and Q were specified as dynamic modifications of amino acid residues; protein N-terminal M loss, N-terminal acetylation, and M-loss + acetylation was set as a variable modification. Carbamidomethyl on C was specified as the static modification. Peptide identifications were filtered to a false discovery rate (FDR) using the Percolator 3.0 decoy analysis algorithm (The et al., 2016). Percolator estimates the number of false positive protein identifications by using a decoy database containing reversed protein sequences from the dm6 protein database. Percolator then goes through the target list of proteins and calculates the FDR that would result if it used the target score of a particular protein as a threshold. The threshold is obtained by dividing the number of target proteins by the number of decoys for every target protein. To account for varying thresholds, Percolator uses the q-value which is defined as the minimum FDR threshold at which a given target would be included in the results. For this analysis the q-value cutoff was 0.05. Proteins were filtered for inclusion by having at least two unique peptide-spectrum matches (PSMs), and one identification per protein group. Protein abundances (NSAFs) between control male accessory gland replicates, mutant male accessory gland replicates, LRT replicated of females mated to control males, and LRT replicates of females mated to mutant males were significantly correlated (Pearson's $r > 0.9$) for all biological replicates (see Supplementary Table 3), indicating high quantitative reproducibility. For quantification, BGLUC homozygous null accessory glands were compared to BGLUC control accessory glands, and *D. melanogaster* Oregon R female LRTs mated to either a BGLUC homozygous null or BGLUC control were compared.

RNA Sequencing Analysis

Raw sequence reads were processed by trimming ten bases from the five-prime end and quality trimmed from the three-prime end to a minimum quality PHRED score of twenty. Following processing, I mapped reads to the *D. melanogaster* transcriptome (Flybase r6.23) with bowtie2, and extracted read counts and normalized abundances using eXpress (Forster et al., 2013; Langmead & Salzberg, 2012). I removed replicates two and three of female 6hr post-mating to BGLUC mutant males. I removed 6hr post-mating replicates two and three from analysis due to their counts being below threshold. I performed all differential expression analyses in R using the packages EdgeR (Robinson et al., 2010) and RUVseq (Risso et al., 2014). I filtered out samples from the data set with cpm < 2 across at least three replicates, leaving 9255 genes in the female reproductive tract samples and 10523 genes in the male accessory gland samples. I used RUVseq to identify k = 2 additional variables added to the linear model in EdgeR for female reproductive tract samples and k = 1 additional variable added to the linear model in EdgeR for male accessory gland samples (see Supplementary Figure 2) based on the residuals from a linear model fitted with the sample variables, these variables were estimated using RUVseq. Transcript abundances (CPM) between control male accessory gland replicates, mutant male accessory gland replicates, control testes replicates, mutant testes replicates, unmated female LRT replicates, LRT replicates of females 1 hr, 3 hrs, and 6hrs post-mating to control males, and LRT replicates of females 1 hr, 3 hrs, and 6hrs post-mating to mutant males were significantly correlated (Pearson's r >0.9) for all biological replicates (see Supplementary Table 4 and 5), indicating high quantitative reproducibility.

Chapter 3.3 Results and Discussion

Male RNA sequencing results and discussion

Mating assays described in Chapter 2 (Figures 1 C & 2 B) showed that BGLUC mutant males fail to sire offspring, and their mates experience reduced refractoriness to remating. In addition, it was shown that BGLUC mutant males transfer a reduced amount of sperm to the female reproductive tract and that the few transferred sperm fail to enter storage (Figure 3 C). To further understand the molecular phenotypes of BGLUC mutants, I performed RNA sequencing on unmated male accessory glands and testes. In addition, shotgun mass spectrometry with label-free quantification was performed on BGLUC mutant males and control male accessory glands. The combination of these two methods will allow me to identify specific genes that are mis-expressed in BGLUC mutant male accessory glands. BGLUC is a predicted glycosylation protein with activity in complex carbohydrate binding and cleavage. Thus, the ablation of this protein may cause errors in post-translational modification of proteins either in the male accessory glands or possible in the mated female LRT.

RNA sequence analysis of BGLUC mutant and control male accessory glands showed significant differences in abundances for several genes between the two samples (Figure 5). This indicates that the BGLUC knockout mutation is causing the misexpression of these genes' RNA transcripts in addition to *BGLUC* transcripts in the male accessory glands. The genes with greatest transcript abundance in the BGLUC mutant, are *HSPBAP1*, *CG13965*, and *SFP79*. *HSPBAP1* is predicted to enable 2-oxoglutarate-dependent dioxygenase activity and is expressed in spermatozoon (Holowatyj et al., 2015). *HSPBAP1* role in sexual reproduction has yet to be characterized, however expression of this gene in spermatozoon indicates that the greater transcript abundance of this gene may be causing the reduction in sperm transfer observed in

BGLUC mutant males. *CG13965* and *SFP79* are uncharacterized SFPs predicted to be involved in sexual reproduction (Findlay et al., 2008a). The greater transcript abundance of these two SFP genes may have a role in causing the sterility phenotype observed in BGLUC mutant males. Additionally, several other genes had significantly different transcript abundance in the BGLUC mutant male accessory glands (Supplementary Figure 3) and their heightened abundances may also have roles in producing the phenotypic defects observed in BGLUC mutant males.

Several genes had greater transcript abundance in control male accessory glands compared to BGLUC mutant male accessory glands. The genes with the greatest transcript abundance in control male accessory glands were *Cdc7*, *dnd*, and *IncRNA:CR45823*. *Cdc7* is a serine-threonine kinase that phosphorylates components of the pre-replication complex during DNA replication initiation (Stephenson et al., 2015). *dnd* (*dead end*) encodes an Arf-like3 GTPase that controls the targeting of exocytosis machinery to specific apical domains in fusion cells during the tracheal branch fusion process (Jiang et al., 2007). *IncRNA:CR45823* (*long non-coding RNA CR45823*) molecular and biological functions are currently unknown (Nyberg & Machado, 2016). The functions of these genes with elevated transcript abundances in control male accessory glands compared to BGLUC mutant male accessory glands does not provide any indications that their lower abundances in the BGLUC mutant male accessory glands have roles in producing the phenotypic defects observed in mutant males.

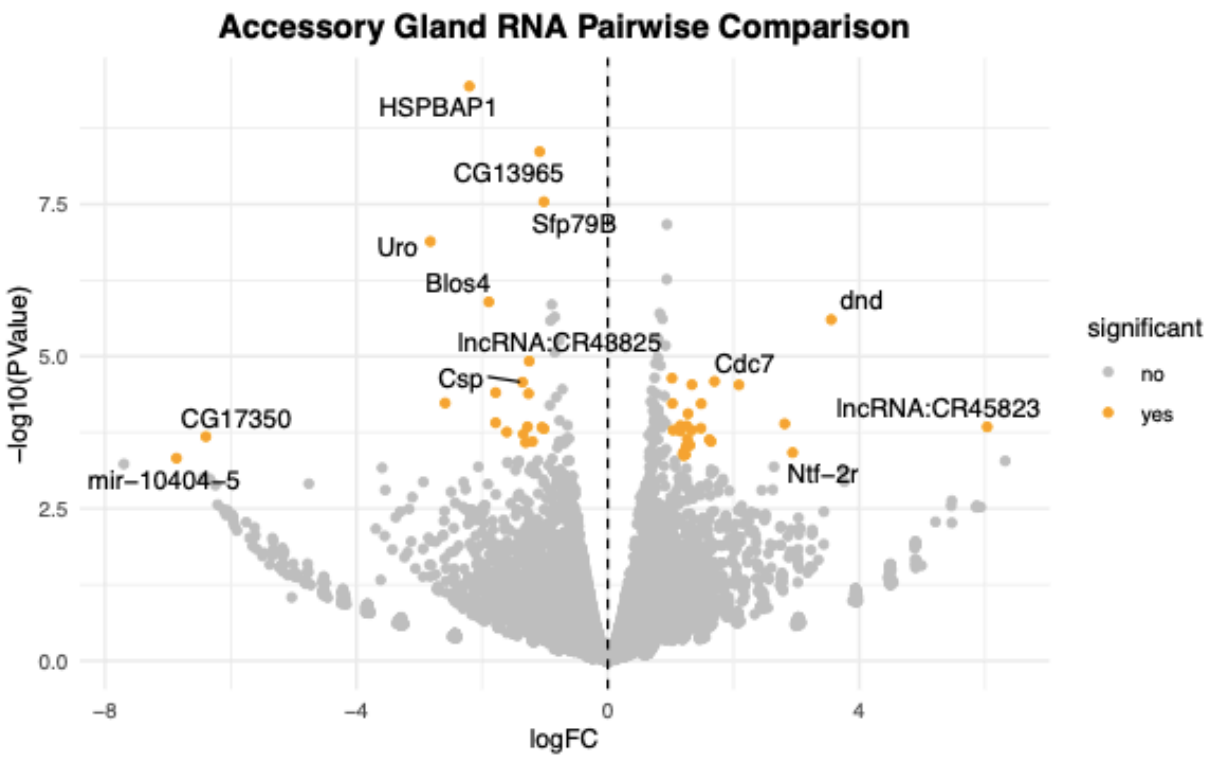


Figure 5. BGLUC mutant and control male accessory gland transcript abundance ratio comparison. The X-axis is the logFC between samples. Genes with a negative logFC have a greater abundance of transcript in BGLUC mutant samples compared control samples. Genes with positive logFC have a greater abundance transcript in control samples than in BGLUC mutant samples. The -log(10) PValue is the Y-axis, with the most significant genes present further up the y-axis. The significant gene cutoff was FDR values being less than 0.05, but genes with logFC > 1 are highlighter in yellow.

RNA sequence analysis of BGLUC mutant and control testes transcript abundances showed significant differences in transcript abundances of only four genes between the two samples (Figure 6). *CG42789* was the only gene with significantly elevated transcript abundance in control testes compared to BGLUC mutant testes (Li et al., 2022). *CG42789* is uncharacterized but highly expressed in male accessory glands with little expression in other tissues. In the BGLUC mutant testes *Tsf1, CG16772*, and *CG4757*, had significantly greater transcript abundances compared to control testes. Tsf1 encodes iron binding in proteins induced during immune responses (Cardoso-Jaime et al., 2022). *CG16772* molecular and biological

functions are currently unknown. *CG4757* enables serine hydrolase activity(Kumar et al., 2021). The results from the testis RNA sequencing show that BGLUC knockout has little effect on transcript abundances in the testes. A complete list of genes with significantly different testes transcript abundances can be found in (Supplementary Table 5).

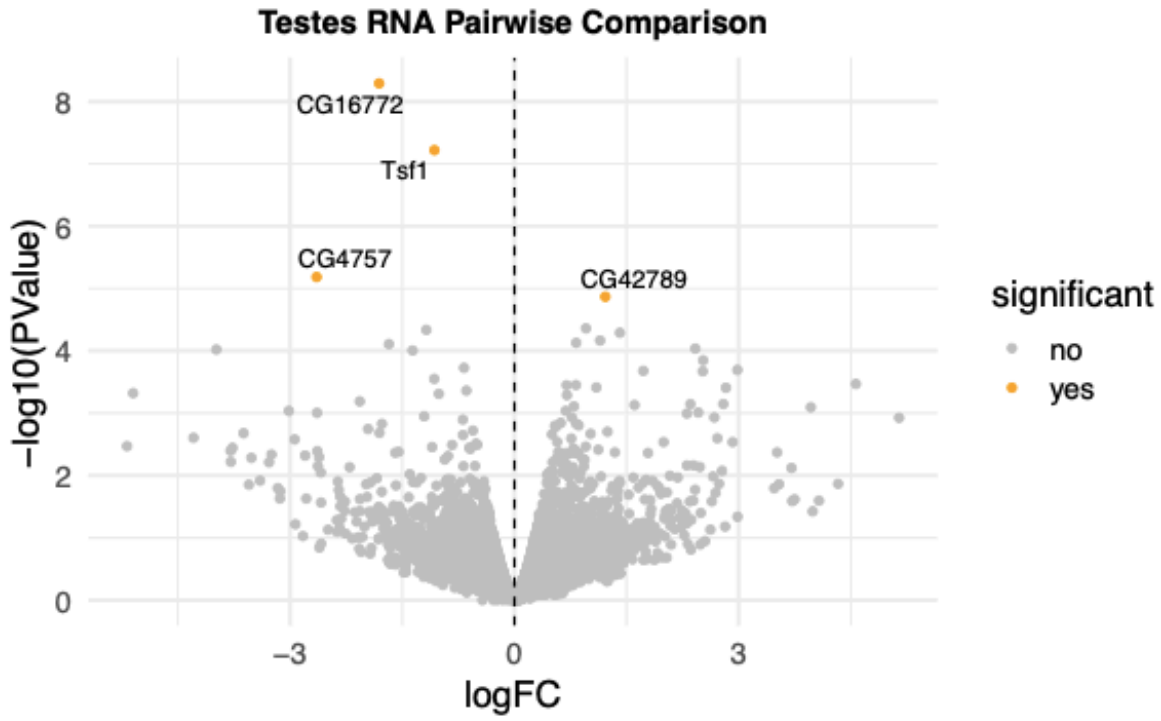


Figure 6. BGLUC mutant and control male testis transcript abundance ratio comparison. The X-axis is the logFC between samples. Genes with a negative logFC have a greater abundance of transcript in BGLUC mutant samples compared to control samples. Genes with positive logFC have a greater abundance of transcript in B control samples than in BGLUC mutant samples. The -log(10) PValue is the Y-axis, with the most significant genes present further up the y-axis. The significant gene cutoff was FDR values being less than 0.05.

Post mating female RNA-Seq

RNA-Seq analysis of female LRTs after mating to either BGLUC mutant or control males show significant transcript abundance differences at one hour, three hours, and six hours post-mating in both sample groups (Figure 7). At the 1hr post-mating time point, transcript abundances are only significantly elevated in control mates for the gene *S-lap* compared to mates

of BGLUC mutant males (Figure 7, 8D). *S-lap* is a component of sperm and is predicted to enable manganese binding and/or ion binding activity (Dorus et al., 2006, 2011; McCullough et al., 2022). At 3hr post-mating transcript abundances are significantly elevated in control mates for several genes compared to mates of BGLUC mutant males. Three of these genes, *CecB, CecC, and Rel*, are involved in the immune response (Figure 7, 8A). The increased abundance of immune gene transcript in the LRT of females 3hrs following mating BGLUC mutant males is consistent with previous findings in several *Drosophila* species (Schnakenberg et al., 2011) where it was shown that postmating transcriptome-level response is determined in part by the genotype of the male. In this study, immune genes' transcript abundances were higher in female LRTs 3hrs after heterospecific mating than in female LRTs 3hrs after conspecific matings.

At 6hr post-mating transcript abundances are elevated in BGLUC mutant mates for several genes compared to mates of control males. This is inversely related to transcript abundance ratios of females at the two earlier post-mating time points. Two of these genes are *CG34227*, which is up in transcript abundances in control mated female reproductive tracts at 6 hrs post-mating (Figure 8 C), and *CG3604*, which is up in transcript abundances in BGLUC mutant mated FRTs six-hours post-mating. Both genes have yet to be characterized. However, this may be a result of there only being a single replicate used in analysis of LRTs from females mated to BGLUC mutant males at the six-hour post-mating time point. Complete lists of significant genes that have increased transcript abundances in the one-hour, three-hour, and or six-hour post-mating time points can be found in (Supplementary Table 3).

