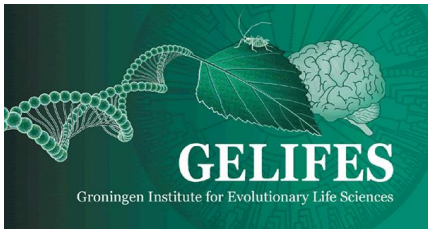
Toxic Love

Evolutionary genomics of the enigmatic Sex Peptide

Jessy Rouhana



This research has been carried out at the Groningen Institute for Evolutionary Life Sciences (GELIFES) of the University of Groningen (The Netherlands) and at the School of Biology of the university of East Anglia (United Kingdom), according to the requirements of the Graduate School of Science (Faculty of Science and Engineering, University of Groningen).

This research was funded by Ubbo Emmius PhD scholarship from the university of Groningen, a PhD scholarship from the university of East Anglia, grants from the John and Pamela Salters charitable trust and the Leverhulme Trust.

university of groningen

University of East Anglia

Toxic Love

Evolutionary genomics of the enigmatic Sex Peptide

PhD thesis

to obtain the degree of PhD of the
University of Groningen
on the authority of the
Rector Magnificus Prof. E. Sterken
and in accordance with
the decision by the College of Deans.

and

to obtain the degree of PhD of the
University of East Anglia
on the authority of the Vice Chancellor
Prof. D. Richardson and in the accordance with
the decision by the Science Faculty and Associate
Dean for Post Graduate Studies Prof A. Grant.