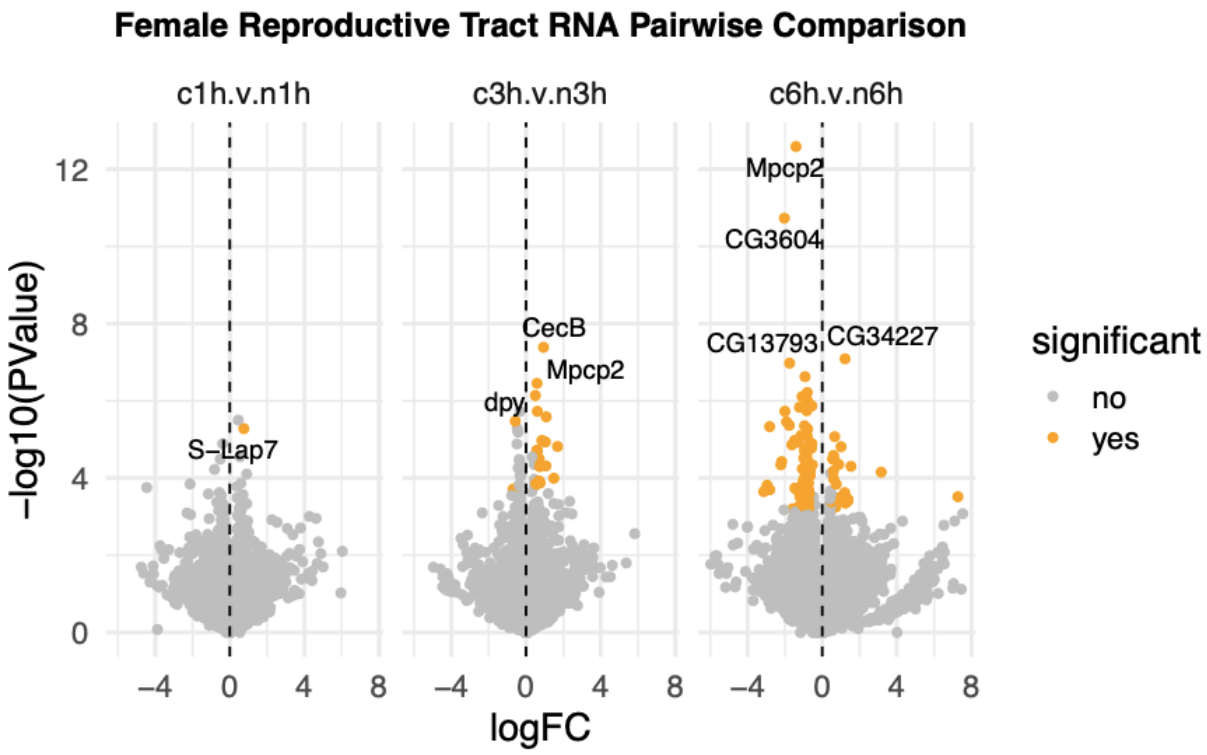


Figure 7. Post-mating female reproductive tract transcript abundance ratio time series. Post-mating female samples at the same post-mating time points were compared based on male genotype. The X-axis is the logFC between samples. Genes with a negative logFC have a greater abundance of transcript in BGLUC mutant samples compared to control samples. Genes with positive logFC have a greater abundance of transcript in control samples than in BGLUC mutant samples. The significant gene cutoff was FDR values being less than 0.05. The -log(10) PValue is the Y-axis, with the most significant genes present further up the y-axis.

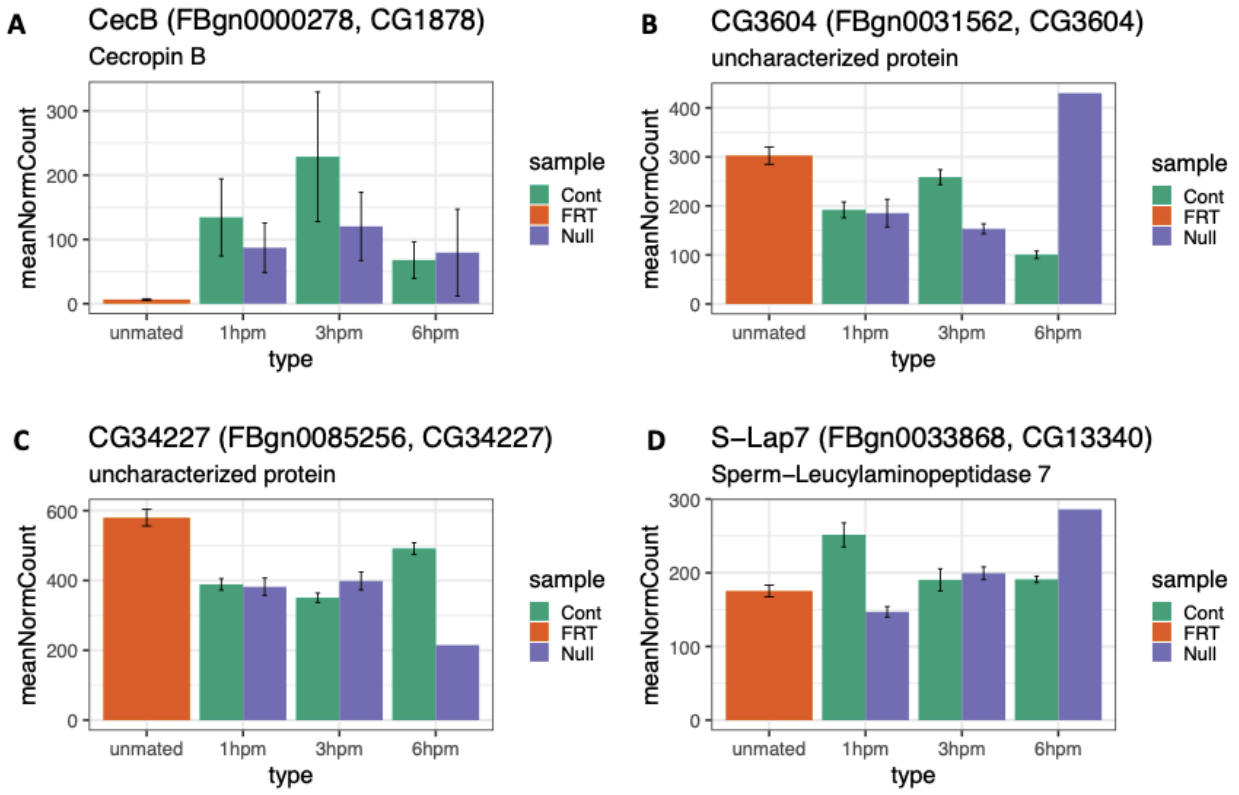


Figure 8. Single gene transcript abundance in female reproductive tracts post-mating to BGLUC mutant males control males. Orange bars represent unmated female LRTs, green bars represent LRTs from females mated to control males, and purple bars represent LRTs from females mated to BGLUC mutant males. (A) Single gene plot of *CecB*. (B) Single gene plot of *CG3604*. (C) Single gene plot of *CG4227*. (D) Single gene plot of *S-Lap7*.

RNA sequence analysis of LRTs of females following mating to either BGLUC mutant or control males compared to unmated females showed significant transcript abundance differences of genes at 1hr, 3hrs, and 6hrs post-mating (Figure 9). At all three post-mating time points, females mated to either BGLUC mutant or control males had significantly elevated transcript abundance ratios of the same genes compared to unmated females at the same post-mating time points. Notably, although there was only one replicate of female LRTs following mating to BGLUC mutant males the same genes had significantly elevated transcript abundance ratios compared to unmated females. This supports the accuracy of this singular replicate.

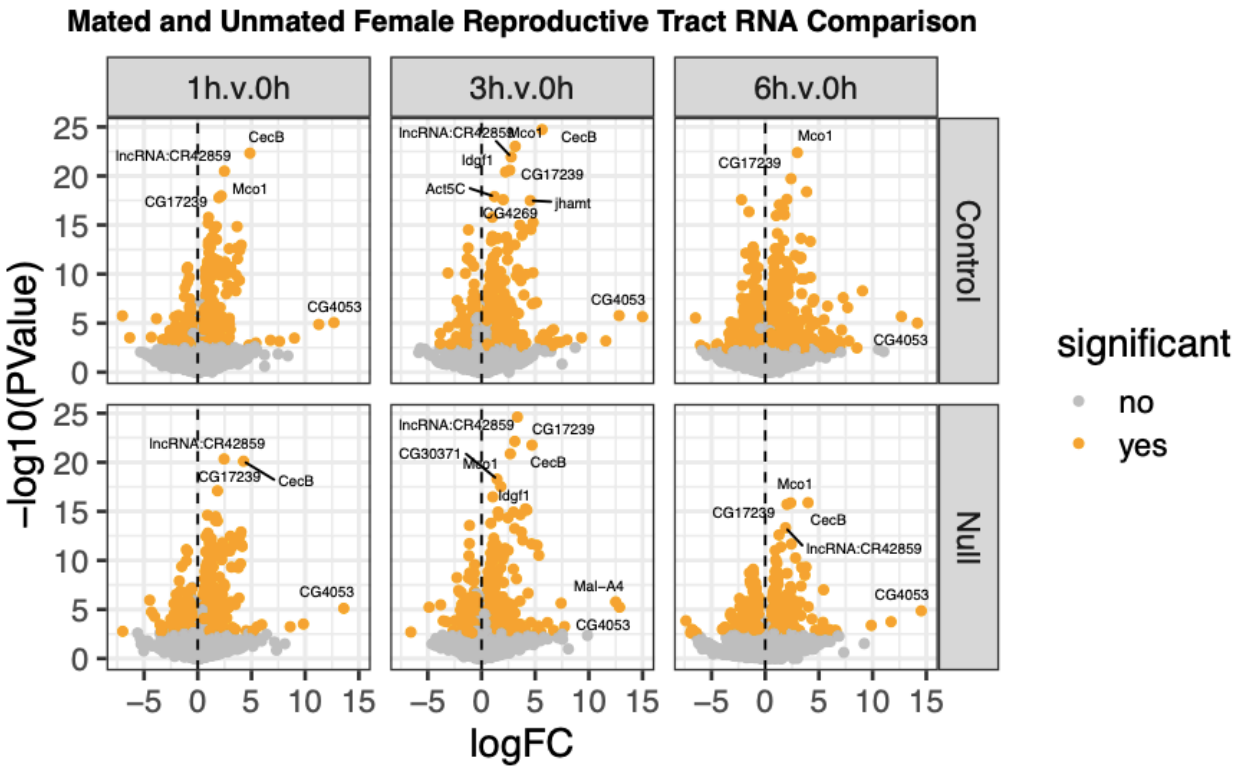


Figure 9. Female LTR post-mating vs unmated transcript abundance ratio. X-axis is the log fold change between samples, y-axis is the -log10(Pvalue) between sample, orange represents genes with significantly different (Pvalue < 0.05) transcript abundance between samples. 0h represents the unmated female samples. 1h, 3h, and 6h represents post-mating time point of female samples Columns from left to right compare 1hr post-mating lower FRTs to unmated lower FRTs, three hours post-mating lower FRTs to unmated lower FRTs, and six hours post-mating lower FRTs to unmated lower FRTs. The top row is females mated to control males while the bottom row is females mated to BGLUC mutant males.

Comparing transcript abundances in females mated to BGLUC mutants 1hr post-mating to unmated females, gene ontology enrichment shows enrichment genes in the significant set involved in immune response (Figure. 10). This was expected since upregulation of immune genes occurs in females after mating (Fedorka et al., 2007; Kapelnikov et al., 2008). Gene ontology enrichment at 3hrs post-mating also shows significant enrichment for genes having roles immune response (Figure 11). The enrichment of genes having roles in immune response in female LRT samples following mating to BGLUC mutant males may be experiencing this enrichment as a response to the mutant male genotype. Comparing gene transcript abundances in

BGLUC mutant mated female LRTs 6 hrs post-mating to unmated female LRTs, gene ontology enrichment shows significant enrichment for genes having roles in cellular amino acid metabolic process and defense response to gram-negative bacterium (Figure. 12).

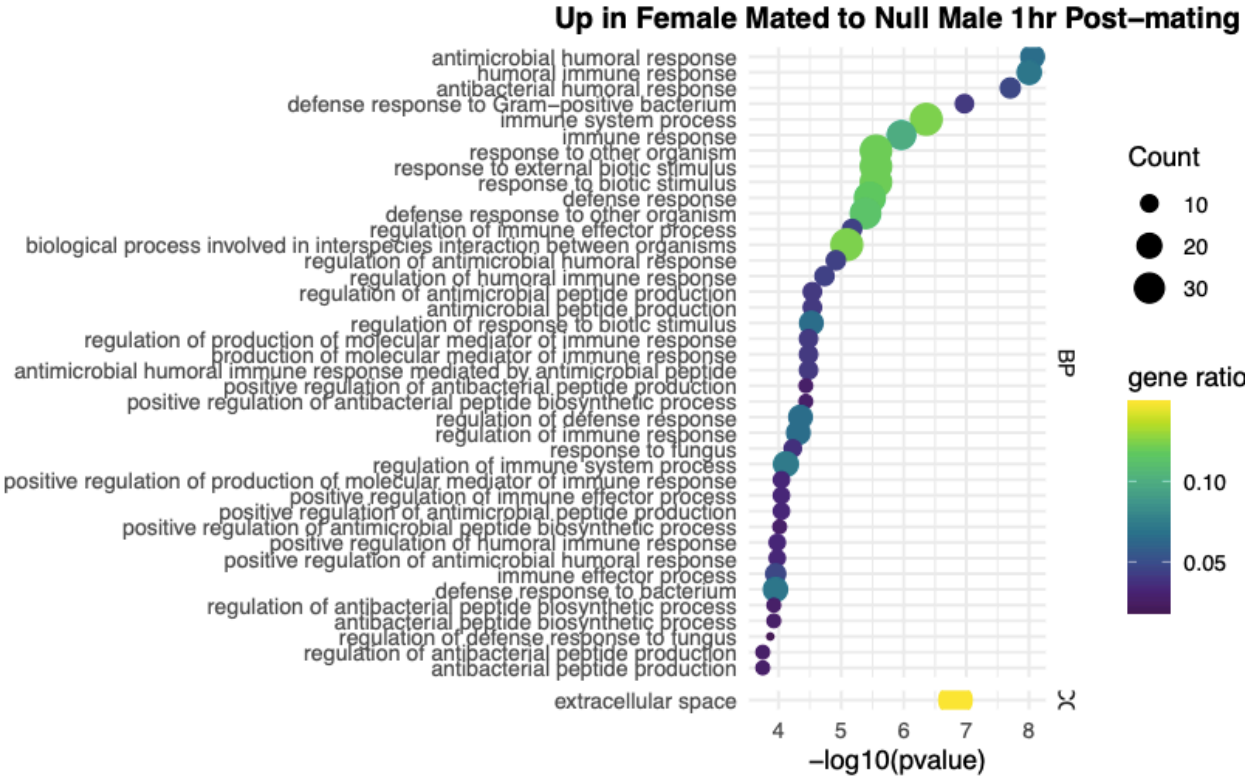


Figure 10. Female reproductive tracts one-hour post-mating to BGLUC mutant males gene ontology enrichment. The most significantly elevated gene ratio is represented by lighter colors (yellow and green) while darker colors (blue and purple) represent less significantly elevated gene ratios. Counts of genes are represented by the size of the dot.