Double PhD degree

This thesis will be defended in public on

Monday 07 January 2018 at 11:00

By

Jessy Rouhana

born on 02 July 1989
In Bikfaya, Lebanon

Supervisors

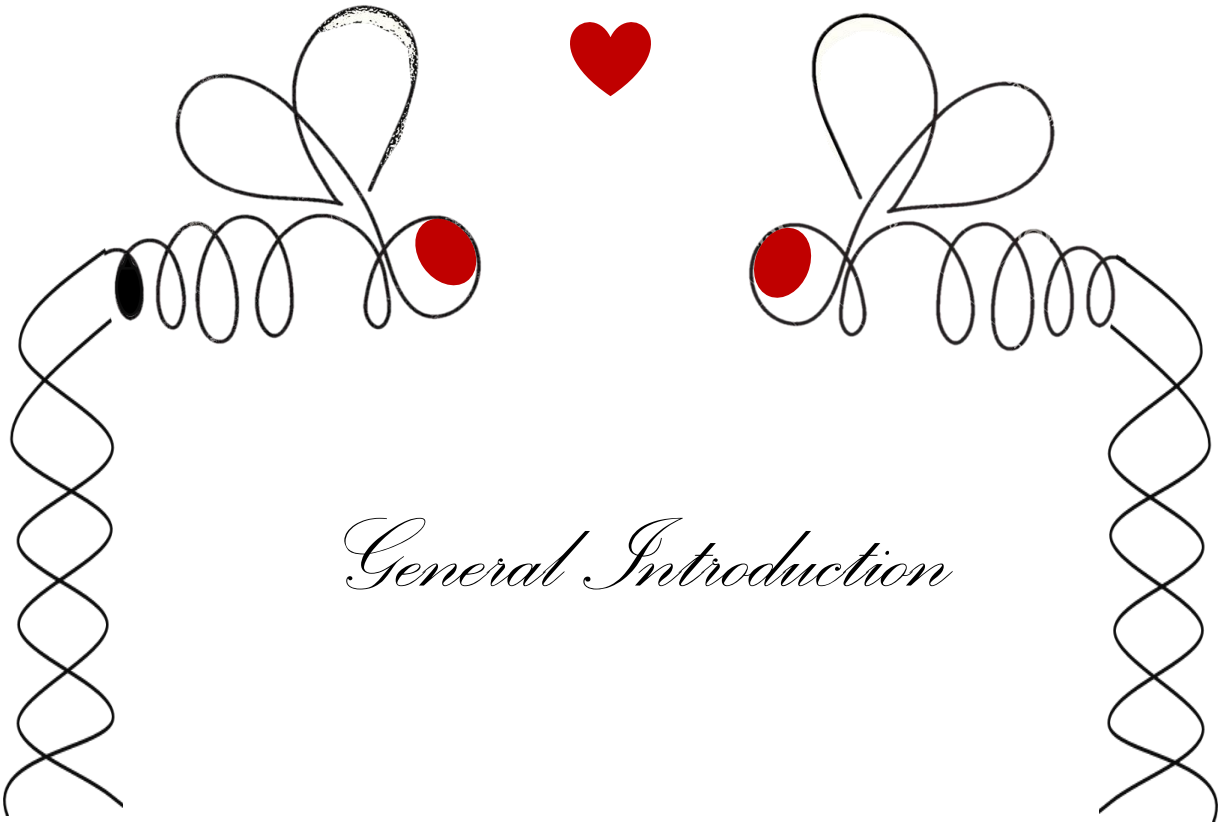
Prof. B. Wertheim
Prof. T. Chapman

Assessment committee

Prof. J.C. Billeter
Prof. D. Richardson
Prof. M. Wolfner
Prof. C. Wilson

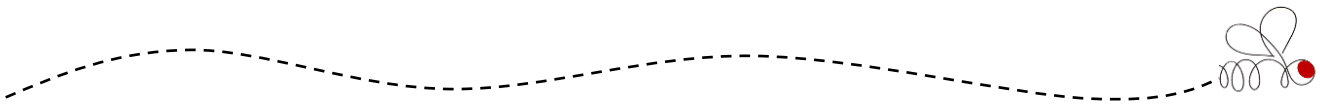
Table of contents

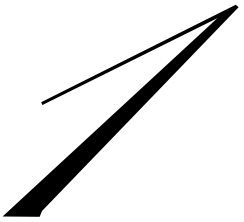
Chapter 1	General introduction	**7**
Chapter 2	A novel immuno-Quantitative PCR assay for quantifying Sex Peptide protein in *Drosophila melanogaster*	**39**
Chapter 3	Genetic variation in immune responses to Sex Peptide, *Drosophila melanogaster*	**75**
Chapter 4	Sex peptide “master regulator” of female Receptivity, Egg laying and starvation lifespan in *Drosophila melanogaster*	**119**
Chapter 5	General discussion	**179**
	Bibliography	**196**



General Introduction

Jessy Rouhana





Abstract:

The 'battle for life' can act on different levels, causing individuals of distinct species, as well as individuals of the same species, to compete over a variety of limiting resources such as food, breeding sites and mates. An important form of competition is driven by sexual conflict and often occurs when reproductive strategies between males and female diverge. This has led to the evolution of different strategies to overcome manipulations by the other sex, while maintaining a level of cooperation sufficient to ensure successful reproduction. The manipulation of one sex by the other through molecular interactions has been illuminated by studies of *Drosophila melanogaster.* Males tend to maximize their chances at fatherhood by releasing sperm and semen proteins into the females. The semen proteins can benefit both sperm and eggs, however they can also sometimes favour the interests of males whilst generating costs in females. One enigmatic semen protein that falls into this category is the 'Sex Peptide', which generates strikingly diverse changes in behavior, reproductive and immune system of females.

In most studies of the effects of Sex peptide, genetic variation has been minimized to delineate its' function. However, to understand the evolutionary processes and dynamics that characterise Sex Peptide-mediated interactions between both sexes, it is important to study this genetic variation. In this thesis, I trace the impact of Sex Peptide on the consequences of sexual conflict in the *Drosophila melanogaster* genome. An in-depth investigation was performed to measure female post-mating phenotypic traits (immunity, egg laying, receptivity to re-mating and starvation lifespan) in response to receipt of Sex Peptide, using 32 Drosophila Genome Reference Panel (DGRP) lines. I also developed a novel quantification technique to measure variation in the amount of Sex Peptide transferred among males. A genome wide association (GWAS) and functional annotation study of the tested phenotypic traits revealed significant genetic variation involved in the transfer of, and the post-mating responses to, Sex Peptide. This investigation of natural variation allowed me to map phenotypes onto underpinning genomic variation, and to determine the likely impact of sexual conflict on genome evolution.