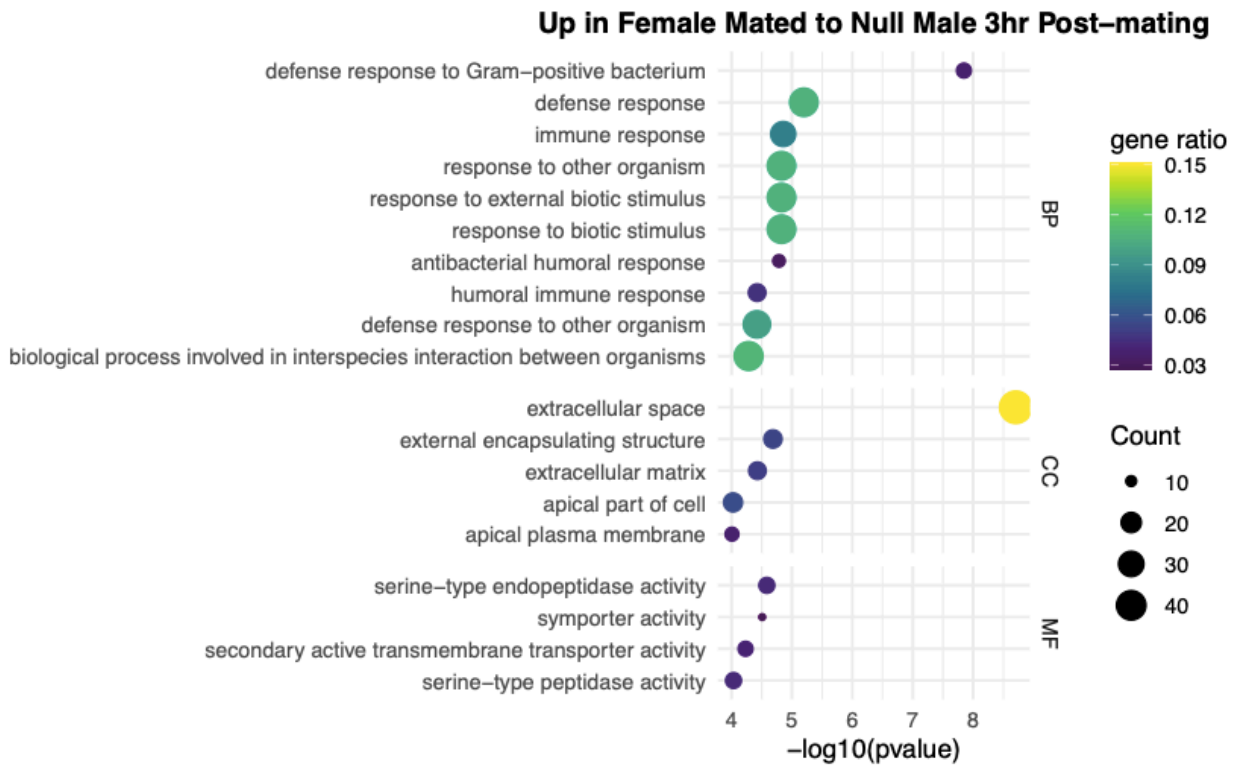


Figure 11. Female reproductive tracts three hours post-mating to BGLUC mutant males gene ontology enrichment. The most significantly elevated gene ratio is represented by lighter colors (yellow and green) while darker colors (blue and purple) represent less significantly elevated gene ratios. Counts of genes are represented by the size of the dot.

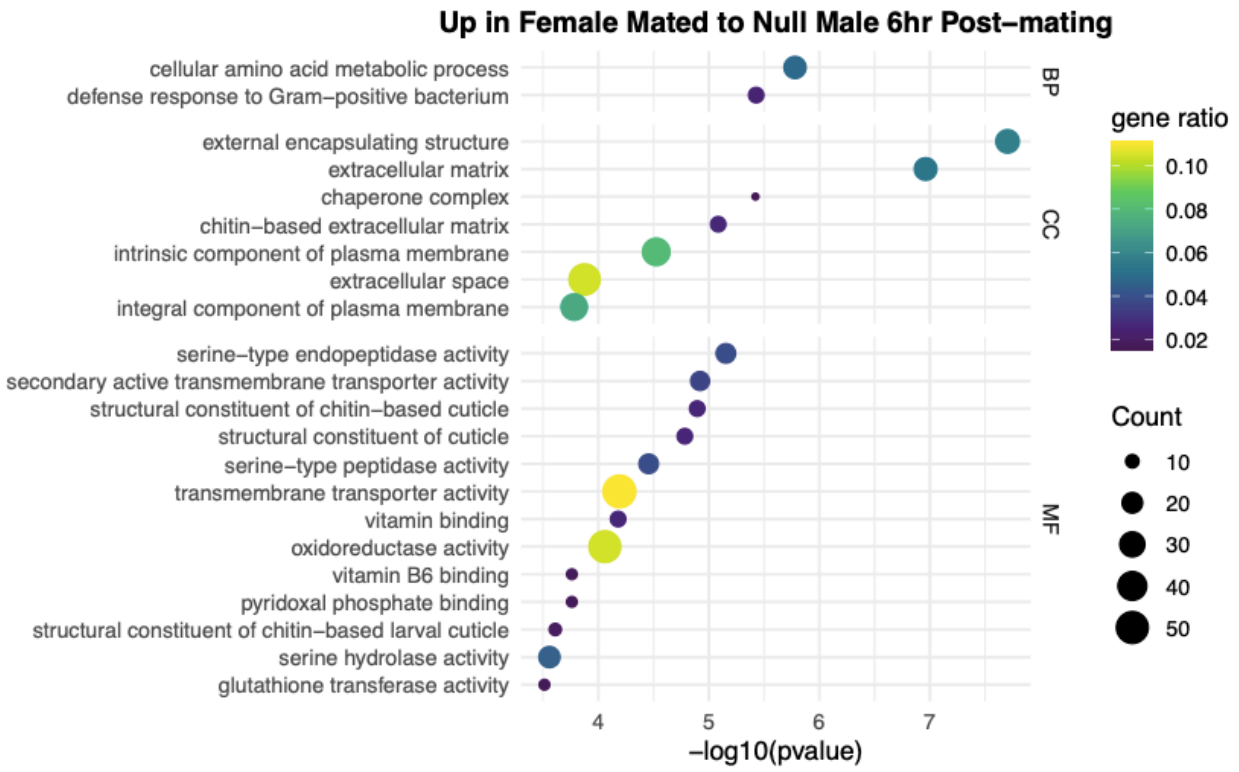


Figure 12. Female reproductive tracts six hours six hours post-mating to BGLUC mutant males gene ontology enrichment. The most significantly elevated gene ratio is represented by lighter colors (yellow and green) while darker colors (blue and purple) represent less significantly elevated gene ratios. Counts of genes are represented by the size of the dot.

Male proteomic analysis

Proteomic analysis of unmated BGLUC mutant and control male accessory glands allowed for the relative quantification of the accessory gland proteome. By comparing protein abundances between the two male genotypes, proteins that differ in abundance compared to control males can be identified in the BGLUC mutant male samples. Identifying these differences might aid in understanding BGLUC's role in male fertility. Several non-SFP abundances were shown to differ between male genotypes (Figure 13). Gene ontology analysis of non-SFP significantly abundant in BGLUC mutant male accessory glands showed enrichment for genes having function in the biological process of oxidoreductase activity (Supplementary

Figure 5). Oxidoreductase enzymes are essential in a number of metabolic pathways (Legesse Habte & Assefa Beyene, 2021). Gene ontology analysis of non-SFP significantly abundant in control male accessory glands showed enrichment for genes having functions in the biological process of reactive oxygen metabolic processes (ROS) (Supplementary Figure 5). ROS known for their role in mediating both physiological and pathophysiological signal transduction and compartments that typically produce ROS are associated with metabolic regulation (Forrester et al., 2018). The absence of BGLUC protein in mutant male accessory glands is likely resulting in the misregulation of protein abundance of genes associated with this function in metabolic pathways. This misregulation of proteins having roles in metabolic pathways may be causing the reduction in sperm transfer to females during mating observed in BGLUC mutant males (Figures 3 D and C). There appears to be no bias towards one male genotype of increased non-SFP protein abundances between the male samples. This data shows there is overall misexpression of non-SFPs in the BGLUC mutant male accessory glands compared to control male accessory glands. A list of non-SFP genes with the most significantly different protein abundances between male genotypes can be found in (Supplementary Table 9).

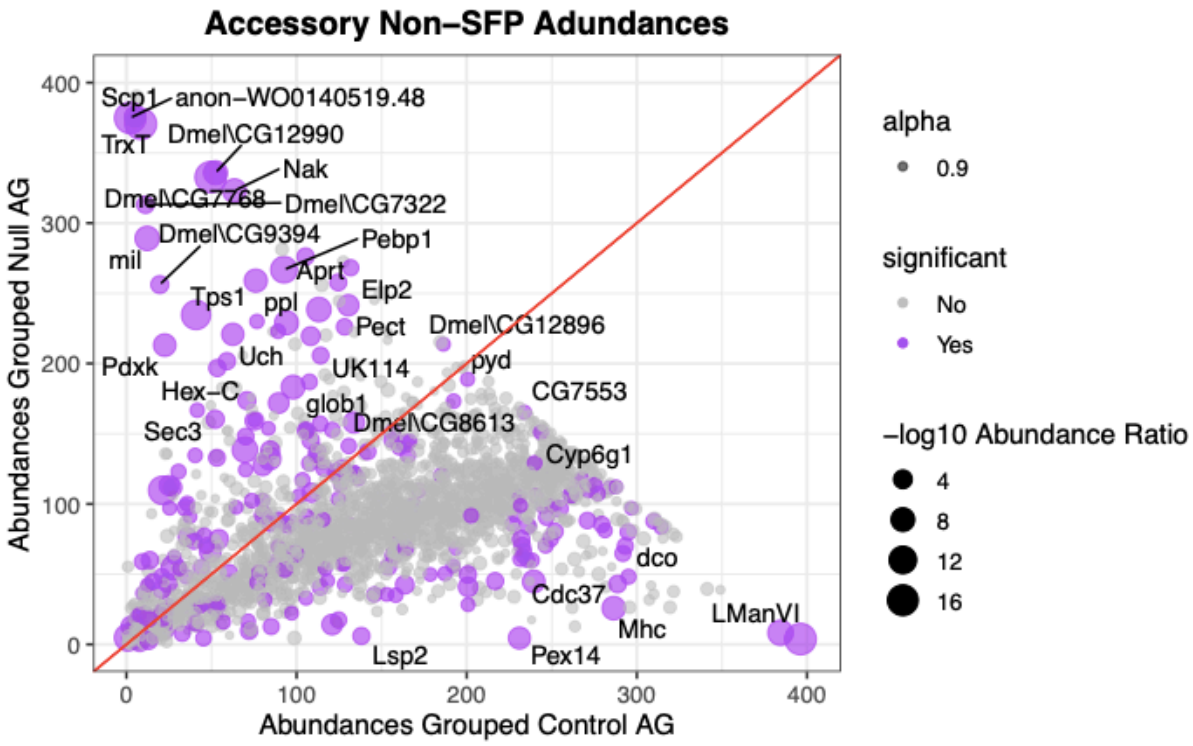


Figure 13. Comparison of BGLUC mutant and control accessory gland protein abundances excluding SFPs. The red line indicates an equal abundance ratio. Significant genes are purple, and non-significant genes are gray. The size of the dot indicated -log10 abundance ratio between the two samples.

Regarding differences in SFP NSAFs grouped abundances between the male accessory gland samples, there are more SFP genes with significantly increased protein abundance in BGLUC control accessory glands than in BGLUC homozygous null accessory glands (Figure 14). Of these SFP genes with protein abundance elevated in BGLUC control samples, several have been shown to have significant roles in reproduction, but the majority are yet to be characterized. *Acp36DE*, *Acp62F*, and *Acp70A* are some of the SFPs with the most significant differences in abundance between the male samples (Figure 15 B-D). *Acp36DE* has reduced protein abundance in BGLUC homozygous null male accessory glands compared to BGLUC control male accessory glands. *Acp36DE* is a glycoprotein produced in male accessory glands and is transferred to females during mating and also causes changes in the female bursa, which

assists the female in moving sperm into sperm storage organs (Avila & Wolfner, 2009, 2017; Bertram et al., 1996). If BGLUC is responsible for the posttranslational modification of *Acp36DE*, the absence of BGLUC in mutant males may result in reduced protein abundances of *Acp36DE*. Provided BGLUC homozygous null males fail to store sperm, the reduced amount of *Acp36DE* protein in mutant male accessory glands may be a likely target causing this sperm storage defect.

Acp62F has reduced protein abundance in BGLUC homozygous null male accessory glands compared to BGLUC control male accessory glands. *Acp62F* encodes a trypsin inhibitor synthesized in male accessory glands, is transferred to females during mating and influences sperm storage in mated females (Lung et al., 2002). In addition to *Acp36DE*, a reduced amount of *Acp62F* protein in mutant male accessory glands may additionally be influencing the sperm storage defect seen in mates of BGLUC mutant males.

Acp70A has increased protein abundance in BGLUC mutant male accessory glands compared to control male accessory glands. *Acp70A* (famously known as Sex peptide, SP) encodes a small peptide produced by the male accessory glands. In mated females, it induces post-mating responses, including increased egg production, decreased mating receptivity, female longevity, and sperm release from storage (T. Chapman et al., 2003). In contrast to what I expected based on BGLUC mutant males' fertility defects, SP abundances are higher in these males' accessory glands.

BGLUC protein had the most significant reduction in protein abundance in BGLUC mutant male accessory glands compared to control male accessory glands (Figure 15 A). However, BGLUC protein was still present in BGLUC mutant male accessory glands, indicating that there was BGLUC heterozygous null male contamination in the accessory gland samples

despite efforts to screen for these males. The presence of these males in samples suggests that many of these protein abundance differences would be more extreme, or there would be no protein detected for several genes if there were no contamination. A complete list of SFP genes with significantly different protein abundances between male genotypes can be found in (Supplementary Table 8).

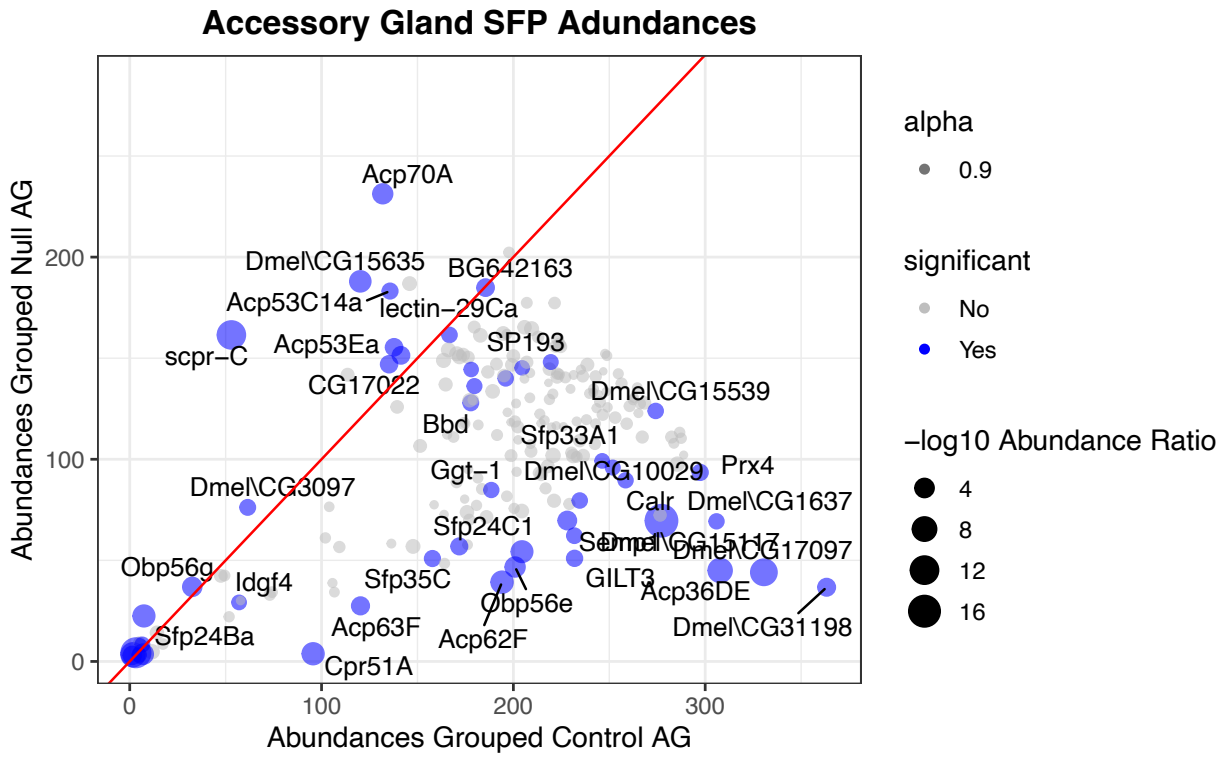


Figure 14. Comparison of BGLUC mutant and control male accessory gland SFP abundances. The red line indicates an equal abundance ratio. Significant genes are purple, and non-significant genes are gray. The size of the dot indicated -log10 abundance ratio between the two samples. There is an increased occurrence of SFPs with greater abundances in the control samples compared to BGLUC mutant samples.