Sexual conflicts:

The primary cause of sexual conflict is differences in the evolutionary interests of males and females. This starts with fundamental differences, (e.g. **anisogamy**) of sexual reproduction, in which females generally produce a small number of immobile **macrogametes** (ovules) rich with energy, while males produce an excess of mobile **microgametes** (sperm)(Parker *et al*., 1972). Consequently, this leads to an intense competition between the male gametes for the fertilization of the more "limited" female gametes. While males seek to increase their reproductive success by increasing the number of mates, females tend to limit their partners to one or few "high quality" mates (Bateman, 1948). Trivers (1972) stated that the difference of gamete sizes is proportional to amount of parental investment made by each sex. This concerns both the production of the gametes, and possibly the nourishing of the developing embryos. By producing small gametes, each male can potentially produce more offspring than each female, particularly if they are only minimally involved in caring for progeny. However, many males may not mate at all, with the result that a male's contribution to the next generation is generally more variable than is true for females. In some species the so-called 'sex roles' roles are even reversed, with males providing nutrition to their mates and caring for offspring, whilst rivalry between females occurs for access to males (Huxley, 1938; Gwynne, 1991). In this scenario, females compete for mates whereas males carefully choose their mate. This phenomenon is known as **"sex role reversal"** and has been reported in several species such as birds, frogs, fishes, crustaceans and insects (Alcock, 2005). The underlying key point is that, whenever the potential rate of reproduction differs between the sexes, both sexes will aim to maximize their reproductive success through different reproductive strategies.

Male reproductive success is primarily determined by the fertilization of as many females as possible; this selects for **competition between males** to gain access to females (Huxley, 1938)**.** Therefore, males invest time, risk, energy etc. in producing **secondary sexual characters** with which to dominate other males (**intra-sexual selection**) and to attract females (**inter-sexual selection**) (Zahavi, 1975). Classical examples of such elaborate differences are the train of peacocks, the ornaments of the birds of paradise, the antlers of male deer, and the manes of lions. In extreme forms, inter-sexual selection can lead to a **"run-away"** process, or an evolutionary association between female preference for ever larger exaggerated male

characters, even if it endangers male survival (Fisher, 1915). Nevertheless, females may favour such characteristics because their sons may benefit through increased attractiveness.

Sexual conflict and mating strategies:

Behaviour is an important component of an individual's reproductive success and sexual selection tends to favour animals that are efficient in mate selection, avoiding competition, copulating and parental care. Sexual conflict can occur when both sexes have contrasting strategies to optimize the reproductive fitness, especially over the mode and frequency of mating, potentially leading to an evolutionary arms race between males and females (Chapman *et al.*, 2003).

In many animal species, males compete with each other for mating opportunities through visual displays to intimidate other males, using bright colours, songs or other ornaments. This mate competition can take a more brutal turn, when males employ weapons such as horns or tusks in their fights, which might inflict injuries or lead to the death of the opponent (Futuyma, 2009). Although intra-sexual selection can favour the lifetime reproductive success of males that are well equipped for fighting, such males may also exhibit heightened vulnerability, for example by increasing the male's vulnerability to predators. There may also be cost of maintaining weaponry in terms of energy and time. Intra-sexual selection is most commonly seen in males, yet females can also experience intra-sexual competition in the case of sex role reversed species or when access to mates is limited.

Intra-sexual competition can take several forms and can favour a wide range of attributes and strategies (Andersson, 1994; Andersson and Iwasa, 1996). One example is contest competition, in which males engage in direct confrontations in order to gain access to fertile females (Alberts et al., 2003). The contrasting strategy is 'scramble', in which males use enhanced speed to find mates before rivals. This may lead males to emerge or even mature and become reproductively active before females (Bulmer, 1983; Morbey and Ydenberg, 2001). An endurance strategy is one in which males retain the ability to remain reproductively active during the entire breeding season, in order to increase mating opportunities (Higham *et al.*, 2011). In coercion, males use threats or force to increase their chance to mate with a female (Clutton-Brock and Parker, 1995). An important point is that sexual conflicts do not always cease at mating and may

continue in several guises, one of which is via sperm competition, commonly seen in polyandrous species, in which females have multiple mates**.** Sperm competition is taxonomically widespread and has been reported in several vertebrate (Snedden, 1990) and invertebrate species (Corderos and Miller, 1992) as well as in plants (Marshall and Ellstrand, 1986). This sperm competition can lead to various traits that are advantageous for fertilization, such as sperm morphology to favour more rapid transit to the ovule, male sexual organs that deliver sperm closer to the fertilization site, chemical composition of the ejaculate to enhance the success of an individual male in gaining fertilization or behaviours to guard females after mating (Simmons, 2001). Many of these male adaptations are selected to enhance the success of a male's own sperm against that of a rival.

To reduce the risk of rivalry and to increase their chances of reproduction, males have evolved numerous "defensive adaptations" to avoid engagement in sperm competition, and "offensive adaptations" to reduce the reproductive success of any rival males (Simmons, 2001). "Defensive adaptations" can take several forms. These include mate guarding, in which males guard receptive females (in their fertile period) both before and after copulation (Parker, 1974). The use of physical barriers, such as mating plugs, or in extreme case whole-body mating plugs, can also significantly reduce the probability of fertilisation by rival males and therefore reduce sperm competition (Dickinson and Rutowski, 1989; Polak *et al.*, 2001; Foellmer and Fairbairn, 2003). A third strategy is the use of chemical barriers, in which males release pheromones onto their mates that act as anti-aphrodisiacs, repelling other males from mating with those females (Jallon, 1984). Finally, seminal fluids can manipulate female reproduction and physiological behaviour, including by decreasing female receptivity to other males (Chapman, 2001). Sexual selection can also affect the evolution and diversification of male's genitalia to influence sperm competition (Arnqvist, 1998). Both sperm removal and internal fertilization can increase a male's chances in fertilizing the females' ova, and this may be part of the reason for the evolution of complex genital morphology. The evolution of internal fertilization is thought to reduce the absolute risk of sperm competition (Parker, 1970; Smith, 1984) and to provide females with a mechanism for selecting "good" motile sperm (Keller, 1999). Consistent with the idea that sexual selection can drive reproductive trait evolution, it is found that penis morphology is more complex and elaborated in species in which females are more likely to mate with more than one partner (Waage, 1979). Following this idea, males of some such

species have evolved penises / intromittent projections endowed with backward facing spines or hairs to entrap stored sperm of rival males (Corderos and Miller, 1992).

Sperm competition will occur only when sperm from different males overlap spatially and/or temporally near the fertilization site (Wigby and Chapman, 2004b). Hence males have adapted offensive strategies to promote their sperm success by destroying or inactivating the sperm of previous males. Such offensive adaptations include any variation in sperm morphology, sperm quality (such as sperm mobility, sperm longevity and viability; Birkhead *et al.*, 1999; Hunter and Birkhead, 2002; Snook, 2005), sperm quantity (Stockley *et al.*, 1997; DelBarco-Trillo and Ferkin, 2004) and sperm size (Gomendio and Roldan, 1991; Stockley *et al.*, 1997) that is advantageous for a male's reproductive success. Sperm removal is an additional offensive adaptation that occurs when males remove the sperm of rival males from the storage organs of mated females. Based on the phenomenon of "last male sperm precedence", some males can remove or somehow displace sperm from previous males by ejaculating new sperm into the female, thus hindering successful insemination opportunities of the previous male (Smith, 1979). In this sense infanticide can also be considered a type of offensive strategy in the context of male-male competition. For example, in several species with internal gestation brood or offspring care, males can resort to infanticide by inducing abortion or killing offspring to render females receptive to them, while reducing the paternity of male rivals (Crook and Shields, 1985).

Inter-sexual selection is often considered as female choice, in which female preferences play an essential role in shaping the evolution of male behavior and of male conspicuous secondary sex traits. Usually, males compete, and females choose, but in the case of sex role reversal i.e. when males are the sex investing more time and energy in caring for offspring, females may compete with each other and be chosen by males. However, for convenience I generally refer to the choice aspect of inter-sexual selection as “female choice”.

Females of many species mate preferentially with males that have larger, more intense, or more exaggerated characters, such as colour patterns, ornaments, vocalization, or display behaviours. Some preferred male characters can be ecologically disadvantageous and can even reduce male survival (Zuk *et al.*, 2006). Female preferences affecting male traits could have direct benefits, such that the male ornaments could reflect a male’s ability to provide material

advantages. Therefore, by choosing a mate with exaggerated ornaments, females may gain direct benefits, such as a superior territory with resources for rearing offspring, or increased parental care and protection (Andersson and Simmons, 2006). In such cases the ornaments are honest signals of male quality and are subject to positive sexual selection by female choice (Andersson, 1994). Indirect benefits can also provide an explanation for the evolution of female preferences and in this scenario, the male provides no direct benefits either to the female or to her offspring and contributes only his presumably high-quality genes. The preferred male may provide genes that increase the survivorship or mating success of offspring in comparison to those provided by less desirable males (Fisher, 1930). Females thus select mates on the basis of indicators of male genetic quality that predict higher offspring fitness.