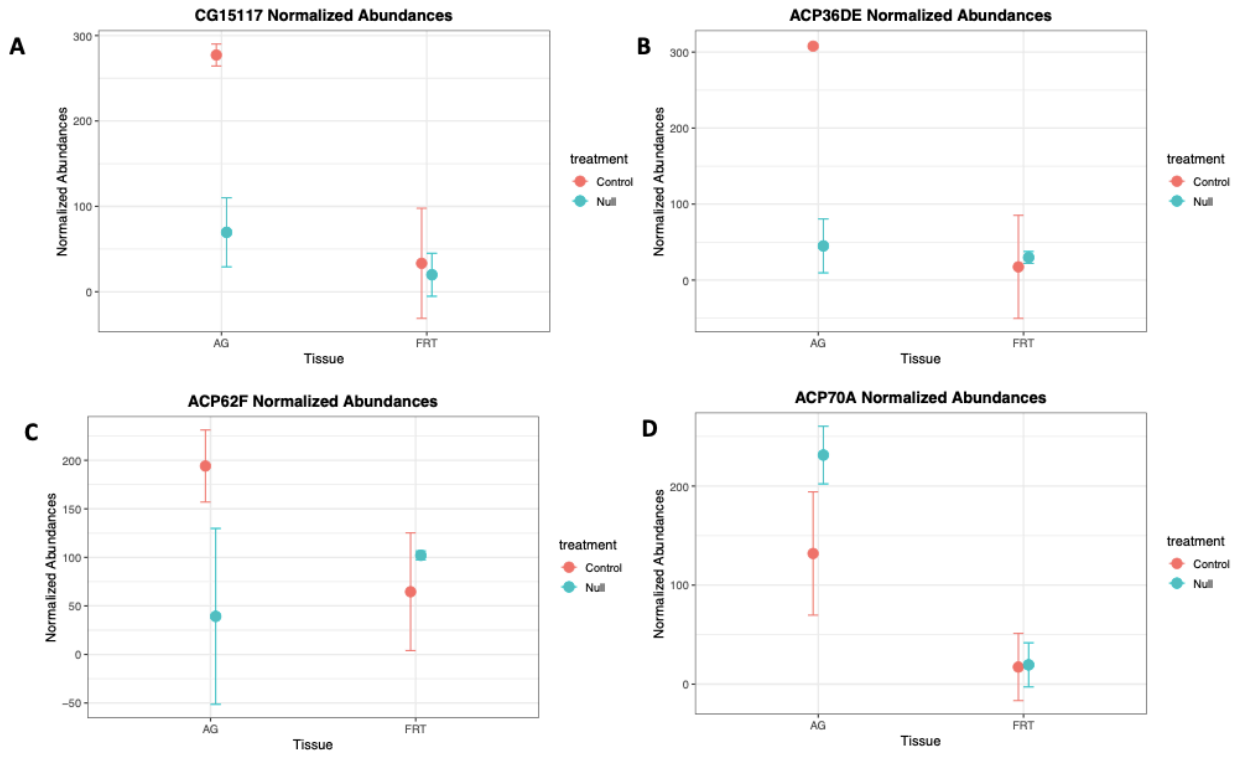


Figure 15. Normalized protein abundances in BGLUC mutant and control accessory glands and female reproductive tracts immediately following mating to either BGLUC mutant or control males. (A) CG15117 (BGLUC) normalized protein abundance is significantly reduced in BGLUC mutant male accessory glands compared to B control male accessory glands. (B) *Acp36DE* normalized protein abundance is significantly reduced in BGLUC mutant male accessory glands compared to control male accessory glands. (C) *Acp62F* normalized protein abundance is significantly reduced in BGLUC mutant male accessory glands compared to control male accessory glands. (D) *Acp70* (sex-peptide) normalized protein abundance significantly increases in BGLUC mutant male accessory glands compared to control male accessory glands

Post-mating female proteomic analysis

Proteomic analysis of female LRTs immediately following mating to either BGLUC mutant or control males allowed for quantifying male SFPs transferred to the female during mating and female protein abundances after mating. Quantifying SFPs in the female LRT allowed for the identification of SFPs that may be at typical abundance in the male accessory gland yet fail to be transferred to the female during mating. Quantifying non-SFPs in the female LRT allows for the identification of female proteins that are misexpressed following mating to BGLUC mutant males.

Many non-SFP abundances differed between lower female reproductive tracts of females mated by either male genotype (Figure 16). Gene ontology analysis of non-SFP significantly abundant in the LRTs of females mated by BGLUC mutant males showed enrichment for genes having functions in the biological processes of transmembrane transport, organic acid transport and anion transport. Gene ontology analysis of non-SFP significantly abundant in the LRTs of females mated by control males showed enrichment for genes having products active in the cellular components of the Golgi medial cisterna and integral component Golgi membrane. The Golgi apparatus has a central roles in post-translational modification and in the secretion of membrane and secretory proteins (Yamamoto-Hino et al., 2012). Given BGLUC is a glycosylation protein (Baycin-Hizal et al., 2011), the enrichment of genes with products active in in the cellular components of the Golgi in LRTs of females mated by control males suggests that the absence of BGLUC in mates of mutant males causes reduced protein abundance of gene with glycosylation-related proteins. Reductions in glycosylation-related proteins in females may be causing the sperm storage defect observed in mates of BGLUC males as errors in posttranslational modification of proteins involved in the sperm storage in the female may be occurring. A list of non-SFP genes with the most significantly different protein abundances between male genotypes can be found in (Supplementary Table 7).

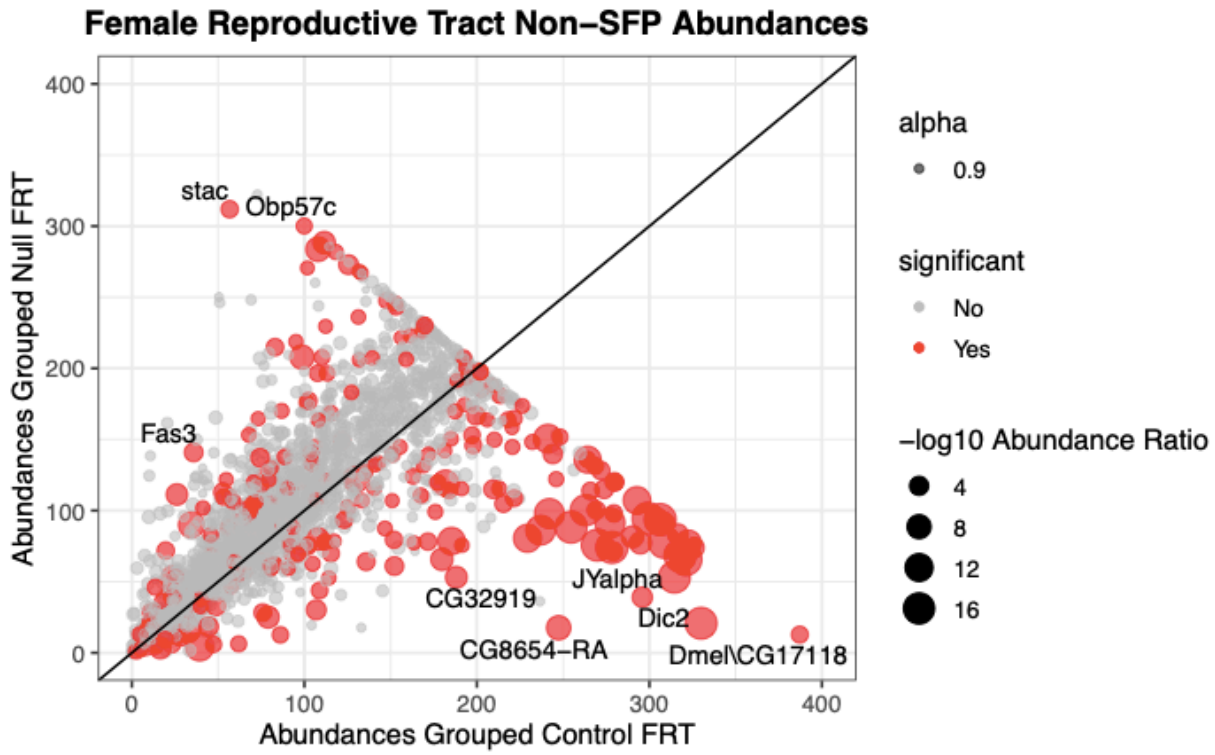


Figure 16. Comparison of protein abundances excluding SFPs in female reproductive tracts immediately following mating of females to BGLUC mutant or control males. The black line indicates an equal abundance ratio. Significant genes are red, and non-significant genes are gray. The size of the dot indicated -log10 abundance ratio between the two samples.

BGLUC abundance did not significantly differ between LRTs of females mated by either male genotype, however several other SFPs abundances were significantly different between samples (Figure 17). BGLUC abundance was detected in low amounts in LRTs of females mated by BGLUC mutant and control males (Figure 15A). This may be the result of a combination of low abundance of BGLUC that is present in the female LRT before mating and an indication that BGLUC is transferred to female in low amounts. Of the SFPs that had the most significant differences between the female LRT samples are *Acp36DE* and *EBPII*. *Acp36DE,* as previously described, has a considerable influence on male fertility. The significant *Acp36DE* protein abundance decreased in mates of BGLUC mutant males compared to control mates suggests that not only is it reduced in abundance in mutant male accessory

glands, but it is either restricted in transfer to the female. *EbpII* (ejaculatory bulb protein II) is a significant component of the posterior mating plug (Avila et al., 2015). It shows decreased abundance in the LRTs of females mated to BGLUC mutant males compared to BGLUC control male mates. No defects in the mating plug formation are seen in (Figure 3 C), however, further analysis may be required. A complete list of non-SFP genes with significantly different protein abundances between male genotypes can be found in (Supplementary Table 6).

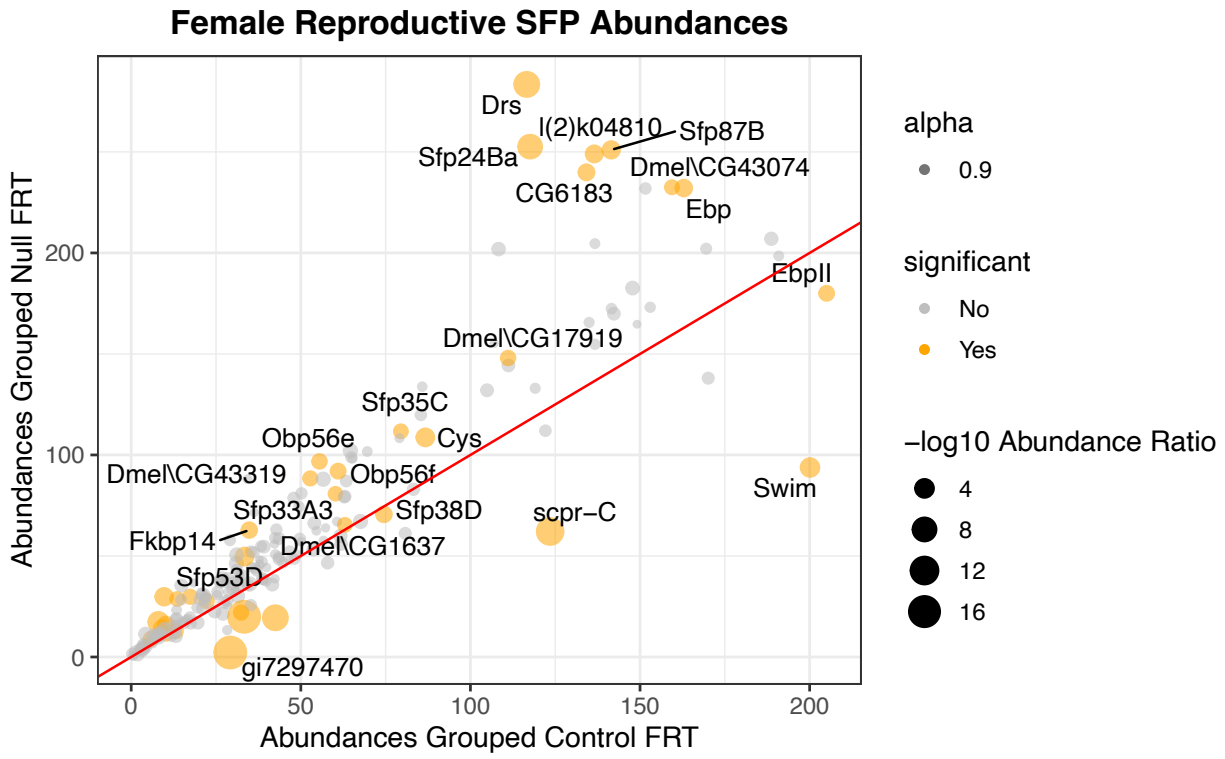


Figure 17. Comparison of SFP abundances in female reproductive tracts immediately following mating of females to BGLUC mutant or control males. The red line indicates an equal abundance ratio. Significant genes are orange, and non-significant genes are gray. The size of the dot indicated -log10 abundance ratio between the two samples.

Lastly, proteomic analysis of LRTs immediately following mating to either BGLUC mutant or control males allowed for the quantification of male sperm proteins. Imaging of GFP sperm of BGLUC mutant males within the female reproductive tract showed very few sperm

(Figure 3C). Through quantification of sperm proteins in females following mating, quantities of sperm transferred are indicated. 48 sperm proteins have protein abundance greater in the LRTs of females mated to control males than in the LRTs of females mated to BGLUC mutant males. While only 11 sperm proteins had greater abundances in LRTs of females mated to BGLUC mutant males compared to LRTs of females mated to control males (Figure 18). This indication of reduced sperm abundance in mates of BGLUC mutant mates further supports that BGLUC is involved in sperm transfer and retention.

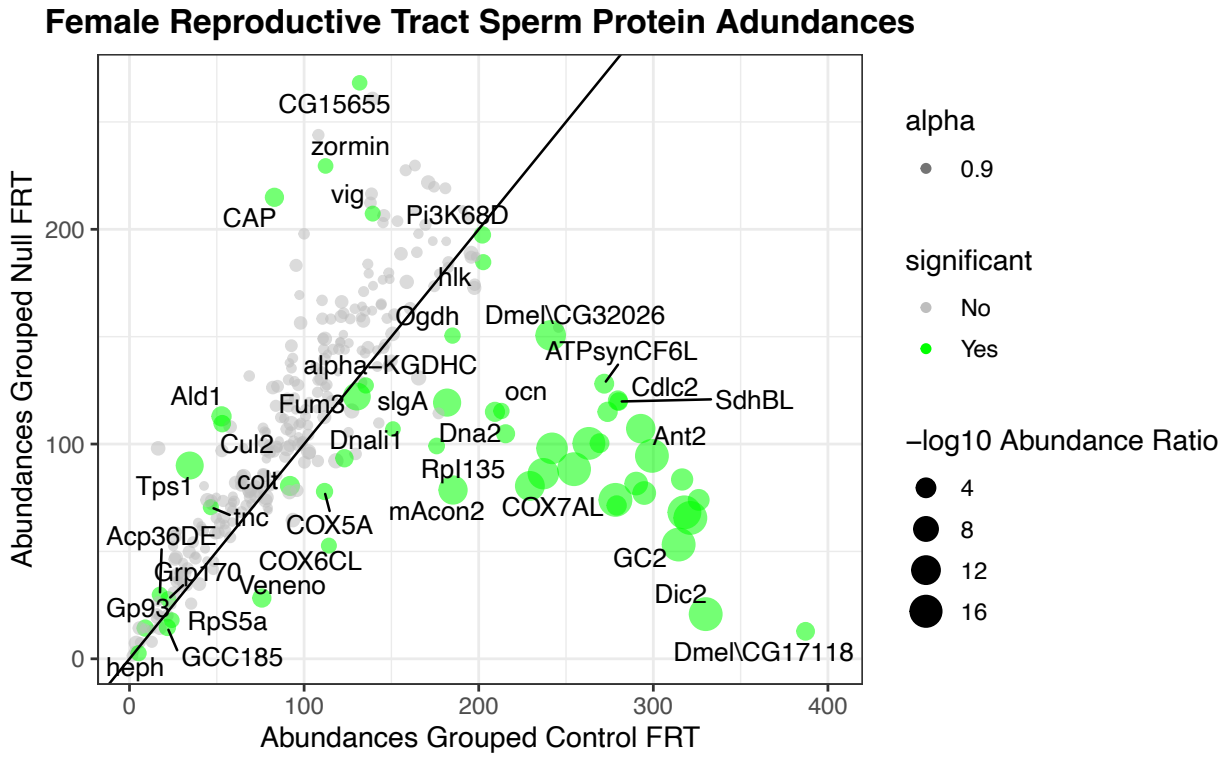


Figure 18. Comparison of sperm protein abundances in female reproductive tracts immediately following mating of females to BGLUC mutant or control males. The black line indicates an equal abundance ratio. Significant genes are red, and non-significant genes are gray. The size of the dot indicated -log10 abundance ratio between the two samples.