***Drosophila melanogaster*:**

History:

Drosophila melanogaster, originally known as *Drosophila ampelohila,* was used for the first time as a research organism in early 1900s by W. E. Castle. Yet it was not until the work of Thomas Hunt Morgan and his students Sturtevant, Bridges and Muller, that *Drosophila* started to become one of the most widely used laboratory animal systems for studies of genetics. In 1933 Morgan was awarded with the Nobel prize for proving that chromosomes contain genes that play a major role in heredity. In addition, Morgan's team were the first to map *Drosophila* genes, and to discover the existence of genetic recombination and sex-linkage (Rubin and Lewis, 2000). In 2000, the *D. melanogaster* genome was fully sequenced (Consortium, 2000), which then allowed researchers access to genomic resources to enable genetic manipulations. All these discoveries made *D. melanogaster* an excellent model organism for genetic analysis. Furthermore, *Drosophila* fruitflies are cheap and easy to culture and maintain in the laboratory. They have a short life cycle and also produce a large number of eggs and progeny, which is useful for developmental studies.

The Drosophilid family consists of approximately 3500 species. Most of these flies breed in rotting plants and fungal material and use microorganisms as their main nutritional source. Of all the Drosophilids, the *Drosophila* genus is the best studied in terms of evolutionary genetics. The genus *Drosophila* originated from the tropics in Southeast Asia, and dates back 80-120 million years. The first major split of the *Drosophila* genus into two sub-genera, *Sophophora* and *Drosophila,* occurred approximately 50 million years ago in the Old-World tropics. Following this split the two sub-genera in turn split again into Old and New World lineages. *Sophophora* gave rise to the present-day Old-World *melanogaster* and New World *willistoni* and *saltans* groups, while the *obscura* group evolved in the African tropics. The subgroup *Drosophila* gave rise to the Old World *virilis* and the New World *repleta* (Powell, 1997). Thus, *D. melanogaster,* with its Afrotropical origin, belongs to the *melanogaster* sub-group of the sub-genus *Sophophora (*Jeffs *et al*., 1994).

D. melanogaster is widely used in studies of genetics, development and molecular biology and is, arguably, the most extensively and intensively studied model organism. Furthermore, *D. melanogaster* represents a powerful model system for studying sexual selection. *D.*

melanogaster has a complex repertoire of mating behaviours, including male courtship rituals to stimulate females (Bastock and Manning, 1955) and female choice behaviours. These features allow the potential for elevated offspring genetic diversity through increased numbers of mates or via mate discrimination (Billeter *et al.*, 2012).

The use of *D. melanogaster* in evolutionary studies of sexual selection was initiated by Frank. E. Lutz in 1911. Lutz showed for the first time that female *D. melanogaster* have mating preferences for certain type of males. In his experiments he noticed that females tend to choose normal males over abnormal extra wing-veined males and this was later recognized as inter-sexual selection. In 1915, Sturtevant described how in *D. melanogaster* male courtship behavior could stimulate females to copulate, and how tactile and olfactory senses were probably involved in this process. Sturtevant (1920) also found that males court females of some closely relates species, but that females prefer to mate with males of their own species, highlighting the fact that female *D. melanogaster* exert choice in mate selection. Sexual selection studies of *D. melanogaster* grew rapidly from these early beginnings and have attracted the attention of many researchers, leading to numerous novel discoveries in the study of sexual selection, as summarised below.