Chapter 3.4 Conclusions

Analysis of transcript and protein abundances in the accessory glands of BGLUC mutant males, as well as LRTs of females mated to BGLUC mutant males, indicated overall misexpression of several genes that have significant roles in sexual reproduction in *Drosophila*. Transcript abundances in BGLUC mutant male accessory glands showed significant misexpression of many genes, with several having roles in reproduction. In the LRTs of females mated to BGLUC mutant males, there was increased transcript abundance for genes related to immune response at all three post-mating time points. This suggests that males lacking BGLUC protein coding sequence may trigger an increased immune response in females post-mating compared to females.

Gene ontology analysis of non-SFP significantly abundant in control male accessory glands showed enrichment for genes having functions in reactive oxygen metabolic processes (ROS). ROS processes have known roles in mediating both physiological and pathophysiological signal transduction and compartments that typically produce ROS are associated with metabolic regulation (Forrester et al., 2018). This misregulation of proteins having roles in metabolic pathways may be causing the reduction in sperm transfer to females during mating observed in BGLUC mutant males.

Additionally, protein abundances of several SFP genes were found to be significantly decreased in BGLUC mutant male accessory glands compared to control male accessory glands as well as in the LRTs of females mated to BGLUC mutant males compared to LRTs of females mated to control males. Among these genes, several have significant roles in sexual reproduction. Two of these genes, *Acp36DE* and *Acp62F*, have been characterized as having significant roles in influencing sperm storage in females and are likely contributing to the sperm

storage defects observed in (Figure 3 C). Neither shows significant transcript abundance differences in either male accessory gland or post-mating female LRT comparisons, therefore BGLUC probably plays a role in the posttranslational modification of these genes or intermediate glycosylation targets. Lastly, gene ontology analysis of non-SFP significantly abundant in the LRTs of females mated by control males showed enrichment for genes having products active in the cellular components of the Golgi medial cisterna and integral component Golgi membrane. The Golgi apparatus has central roles in post-translational modification and in the secretion of membrane and secretory proteins (Yamamoto-Hino et al., 2012). Reductions in glycosylation-related proteins in females are likely causing the sperm storage defects observed in mates of BGLUC males for errors in posttranslational modification of proteins involved in the sperm storage in the female may be occurring.

Chapter 3.5 Future Directions

Several aspects of BGLUC's role in sexual reproduction need to be further explored to characterize its role fully. Given (1) the absence of sperm in storage organs of BGLUC mutant mates, (2) proteomic data showing reductions in *Acp36DE* in null male accessory glands and, (3) *Acp36DE*'s role in stimulating conformational changes in the bursa that facilitate sperm entry into storage. Female bursae need to be imaged and measured for these conformational changes. Imaging at time points closer to the start of copulation, as done in (Avila & Wolfner, 2009), will provide evidence if the absence of BGLUC results in the failure of these conformational changes to occur. Lastly, it is still unknown as to what is causing BGLUC mutant males to transfer very few sperm during copulation. To better understand possible causes for this phenotype, imaging of the anterior and posterior ejaculatory duct may provide insight as to if there are any blockages or defects that may be causing reduced sperm transfer.

Supplementary Tables and Figures

Species 1	Species 2	Ka/Ks	Protein Percent ID
D.mel	*D.sec*	0.0363	98.51
D.mel	*D.sim*	0.03902	98.66
D.mel	*D.ere*	0.04482	97.46
D.mel	*D.yak*	0.03633	97.46

Supplementary Table 1. Pairwise Ka/Ks ratios and protein percent identity of BGLUC between five species of the *Drosophila melanogaster* species group.

Species	D.mel Protein Percent I.D.
Caenorhabditis elegans	39.9
Mus musculus	44.1
Danio rerio	44.4
Homo sapiens	44.6
Scaptodrosophila lebanonensis	77.3
Drosophila busckii	76.1
Drosophila virilis	77.7
Drosophila innubila	77.9
Drosophila pseudoobscura	82.5
Drosophila obscura	83.1
Drosophila suzukii	93.6
Drosophila yakuba	97.5
Drosophila teissieri	97.6

Supplementary Table 2. Protein percent identity of BGLUC between various organisms and *Drosophila melanogaster*.

Sample	Cont_Ag_1	Cont_Ag_2	Null_Ag_1	Null_Ag_2	Cont_Frt_1
Cont_Ag_1	1	0.9464295	0.8376347	0.8330395	0.3204388
Cont_Ag_2	0.9464295	1	0.9196167	0.9211648	0.3270342
Null_Ag_1	0.8376347	0.9196167	1	0.9908728	0.3470227
Null_Ag_2	0.8330395	0.9211648	0.9908728	1	0.3427455
Cont_Frt_1	0.3204388	0.3270342	0.3470227	0.3427455	1
Cont_Frt_2	0.3192551	0.326283	0.3436882	0.3404131	0.9950128
Cont_Frt_3	0.339179	0.3430012	0.3617537	0.3559677	0.9933733
Null_Frt_1	0.3571187	0.3589141	0.3766085	0.371607	0.991838
Null_Frt_2	0.3661924	0.3619713	0.3683519	0.3658194	0.989443
Null_Frt_3	0.3202639	0.3198796	0.3288045	0.3263144	0.996655
Sample	Cont_Frt_2	Cont_Frt_3	Null_Frt_1	Null_Frt_2	Null_Frt_3
Cont_Ag_1	0.3192551	0.339179	0.3571187	0.3661924	0.3202639
Cont_Ag_2	0.326283	0.3430012	0.3589141	0.3619713	0.3198796
Null_Ag_1	0.3436882	0.3617537	0.3766085	0.3683519	0.3288045
Null_Ag_2	0.3404131	0.3559677	0.371607	0.3658194	0.3263144
Cont_Frt_1	0.9950128	0.9933733	0.991838	0.989443	0.996655
Cont_Frt_2	1	0.9933056	0.9913226	0.9816955	0.9935573
Cont_Frt_3	0.9933056	1	0.9918259	0.9832079	0.9913048
Null_Frt_1	0.9913226	0.9918259	1	0.9869719	0.9890111
Null_Frt_2	0.9816955	0.9832079	0.9869719	1	0.9914229
Null_Frt_3	0.9935573	0.9913048	0.9890111	0.9914229	1

Supplementary Table 3. Proteomics Pearson's correlation analysis between biological replicates in male and female reproductive tissues. *r* values > 0.9 indicate high quantitative reproducibility.

Sample	Cont_AG_1	Cont_AG_2	Cont_AG_3	Null_AG_1	Null_AG_2	Null_AG_3
Cont_AG_1	1	0.99665443	0.99386028	0.98818021	0.99370961	0.99005896
Cont_AG_2	0.99665443	1	0.99270352	0.98413043	0.99398814	0.99061664
Cont_AG_3	0.99386028	0.99270352	1	0.99715407	0.99852081	0.99771992
Null_AG_1	0.98818021	0.98413043	0.99715407	1	0.99615751	0.99652303
Null_AG_2	0.99370961	0.99398814	0.99852081	0.99615751	1	0.99943733
Null_AG_3	0.99005896	0.99061664	0.99771992	0.99652303	0.99943733	1
Cont_testes_1	0.20548201	0.20019514	0.2063847	0.20264593	0.2030579	0.20190732
Cont_testes_2	0.07826701	0.07551219	0.07849566	0.07477292	0.07692767	0.07593623
Cont_testes_3	0.13108197	0.12660829	0.13150126	0.12683798	0.12885403	0.12713811
Null_testes_1	0.09664036	0.09329982	0.09690848	0.09244182	0.09530082	0.09392506
Null_testes_2	0.10177213	0.0987255	0.10248077	0.09806074	0.10079365	0.09955389
Null_testes_3	0.15082361	0.14645411	0.15140608	0.14728272	0.14877546	0.14715705
Sample	Cont_testes_1	Cont_testes_2	Cont_testes_3	Null_testes_1	Null_testes_2	Null_testes_3
Cont_AG_1	0.205482	0.07826701	0.131082	0.09664036	0.10177213	0.1508236
Cont_AG_2	0.2001951	0.07551219	0.1266083	0.09329982	0.0987255	0.1464541
Cont_AG_3	0.2063847	0.07849566	0.1315013	0.09690848	0.10248077	0.1514061
Null_AG_1	0.2026459	0.07477292	0.126838	0.09244182	0.09806074	0.1472827
Null_AG_2	0.2030579	0.07692767	0.128854	0.09530082	0.10079365	0.1487755
Null_AG_3	0.2019073	0.07593623	0.1271381	0.09392506	0.09955389	0.147157
Cont_testes_1	1	0.95869171	0.9676793	0.93697062	0.97222497	0.9768795
Cont_testes_2	0.9586917	1	0.9453382	0.95305545	0.96495724	0.9578088
Cont_testes_3	0.9676793	0.94533817	1	0.9758292	0.98811356	0.9942468
Null_testes_1	0.9369706	0.95305545	0.9758292	1	0.97623687	0.9795204
Null_testes_2	0.972225	0.96495724	0.9881136	0.97623687	1	0.991565
Null_testes_3	0.9768795	0.95780875	0.9942468	0.97952038	0.99156501	1

Supplementary Table 4. RNA seq Pearson's correlation analysis between biological replicates in male testes. *r* values > 0.9 indicate high quantitative reproducibility.

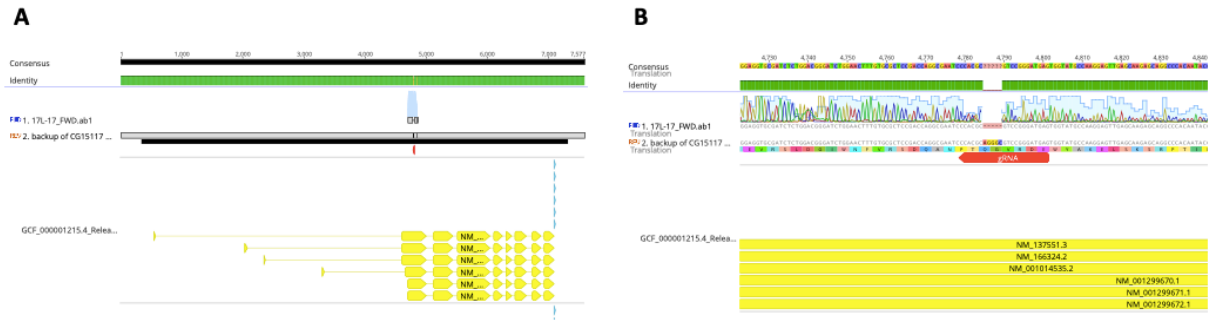
Sample	FRT_unmated_1	FRT_unmated_2	FRT_unmated_3	Cont_1hpm_1	Cont_1hpm_2	Cont_1hpm_3
FRT_unmated_1	1	0.9893728	0.9679208	0.9859374	0.9883358	0.9946076
FRT_unmated_2	0.9893728	1	0.9802212	0.9826425	0.9880189	0.9892428
FRT_unmated_3	0.9679208	0.9802212	1	0.9778429	0.9802017	0.9724333
Cont_1hpm_1	0.9859374	0.9826425	0.9778429	1	0.9900704	0.9892248
Cont_1hpm_2	0.9883358	0.9880189	0.9802017	0.9900704	1	0.9965134
Cont_1hpm_3	0.9946076	0.9892428	0.9724333	0.9892248	0.9965134	1
Cont_3hpm_1	0.9766459	0.9814714	0.9750113	0.9836631	0.9932212	0.988523
Cont_3hpm_2	0.980974	0.9858431	0.9727675	0.9871144	0.9940435	0.9910676
Cont_3hpm_3	0.9816836	0.9771946	0.9712508	0.9865555	0.9951913	0.9919251
Cont_6hpm_1	0.9791539	0.9748243	0.9402192	0.962188	0.9783625	0.9852009
Cont_6hpm_2	0.9792451	0.9752621	0.9536618	0.9679961	0.984218	0.9845498
Cont_6hpm_3	0.9836806	0.9787195	0.9588247	0.9766604	0.9879923	0.9876575
Null_1hpm_1	0.9880068	0.9872892	0.9615013	0.985423	0.9875128	0.9897005
Null_1hpm_2	0.9931136	0.988799	0.9744914	0.9884361	0.9969297	0.9990906
Null_1hpm_3	0.9941299	0.9818813	0.9612993	0.9873638	0.9881079	0.9945742
Null_3hpm_1	0.9643545	0.9729909	0.9756511	0.9736165	0.9876625	0.9799476
Null_3hpm_2	0.9803647	0.9830562	0.9667996	0.9814883	0.9943022	0.9919238
Null_3hpm_3	0.9788531	0.9758463	0.9606353	0.9794599	0.9869302	0.9892472
Null_6hpm_1	0.9763701	0.9787814	0.9694877	0.9764507	0.9875143	0.9830943
Sample	Cont_3hpm_1	Cont_3hpm_2	Cont_3hpm_3	Cont_6hpm_1	Cont_6hpm_2	Cont_6hpm_3
FRT_unmated_1	0.9766459	0.980974	0.9816836	0.9791539	0.9792451	0.9836806
FRT_unmated_2	0.9814714	0.9858431	0.9771946	0.9748243	0.9752621	0.9787195
FRT_unmated_3	0.9750113	0.9727675	0.9712508	0.9402192	0.9536618	0.9588247
Cont_1hpm_1	0.9836631	0.9871144	0.9865555	0.962188	0.9679961	0.9766604
Cont_1hpm_2	0.9932212	0.9940435	0.9951913	0.9783625	0.984218	0.9879923
Cont_1hpm_3	0.988523	0.9910676	0.9919251	0.9852009	0.9845498	0.9876575
Cont_3hpm_1	1	0.995704	0.9916831	0.9775355	0.9878505	0.9897744

Cont_3hpm_2	0.995704	1	0.9937257	0.9787186	0.9833364	0.9866023
Cont_3hpm_3	0.9916831	0.9937257	1	0.9770707	0.9835209	0.9858975
Cont_6hpm_1	0.9775355	0.9787186	0.9770707	1	0.9903278	0.9860244
Cont_6hpm_2	0.9878505	0.9833364	0.9835209	0.9903278	1	0.9967236
Cont_6hpm_3	0.9897744	0.9866023	0.9858975	0.9860244	0.9967236	1
Null_1hpm_1	0.981108	0.9881046	0.9850243	0.9758549	0.9792328	0.981036
Null_1hpm_2	0.9897342	0.9908043	0.9924878	0.9850645	0.9856608	0.9889921
Null_1hpm_3	0.97745	0.9814118	0.9839579	0.9800845	0.9762982	0.981253
Null_3hpm_1	0.9915457	0.988017	0.9900571	0.9683546	0.9768327	0.9789328
Null_3hpm_2	0.9928322	0.9935941	0.9908318	0.984828	0.9844569	0.9884329
Null_3hpm_3	0.986118	0.9857824	0.9880308	0.9868722	0.9799743	0.9823174
Null_6hpm_1	0.9914264	0.9884106	0.9879451	0.9762312	0.9924492	0.9927601
Sample	**Null_1hpm_1**	**Null_1hpm_2**	**Null_1hpm_3**	**Null_3hpm_1**	**Null_3hpm_2**	**Null_3hpm_3**
FRT_unmated_1	0.9880068	0.9931136	0.9941299	0.9643545	0.9803647	0.9788531
FRT_unmated_2	0.9872892	0.988799	0.9818813	0.9729909	0.9830562	0.9758463
FRT_unmated_3	0.9615013	0.9744914	0.9612993	0.9756511	0.9667996	0.9606353
Cont_1hpm_1	0.985423	0.9884361	0.9873638	0.9736165	0.9814883	0.9794599
Cont_1hpm_2	0.9875128	0.9969297	0.9881079	0.9876625	0.9943022	0.9869302
Cont_1hpm_3	0.9897005	0.9990906	0.9945742	0.9799476	0.9919238	0.9892472
Cont_3hpm_1	0.981108	0.9897342	0.97745	0.9915457	0.9928322	0.986118
Cont_3hpm_2	0.9881046	0.9908043	0.9814118	0.988017	0.9935941	0.9857824
Cont_3hpm_3	0.9850243	0.9924878	0.9839579	0.9900571	0.9908318	0.9880308
Cont_6hpm_1	0.9758549	0.9850645	0.9800845	0.9683546	0.984828	0.9868722
Cont_6hpm_2	0.9792328	0.9856608	0.9762982	0.9768327	0.9844569	0.9799743
Cont_6hpm_3	0.981036	0.9889921	0.981253	0.9789328	0.9884329	0.9823174
Null_1hpm_1	1	0.9875877	0.9868552	0.965839	0.9809144	0.9720471
Null_1hpm_2	0.9875877	1	0.993369	0.9831833	0.9930078	0.9904738
Null_1hpm_3	0.9868552	0.993369	1	0.9651999	0.9850928	0.9848005
Null_3hpm_1	0.965839	0.9831833	0.9651999	1	0.9883842	0.9867742
Null_3hpm_2	0.9809144	0.9930078	0.9850928	0.9883842	1	0.9926033

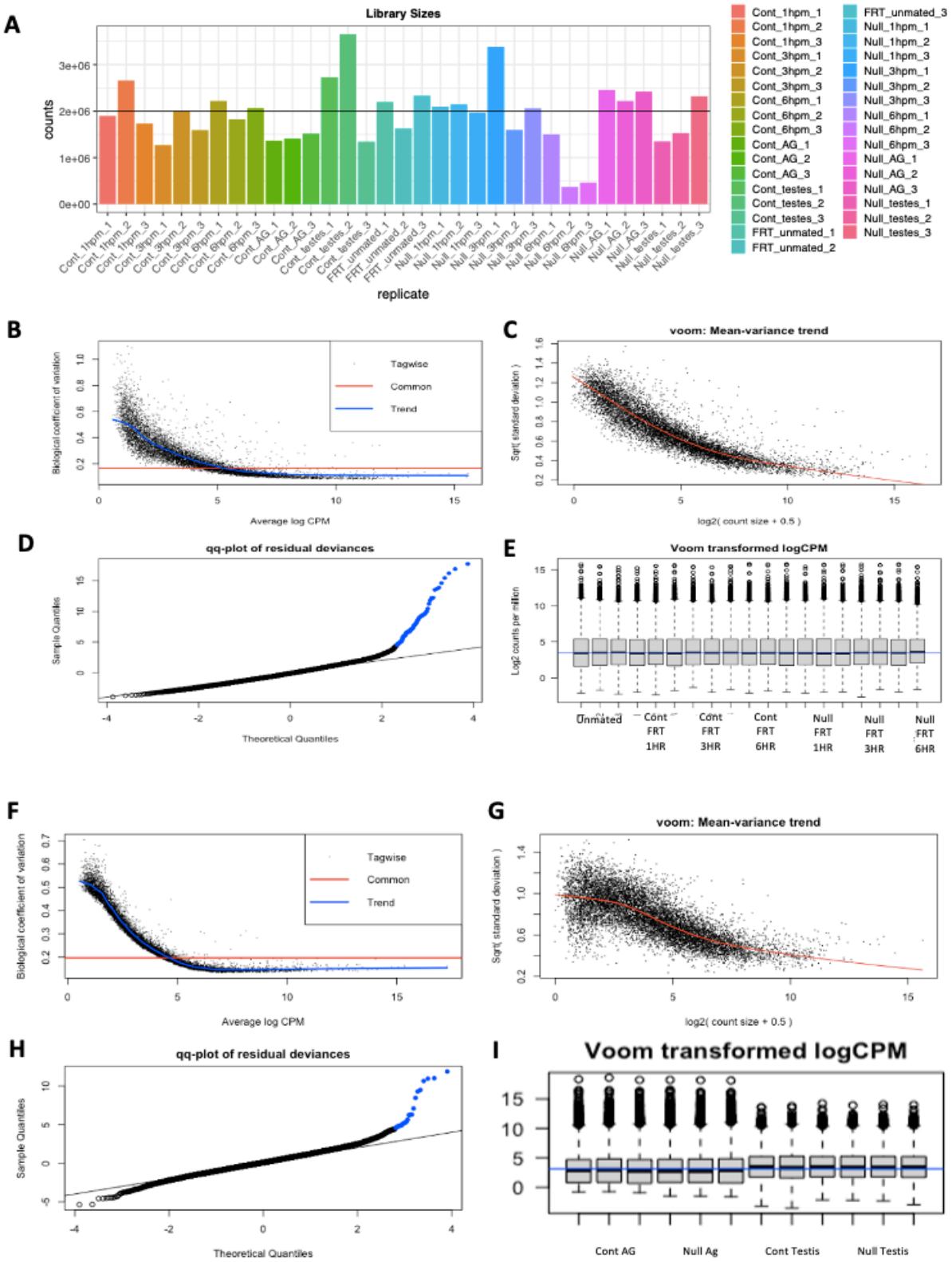
Null_3hpm_3	0.9720471	0.9904738	0.9848005	0.9867742	0.9926033	1
Null_6hpm_1	0.9824374	0.9838511	0.9725576	0.9845413	0.9828421	0.9749933

Sample	Null_6hpm_1
FRT_unmated_1	0.9763701
FRT_unmated_2	0.9787814
FRT_unmated_3	0.9694877
Cont_1hpm_1	0.9764507
Cont_1hpm_2	0.9875143
Cont_1hpm_3	0.9830943
Cont_3hpm_1	0.9914264
Cont_3hpm_2	0.9884106
Cont_3hpm_3	0.9879451
Cont_6hpm_1	0.9762312
Cont_6hpm_2	0.9924492
Cont_6hpm_3	0.9927601
Null_1hpm_1	0.9824374
Null_1hpm_2	0.9838511
Null_1hpm_3	0.9725576
Null_3hpm_1	0.9845413
Null_3hpm_2	0.9828421
Null_3hpm_3	0.9749933
Null_6hpm_1	1

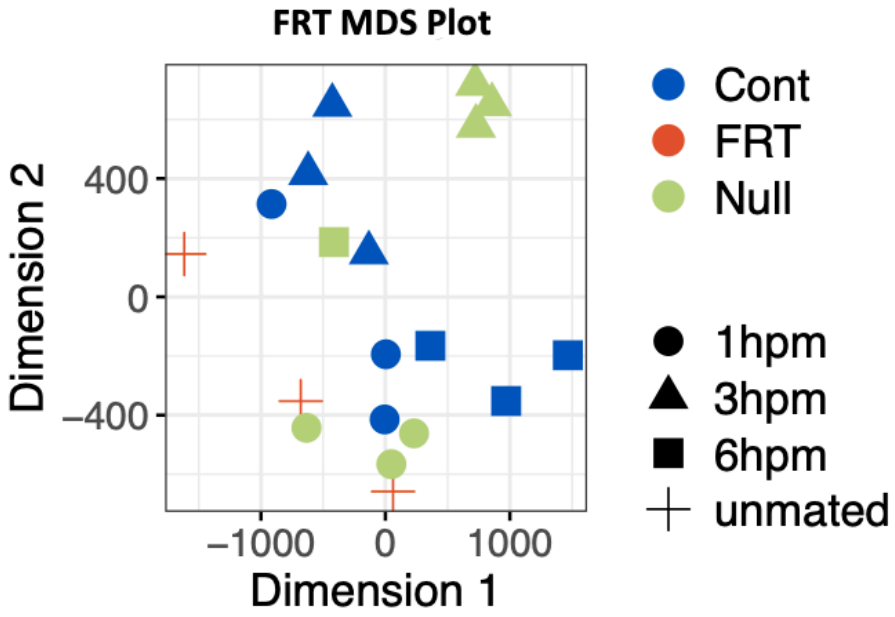
Supplementary Table 5 RNA seq Pearson's correlation analysis between biological replicates in female LRTs. *r* values > 0.9 indicate high quantitative reproducibility.



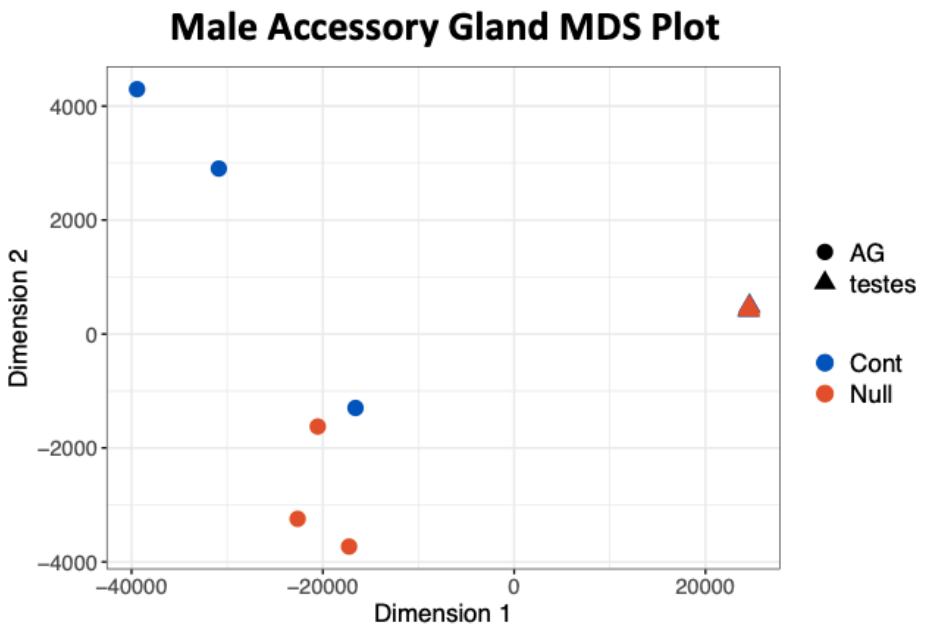
Supplementary Figure 1. *Drosophila melanogaster* BGLUC CRISPR knockout alignment to wild-type D. *melanogaster*. (A) Whole gene alignment of BGLUC knockout. Yellow indicated BGLUC protein coding sequence, red indicates the guide RNA, dashed lines highlighted in red indicate the five base-pair deletion. (B) Zoomed in image of the guide RNA and five base-pair deletion.


A
Library Sizes
counts
0e+00
1e+06
2e+06
3e+06
replicate
Cont_1hpm_1
Cont_1hpm_2
Cont_1hpm_3
Cont_3hpm_1
Cont_3hpm_2
Cont_3hpm_3
Cont_6hpm_1
Cont_6hpm_2
Cont_6hpm_3
Cont_AG_1
Cont_AG_2
Cont_AG_3
Cont_testes_1
Cont_testes_2
Cont_testes_3
FRT_unmated_1
FRT_unmated_2
FRT_unmated_3
Null_1hpm_1
Null_1hpm_2
Null_1hpm_3
Null_3hpm_1
Null_3hpm_2
Null_3hpm_3
Null_6hpm_1
Null_6hpm_2
Null_6hpm_3
Null_AG_1
Null_AG_2
Null_AG_3
Null_testes_1
Null_testes_2
Null_testes_3
B
Biological coefficient of variation
Average log CPM
Tagwise
Common
Trend
C
voom: Mean-variance trend
Sqrt(standard deviation)
log2(count size + 0.5)
D
qq-plot of residual deviances
Sample Quantiles
Theoretical Quantiles
E
Voom transformed logCPM
Log2 counts per million
Unmated
Cont FRT 1HR
Cont FRT 3HR
Cont FRT 6HR
Null FRT 1HR
Null FRT 3HR
Null FRT 6HR
F
Biological coefficient of variation
Average log CPM
Tagwise
Common
Trend
G
voom: Mean-variance trend
Sqrt(standard deviation)
log2(count size + 0.5)
H
qq-plot of residual deviances
Sample Quantiles
Theoretical Quantiles
I
Voom transformed logCPM
Cont AG
Null Ag
Cont Testis
Null Testis

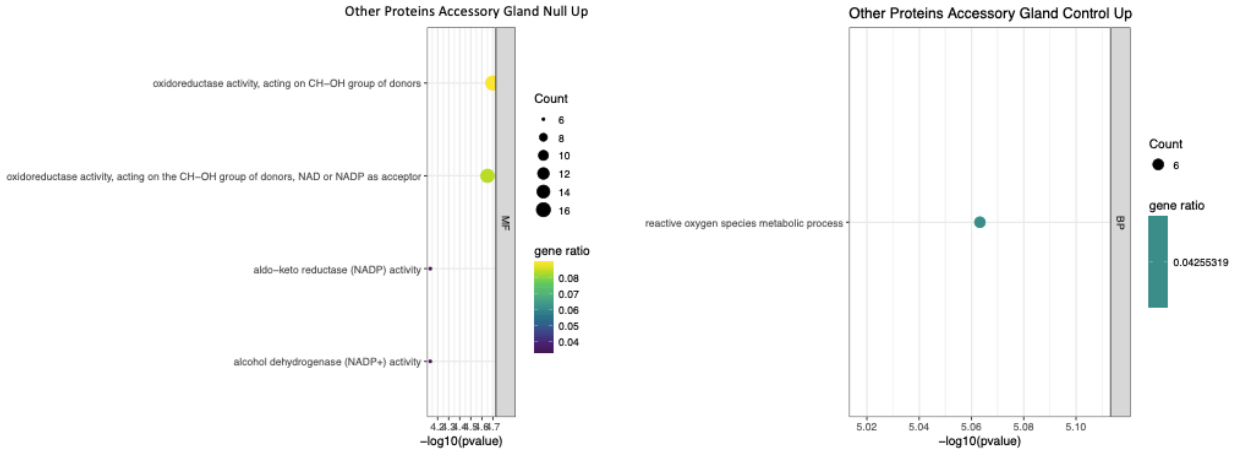
Supplementary Figure 2. RNA sequencing quality analysis of male accessory glands, testes, and female LRTs. (A) Library sizes, from left to right control 1hr post-mating replates 1-3, control 3hr post-mating replates 1-3, control 6hr post-mating replates 1-3, control accessory glands replicates 1-3, control testes replicates 1-3, unmated female LRT replicates 1-3, null 1hr post-mating replates 1-3, null 3hr post-mating replates 1-3, null 6hr post-mating replates 1-3, null accessory glands replicates 1-3, null testes replicates 1-3. (B) Female samples average CPM. (C) Female samples mean-variance trend. (D) Female samples QQ plot of residual deviance. (E) Female samples transformed logCPM. (F) Male samples average CPM. (G) Male samples mean-variance trend. (H) Male samples QQ plot of residual deviance. (I) Male samples transformed logCPM.



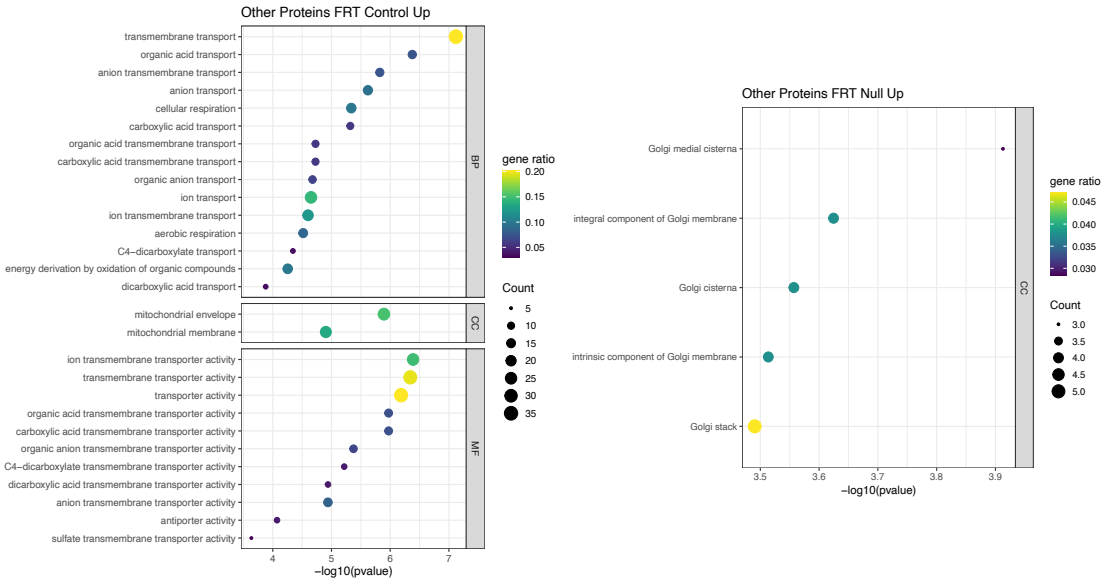
Supplementary Figure 3. Multidimensional scaling plot of post mating female samples. Blue represents female LRTs after mating to control males, green represents female LTRs after mating to BGLUC mutant males and red represents unmated female LRTs. 1hr post-mating samples are circles, three hour post mating samples are triangles and six hour post mating samples are squares.



Supplementary Figure 4. Multidimensional scaling plot of post mating male samples. Blue represents control male samples, red represents BGLUC mutant male samples, circles represent accessory gland samples, and triangles represent testes samples.



Supplementary Figure 5. Gene ontology enrichment analysis of BGLUC mutant and control accessory glands excluding SFPs. The most significantly elevated gene ratio is represented by lighter colors (yellow and green) while darker colors (blue and purple) represent less significantly elevated gene ratios. Counts of genes are represented by the size of the dot.



Supplementary Figure 6. Excluding SFPs, gene ontology enrichment analysis of female reproductive tracts immediately following mating of females to either BGLUC mutant or control males. The most significantly elevated gene ratio is represented by lighter colors (yellow and green) while darker colors (blue and purple) represent less significantly elevated gene ratios. Counts of genes are represented by the size of the dot.

Comparison	SYMBOL	FLYBASE	PValue	logFC	logCPM	FDR
c1h.v.n1h	S-Lap7	FBgn0033868	5.259E-06	0.748507945	6.645791632	0.024335932
c3h.v.n3h	CecB	FBgn0000278	4.099E-08	0.938859842	6.708532407	0.000379406
c3h.v.n3h	Mpcp2	FBgn0026409	3.521E-07	0.588841864	8.461732298	0.001629298
c3h.v.n3h	dpy	FBgn0053196	7.247E-07	0.500182595	8.995918912	0.002235638
c3h.v.n3h	CG7296	FBgn0032283	1.872E-06	0.608961863	8.137540646	0.003481992
c3h.v.n3h	CecC	FBgn0000279	2.594E-06	1.0810517	7.060812826	0.004000494
c3h.v.n3h	lncRNA:CR42859	FBgn0262106	3.355E-06	-0.576613096	8.333775997	0.004436115
c3h.v.n3h	CG10211	FBgn0032685	1.067E-05	0.845965965	5.810613078	0.00826033
c3h.v.n3h	jhamt	FBgn0028841	1.114E-05	0.925728535	6.504190789	0.00826033
c3h.v.n3h	CG13793	FBgn0031935	1.16E-05	1.03757364	6.114460854	0.00826033
c3h.v.n3h	pirk	FBgn0034647	1.526E-05	1.690078157	4.40574624	0.009418145

c3h.v.n3h	wdp	FBgn0034718	1.907E-05	0.576248766	7.244785604	0.010652399
c3h.v.n3h	CG31869	FBgn0051869	1.957E-05	0.615336911	7.071188622	0.010652399
c3h.v.n3h	Rel	FBgn0014018	3.14E-05	0.701262266	7.119964435	0.01524439
c3h.v.n3h	Zip89B	FBgn0038412	4.857E-05	1.064663037	5.452525568	0.019518682
c3h.v.n3h	CG3604	FBgn0031562	5.062E-05	0.734958554	6.831911109	0.019518682
c6h.v.n6h	Mpcp2	FBgn0026409	2.587E-13	-1.404345526	8.461732298	2.3947E-09
c6h.v.n6h	CG3604	FBgn0031562	1.854E-11	-2.028701445	6.831911109	8.57847E-08
c6h.v.n6h	CG34227	FBgn0085256	8.135E-08	1.221431728	7.72728558	0.000244242
c6h.v.n6h	CG13793	FBgn0031935	1.056E-07	-1.748013581	6.114460854	0.000244242
c6h.v.n6h	Acp98AB	FBgn0263597	2.372E-07	-0.921846631	9.41635743	0.000439024
c6h.v.n6h	Mst57Da	FBgn0011668	6.095E-07	-0.801366272	8.856124309	0.000940093
c6h.v.n6h	dj	FBgn0019828	7.781E-07	-1.081775077	6.669623798	0.001028699
c6h.v.n6h	CG31988	FBgn0051988	1.024E-06	-0.774404756	8.268981357	0.001185175
c6h.v.n6h	eIF4A	FBgn0001942	1.356E-06	-0.554438542	11.02805971	0.001357293
c6h.v.n6h	Act57B	FBgn0000044	1.467E-06	-1.219047118	9.506669158	0.001357293
c6h.v.n6h	CG43147	FBgn0262623	1.806E-06	-0.828183819	9.098325288	0.001443829
c6h.v.n6h	Sclp	FBgn0030357	1.872E-06	-2.001781979	5.450809334	0.001443829
c6h.v.n6h	CG5023	FBgn0038774	3.498E-06	-1.911650803	6.64276928	0.002490475
c6h.v.n6h	BomT3	FBgn0038930	4.325E-06	-1.763725198	6.988568764	0.002701556
c6h.v.n6h	CG4836	FBgn0270925	4.438E-06	-0.947707404	6.658490559	0.002701556
c6h.v.n6h	CG42521	FBgn0260396	4.67E-06	-2.816213386	8.382601671	0.002701556
c6h.v.n6h	Actn	FBgn0000667	5.361E-06	-0.782170511	7.655274646	0.002918329
c6h.v.n6h	CG5762	FBgn0039190	7.877E-06	-1.130508091	5.956374665	0.003923331
c6h.v.n6h	Sfp96F	FBgn0261061	8.14E-06	-0.807615413	8.25963605	0.003923331
c6h.v.n6h	RpS20	FBgn0019936	8.478E-06	0.668584986	9.089172463	0.003923331
c6h.v.n6h	GstT4	FBgn0030484	1.059E-05	-1.477174386	6.033200649	0.004668588
c6h.v.n6h	CG9911	FBgn0030734	1.188E-05	-1.280086913	6.08968723	0.004831003
c6h.v.n6h	CG10527	FBgn0034583	1.25E-05	-0.613945033	8.974031249	0.004831003
c6h.v.n6h	eEF1gamma	FBgn0029176	1.288E-05	-0.557227403	9.996158709	0.004831003
c6h.v.n6h	CG42852	FBgn0262099	1.305E-05	-0.702912986	9.600138154	0.004831003

c6h.v.n6h	CG8974	FBgn0030693	1.378E-05	-1.626513824	5.027523878	0.004904876
c6h.v.n6h	AcCoAS	FBgn0012034	1.55E-05	1.0114627	7.285243548	0.005313666
c6h.v.n6h	SP	FBgn0003034	1.639E-05	-0.814538507	7.615900528	0.005318687
c6h.v.n6h	lncRNA:CR40469	FBgn0058469	1.667E-05	-0.56770224	9.321016495	0.005318687
c6h.v.n6h	CG31468	FBgn0047351	1.921E-05	-0.953164126	6.376986535	0.005801193
c6h.v.n6h	Pen	FBgn0267727	1.943E-05	-0.829767234	7.16016519	0.005801193
c6h.v.n6h	Tm2	FBgn0004117	2.253E-05	-0.868510411	10.65763158	0.006516576
c6h.v.n6h	CG43319	FBgn0263024	2.529E-05	-0.834990726	6.845134536	0.007046582
c6h.v.n6h	COX8	FBgn0263911	2.589E-05	0.605519078	8.765489638	0.007046582
c6h.v.n6h	CG17376	FBgn0042189	2.853E-05	-0.930415252	6.298667842	0.007545097
c6h.v.n6h	Acp36DE	FBgn0011559	3.044E-05	-0.983428746	6.45657341	0.007825048
c6h.v.n6h	RpS26	FBgn0261597	3.192E-05	0.554072863	11.56122978	0.007984888
c6h.v.n6h	CG2930	FBgn0028491	3.292E-05	-0.810391689	6.85279989	0.007993907
c6h.v.n6h	CG13124	FBgn0032156	3.369E-05	0.644314957	8.672365604	0.007993907
c6h.v.n6h	scaf	FBgn0033033	3.754E-05	-2.172278066	3.97068746	0.008685553
c6h.v.n6h	CG6910	FBgn0036262	3.882E-05	-0.835884398	7.249131277	0.008763203
c6h.v.n6h	Sfp79B	FBgn0259973	4.075E-05	-0.801703893	6.864672902	0.008779823
c6h.v.n6h	Mst84Db	FBgn0004173	4.117E-05	-0.801060442	6.822780198	0.008779823
c6h.v.n6h	Acp54A1	FBgn0083936	4.329E-05	-0.792566658	7.21245381	0.008779823
c6h.v.n6h	CG44388	FBgn0265538	4.347E-05	-0.813288535	7.094803696	0.008779823
c6h.v.n6h	CG13364	FBgn0026879	4.446E-05	0.88625815	7.521173	0.008779823
c6h.v.n6h	Argk	FBgn0000116	4.544E-05	-0.529193081	9.848178642	0.008779823
c6h.v.n6h	yki	FBgn0034970	4.554E-05	-2.216754975	3.30146464	0.008779823
c6h.v.n6h	CG34132	FBgn0083968	4.971E-05	1.540224127	5.4179615	0.009247813
c6h.v.n6h	CG10252	FBgn0039104	4.996E-05	-0.908508314	6.325789203	0.009247813
c6h.v.n6h	BG642312	FBgn0047334	5.402E-05	-0.774811989	7.399741805	0.009802225
c6h.v.n6h	betaTub85D	FBgn0003889	5.741E-05	-1.068125659	5.69855857	0.010217847
c6h.v.n6h	CG31313	FBgn0051313	6.644E-05	0.596981954	8.672127744	0.01158717
c6h.v.n6h	CG42481	FBgn0259971	6.761E-05	-0.630100912	8.238591516	0.01158717
c6h.v.n6h	GIIIspla2	FBgn0030013	7.026E-05	3.16241428	4.440190657	0.011823399
c6h.v.n6h	fest	FBgn0034435	7.964E-05	-0.898936199	6.290684928	0.012826855

c6h.v.n6h	msopa	FBgn0004414	8.038E-05	-0.782289128	7.431746229	0.012826855
c6h.v.n6h	GlyP	FBgn0004507	8.501E-05	-0.643592155	8.417076375	0.013334848
c6h.v.n6h	Mst57Db	FBgn0011669	8.806E-05	-0.776395136	11.36312035	0.013582842
c6h.v.n6h	CG34168	FBgn0085197	9.617E-05	-0.806611792	6.734248402	0.014590469
c6h.v.n6h	ATPsynO	FBgn0016691	0.0001052	0.613523504	8.185777646	0.015522044

Supplementary Table 6. Genes with significantly different post-mating female LRT transcript abundances at the three post-mating time points between females mated by control males and females mated by BGLUC mutant males.

Gene	logFC	logCPM	PValue	FDR
Dmel_CG43320	-2.202307	4.564518	3.68E-10	3.87E-06
Dmel_CG13965	-1.083948	7.37006	4.36E-09	2.29E-05
Dmel_CG42483	-1.016499	12.692157	2.89E-08	1.01E-04
Dmel_CG7171	-2.823579	3.443609	1.30E-07	2.74E-04
Dmel_CG14149	-1.893694	4.139973	1.28E-06	1.86E-03
Dmel_CG6560	3.555027	3.079017	2.49E-06	2.09E-03
Dmel_CR43825	-1.25145	4.850701	1.20E-05	6.65E-03
Dmel_CG10869	1.021269	8.255293	2.28E-05	1.05E-02
Dmel_CG32742	1.696984	5.674414	2.58E-05	1.12E-02
Dmel_CG6395	-1.358164	5.485272	2.66E-05	1.12E-02
Dmel_CG31281	1.337106	7.619678	2.92E-05	1.15E-02
Dmel_CG11656	2.086397	6.404151	2.95E-05	1.15E-02
Dmel_CR43826	-1.784051	3.788177	3.94E-05	1.37E-02
Dmel_CG7342	-1.261033	4.629512	4.09E-05	1.37E-02
Dmel_CR46086	-2.587736	2.557942	5.85E-05	1.63E-02
Dmel_CG15178	1.026228	8.273913	5.92E-05	1.63E-02
Dmel_CG12479	1.487295	6.844331	6.00E-05	1.63E-02
Dmel_CG30412	1.276387	7.444037	8.74E-05	2.09E-02
Dmel_CG16725	-1.784144	4.870563	1.24E-04	2.59E-02
Dmel_CG14036	2.814237	4.931021	1.28E-04	2.59E-02
Dmel_CG6481	1.154263	7.463536	1.38E-04	2.62E-02
Dmel_CG18418	1.256136	7.272524	1.38E-04	2.62E-02
Dmel_CG9747	-1.27644	4.381836	1.44E-04	2.62E-02
Dmel_CR45823	6.036612	3.852109	1.44E-04	2.62E-02
Dmel_CG6921	-1.048569	5.846288	1.47E-04	2.62E-02

Dmel_CG11373	1.481268	7.034522	1.51E-04	2.66E-02
Dmel_CR44389	-1.016233	10.630612	1.54E-04	2.66E-02
Dmel_CG6262	1.040803	7.785072	1.64E-04	2.69E-02
Dmel_CG7634	1.336682	7.161219	1.64E-04	2.69E-02
Dmel_CG31740	1.155755	7.615177	1.69E-04	2.74E-02
Dmel_CG14767	-1.608157	3.989862	1.75E-04	2.80E-02
Dmel_CG7510	-1.344733	4.102543	1.90E-04	2.94E-02
Dmel_CG17350	-6.394708	3.335591	2.08E-04	3.08E-02
Dmel_CG31806	1.264649	7.216383	2.28E-04	3.20E-02
Dmel_CG5583	1.617621	3.660086	2.34E-04	3.20E-02
Dmel_CG1081	-1.198755	4.816066	2.51E-04	3.22E-02
Dmel_CG5958	1.641325	4.298358	2.51E-04	3.22E-02
Dmel_CG31868	-1.307873	4.574572	2.61E-04	3.27E-02
Dmel_CG6586	1.307125	6.748867	2.88E-04	3.52E-02
Dmel_CG11068	1.244547	7.242138	3.03E-04	3.66E-02
Dmel_CG10174	2.941006	4.47035	3.81E-04	4.26E-02
Dmel_CG7813	1.199946	7.293048	3.81E-04	4.26E-02
Dmel_CG17300	1.235292	6.864855	4.10E-04	4.50E-02
Dmel_CG14735	1.21596	7.276579	4.20E-04	4.53E-02
Dmel_CG14391	1.196362	7.030209	4.22E-04	4.53E-02
Dmel_CR46367	-6.86128	1.316305	4.71E-04	4.86E-02

Supplementary Table 7. Genes with significantly different transcript abundances between control and BGLUC mutant male accessory glands.

Symbol	Gene	logFC	logCPM	PValue	FDR
	CG16772	-1.808241	4.629707	5.11E-09	5.38E-05
Tsfi	CG6186	-1.069467	6.81021	6.04E-08	3.18E-04
	CG4757	-2.645228	2.480274	6.54E-06	2.30E-02
	CG42789	1.215295	9.366357	1.36E-05	3.57E-02

Supplementary Table 8. Genes with significantly different transcript abundances between BGLUC control and mutant male testes.

Gene Symbol	Abundance Ratio Null FRT v Control FRT	Abundance Ratio Pvalue Null FRT v Control FRT
Dmel\CG15117	0.261	1E-17
gi7297470	0.074	1E-17
scpr-C	0.445	1.74383E-11
Drs	2.377	1.01541E-09

Dmel\CG11608	0.497	1.18218E-09
Sfp24Ba	2.13	1.31607E-08
Dmel\CG17097	1.85	1.41833E-05
Swim	0.605	0.000132109
Sfp53D	1.672	0.000377868
Cys	0.615	0.000391035
BcDNA:GM24986	3.29	0.000612734
Sfp87B	1.628	0.000812678
l(2)k04810	1.588	0.00121743
Ebp	1.576	0.001963223
CG6183	1.528	0.004287233
Sfp38D	0.723	0.005847723
Fkbp14	1.472	0.00720937
Gp93	1.487	0.008200933
NUCB1	1.463	0.010059284
EbpII	0.768	0.013006703
Obp56f	1.455	0.013200125
CT41369	1.501	0.017245849
Obp56e	1.434	0.01808744
Dmel\CG17919	0.794	0.020189224
Grp170	1.42	0.022042398
Acp36DE	1.412	0.025003426
Sfp33A1	0.718	0.025443937
Dmel\CG43319	1.416	0.026815387
Dmel\CG43074	1.393	0.032508532
Sfp35C	1.388	0.034959767
Dmel\CG1637	0.763	0.03553696
Dmel\CG17575	0.816	0.035728199
Dmel\CG12093	1.449	0.037012913
Dmel\CG34129	0.767	0.039837724
Sfp33A3	1.371	0.044051508
Dmel\CG11113	100	1E-17
Dmel\CG1637	100	1E-17
intr	100	1E-17

Supplementary Table 9. SFP genes with significantly different post-mating female lower reproductive tract protein abundances between females mated by control males and females mated by BGLUC mutant males.

Gene Symbol	Abundance Ratio Null FRT v Control FRT	Abundance Ratio Pvalue Null FRT v Control FRT
GC2	0.146	1E-17
Dic2	0.201	1E-17
Dmel\CG7514	0.215	1E-17
NEST:bs21e09	0.215	1E-17
ymp	0.284	1E-17
Ant2	0.294	1E-17
Dmel\CG16782	0.309	1E-17
Hex-t2	0.311	1E-17
Cyp4g1	0.315	1E-17
AK-3	0.329	1E-17
Porin2	0.366	2.22045E-16
Dmel\CG7309	0.364	2.44249E-15
Cyt-c-d	0.403	6.88338E-15
Sfxn1-3	0.369	1.39888E-14
Dmel\CG32026	0.342	1.38112E-13
Dmel\CG31493	0.106	2.9643E-13
COX7AL	0.308	7.93143E-13
FBgn 52081	0.365	2.63412E-12
mAcon2	0.379	4.27547E-12
Dmel\CG9254	0.371	9.10938E-12
Obp44a	0.404	9.60543E-12
betaTub85D	0.455	5.67961E-11
Tps1	2.353	8.72136E-11
GstS1	2.33	8.82243E-11
Fum3	0.395	1.19532E-10
CG2337	0.307	3.46955E-10
Dmel\CG10469	2.264	3.47864E-09
CG8654-RA	0.431	4.58103E-09
CG2280	2.222	8.32868E-09
Obp99b	0.484	3.12876E-07
Act42A	0.495	3.53433E-07
aPKC	2.586	4.22262E-07
Dmel\CG15531	0.217	5.87839E-07
Cyt-c1L	0.45	7.37866E-07
Pp1-13C	0.252	8.14402E-07
CG32919	0.488	8.56371E-07
TwdlS	3.56	1.37855E-06
Pebp1	0.462	1.79539E-06

Scp1	0.233	2.02412E-06
Dic3	0.305	8.35739E-06
bs30h03.y1	0.527	9.49911E-06
JYalpha	0.328	1.29297E-05
Ald2	0.498	1.94716E-05
axo	0.584	1.97377E-05
Dmel\CG3803	0.282	2.65715E-05
Dmel\CG32277	1.972	3.11225E-05
MFS9	0.572	6.40448E-05
Tap42	0.276	8.09857E-05
HINT1	0.506	0.000111079
Dmel\CG43788	1.729	0.000135864
Dmel\CG7675	0.628	0.000147787
Dmel\CG7272	0.476	0.000151596
Fas3	3.926	0.000151798
Dmel\CG18130	0.399	0.000190619
ATPsynbetaL	0.322	0.000192724
Ald1	2.143	0.000215397
Dna2	0.55	0.000241462
Nmdmc	1.776	0.000263007
Cdlc2	0.43	0.000273357
colt	0.611	0.000286626
ATPsynCF6L	0.479	0.000362873
Dmel\CG9389	0.406	0.00041059
mge	0.311	0.000414479
mRpS5	0.614	0.0004361
Dmel\CG7910	0.652	0.000451298
Dmel\CG1418	0.368	0.000602201

Supplementary Table 10. Non-SFP genes with significantly different post-mating female lower reproductive tract protein abundances between females mated by control males and females mated by BGLUC mutant males.

Gene Symbol	Abundance Ratio Null AG v Control AG	Abundance Ratio Pvalue Null AG v Control AG
Dmel\CG15117	0.073	1E-17
Sfp87B	4.3	3.17524E-14
scpr-C	2.409	1.64802E-12
Dmel\CG17097	0.141	1.14036E-10
Acp36DE	0.169	2.05013E-08

Ebp	1.923	2.70403E-08
Acp62F	0.197	1.05204E-06
Cpr51A	0.177	1.35497E-06
Sfp24Ba	3.036	1.69656E-06
Dmel\CG43319	0.212	2.46321E-06
Dmel\CG15635	1.564	5.1943E-06
EbpIII	2.621	2.18876E-05
Obp56e	0.24	3.67862E-05
Acp70A	1.362	3.89915E-05
Obp56g	1.24	0.000141331
Dmel\CG2852	0.261	0.00034699
Sfp24Bb	1.508	0.000569241
Acp63F	0.249	0.00088928
Dmel\CG31198	0.17	0.001087031
Dmel\CG30395	1.112	0.00152268
BG642163	1.112	0.001537471
Acp53Ea	1.101	0.001787031
CG17022	1.089	0.002117501
Sfp24C1	0.309	0.003567261
GILT3	0.235	0.007714486
Prx4	0.32	0.008594803
Sfp35C	0.322	0.009266302
Acp53C14a	0.978	0.010099838
Dmel\CG3097	0.977	0.012013835
Bbd	0.951	0.012697397
Semp1	0.27	0.014939114
lectin-29Ca	0.94	0.016973363
Dmel\CG1701	0.35	0.018441089
Dmel\CG15539	0.346	0.019191363
BcDNA:AT19802	0.93	0.019489261
Dmel\CG1637	0.227	0.021228551
Calr	0.344	0.021661552
Ggt-1	0.344	0.021931032
Dmel\CG42467	0.899	0.027741309
Dmel\CG10029	0.354	0.03057082
Dmel\CG33290	0.984	0.032043459
EbpII	1.135	0.034109348
Idgf4	0.36	0.037313938
Sfp33A1	0.349	0.039614043
Dmel\CG43074	0.306	0.040825978
SP193	0.943	0.043403441

Qsox3	0.864	0.045851548
Cpr56F	0.01	1E-17
Sfp24Bc	100	1E-17
betaTry	100	1E-17

Supplementary Table 11. SFP genes with significantly different protein abundances between control and BGLUC mutant male accessory glands.

Gene Symbol	Abundance Ratio Null AG v Control AG	Abundance Ratio Pvalue Null AG v Control AG
AttA	0.039	1E-17
Dmel\CG7768	4.922	1E-17
anon-WO0140519.48	10.984	1E-17
TrxT	14.41	1E-17
Tps1	2.788	7.32747E-15
Jhbp5	3.735	6.88338E-14
Cpr49Ae	4.582	4.89631E-12
Pebp1	2.6	9.00413E-12
Dmel\CG9331	2.352	1.54772E-10
LManVI	0.078	2.186E-10
Obp44a	2.052	1.75228E-09
mil	4.166	2.39325E-09
Mlp60A	1.915	3.16786E-09
Nak	4.375	6.51002E-09
Dmel\CG12990	5.661	7.92682E-09
GstS1	1.941	8.38631E-09
Pgm1	2.09	9.95835E-09
Mhc	0.09	1.57245E-08
ppl	3.396	3.181E-08
ens	0.155	1.00916E-07
Pdxk	3.426	1.06833E-07
Dmel\CG10863	1.833	1.85353E-07
Uch	2.975	2.02778E-07
Pex14	0.075	3.12329E-07
FASN1	1.577	4.97515E-07
dHspB8	1.78	6.13751E-07
AAF47782	0.078	1.00389E-06
Pect	1.932	1.47301E-06
Bacc	1.491	1.66204E-06
Had1	1.516	3.98916E-06

CG8654-RA	0.101	4.14659E-06
Jhbp13	5.236	7.01967E-06
Scp1	2.313	9.12786E-06
Adh-E	1.417	1.74181E-05
Rbp1	4.517	1.98836E-05
nito	0.157	2.76128E-05
BcDNA:RH38235	0.145	3.06898E-05
apolpp	1.376	3.19032E-05
Wdr62	3.243	3.28842E-05
Dmel\CG1648	1.336	3.44026E-05
Akr1B	1.32	7.31693E-05
Dmel\CG6726	1.559	7.54456E-05
Galt	2.201	8.46489E-05
Dmel\CG1674	2.136	0.000100389
Ald1	2.117	0.000102743
Sod3	0.175	0.000121365
IMP	2.258	0.00012592
sti	3.069	0.000231384
Hibch	2.172	0.000243428
Jhbp3	0.17	0.000256634
Gss2	1.393	0.000305641
Dmel\CG9394	1.743	0.000340466
wupA-RA	1.215	0.000343527
Dmel\CG3270	1.778	0.000353702
fabp	1.17	0.000364145
Galk	1.165	0.00039308
Dmel\CG7322	1.862	0.000526044
dmGlut	0.143	0.000557581
Cdc37	0.248	0.000593681
Cyt-c-d	1.73	0.000612083
Der-2	0.215	0.000654019
Pgls	1.75	0.000664989
Dmel\CG10911	0.19	0.000763742
CG6316	0.109	0.000778755

Supplementary Table 12. Non-SFP genes with significantly different protein abundances between control and BGLUC mutant male accessory glands.

Gene Symbol	Abundance Ratio Null FRT v Control FRT	Abundance Ratio Pvalue Null FRT v Control FRT
GC2	0.146	1E-17
Dic2	0.201	1E-17
Dmel\CG7514	0.215	1E-17
NEST:bs21e09	0.215	1E-17
Ant2	0.294	1E-17
Hex-t2	0.311	1E-17
AK-3	0.329	1E-17
Porin2	0.366	2.22045E-16
Cyt-c-d	0.403	6.88338E-15
Sfxn1-3	0.369	1.39888E-14
Dmel\CG32026	0.342	1.38112E-13
COX7AL	0.308	7.93143E-13
FBgn 52081	0.365	2.63412E-12
mAcon2	0.379	4.27547E-12
betaTub85D	0.455	5.67961E-11
Tps1	2.353	8.72136E-11
Fum3	0.395	1.19532E-10
Cyt-c1L	0.45	7.37866E-07
Pp1-13C	0.252	8.14402E-07
bs30h03.y1	0.527	9.49911E-06
Ald2	0.498	1.94716E-05
Dmel\CG18130	0.399	0.000190619
ATPsynbetaL	0.322	0.000192724
Ald1	2.143	0.000215397
Dna2	0.55	0.000241462
Cdlc2	0.43	0.000273357
colt	0.611	0.000286626
ATPsynCF6L	0.479	0.000362873
Dmel\CG9389	0.406	0.00041059
Veneno	0.373	0.000855288
CAP	2.591	0.000870846
Wdr62	0.637	0.001088455
Dmel\CG17118	0.388	0.001579575
SdhBL	0.428	0.002124604
Dnali1	0.578	0.002626417
Pi3K68D	0.749	0.007308454
Cul2	2.061	0.007321343
Gp93	1.487	0.008200933
COX5A	0.741	0.008893821
GCC185	0.616	0.00906876

RpI135	0.563	0.016237868
Grp170	1.42	0.022042398
ocn	0.559	0.0227183
Ogdh	0.782	0.023260727
alpha-KGDHC	0.791	0.023896415
heph	0.501	0.024971687
Acp36DE	1.412	0.025003426
hlk	0.803	0.027905839
tnc	1.401	0.028900485
COX6CL	0.698	0.0319247
vig	1.432	0.033118319
RpS5a	0.817	0.039599091
zormin	1.356	0.043474938
slgA	0.579	0.045076574
CG15655	2.034	0.046501829
Vha68-3	0.01	1E-17
Nbr	0.01	1E-17
Dmel\CG18662	0.01	1E-17
Indy-2	0.01	1E-17

Supplementary Table 13. Sperm protein genes with significantly different protein abundances between control and BGLUC mutant male accessory glands.

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RESEARCH INTERESTS

- Experimental Evolution, Evolutionary Genetics, Molecular Basis of Reproductive Interactions, Computational Genomics, Population Genetics, Speciation

SKILLS

- Handling and maintaining large laboratory populations of *Drosophila*
- Molecular techniques such as PCR, Genomic DNA Extraction, Total RNA Extraction; site directed mutagenesis such as, CRISPR knockouts, RNAi knockdowns, Plasmid Preparation, and *Drosophila* embryo micro injections
- Computational skills in Unix computing, R programing, Geneious and Image J

MANUSCRIPTS UNDER PREPARATION

- **Hockenbury D**, Ahmed-Braimah Y: Knockout of Highly Conserved Seminal Fluid Protein Ablates Male Fertility in *Drosophila melanogaster*

RESEARCH EXPERIENCE

- **Computational Genomic Analysis of The *Drosophila melanogaster* Species Group for Genetic Signatures of Rapid Evolution**
  April 2022 - Current
  Project Guide: Dr. Yasir Ahmed-Braimah
  At: Syracuse University, Syracuse NY
  Contribution: Design and execution of Experiment, Data Analysis

- **Functional Characterization of Rapidly Evolving Seminal Fluid Proteins in *Drosophila melanogaster* through Genetic Manipulation**
  September 2020 - Current
  Project Guide: Dr. Yasir Ahmed-Braimah
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  Contribution: Designed and executed experiment

- **The Role of the Y Chromosome in *Drosophila virilis* x *Drosophila novamexicana* Hybrid Sterility**
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TEACHING EXPERIENCE

- Teaching Assistant: **Introductory Biology Laboratory -** Undergraduate Course *Syracuse University*
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PROFESSIONAL SERVICE

- Biology Graduate Student Organization Representative, 2021-2022 Curriculum Committee
  - Aided in the revision of Syracuse University's Undergraduate Biology Curriculum
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COMMUNITY SERVICE

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