Reproductive system:

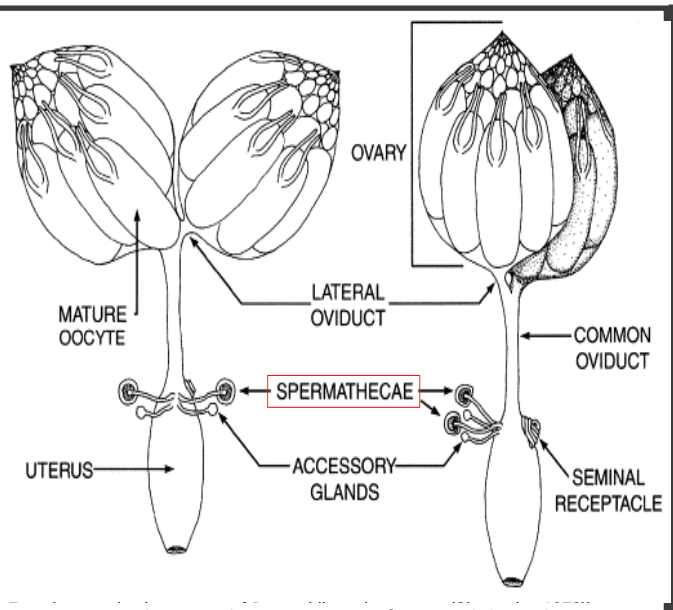


Figure 1: Female reproductive system of *Drosophila melanogaster* (Shorrocks, 1972)

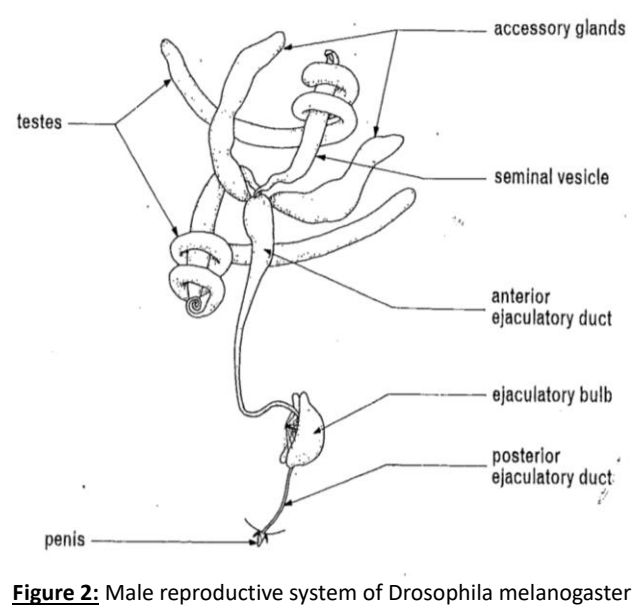


Figure 2: Male reproductive system of Drosophila melanogaster (Shorrocks, 1972)

The reproductive system of female *D. melanogaster* consists of a pair of two *ovaries,* each composed of 10 to 20 *ovarioles.* The base of each *ovariole* forms a small duct, and all these ducts are connected to two *oviducts* that enlarge posteriorly to form a uterus. The uterus is a heavily muscularized and innervated structure that receives sperm during mating and also holds the egg in position for fertilization. At the anterior end of the uterus are three organs that maintain sperm survival and / or store the spermthemselves. These are the paired female *accessory glands,* which produce a variety of substances to facilitate sperm maintenance and egg protection, two *spermathecae* (for long-term storage), and a *seminal receptacle* (the primary sperm storage organ) (Figure 1).

The reproductive system of male *D. melanogaster* consists of two testes each containing spermatozoa in various stages of development. The anterior ends of the testes comprise two *vasa deferentia,* which are the swollen regions of each testis that form the seminal vesicles. In addition, two *accessory glands*, to which the testes are joined, secrete seminal fluids that are key for sperm maintenance, for egg fertilization and for inducing female post mating responses. The duct of the accessory glands, together with *vasa differentia,* open into the ampullary part of the ejaculatory duct. During copulation the sperm is stored in the seminal vesicles, they enter the ejaculatory duct where they are mixed with the accessory gland protein, and thereafter transferred to the female genital canal (Shorrocks, 1972; Chen, 1984) (Figure 2).

Reproduction:

Mating initiates a series of events within the female reproductive tract, including ovulation and sperm storage. Female *D. melanogaster* receive approximately 4000 sperm per insemination. Some of these sperm are stored in the seminal receptacle (main sperm storage organ) and in the spermathecae. The migration of the sperm from the genital chambers to the storage organs is very rapid and is stimulated by the secretions of the accessory glands (Powell, 1997). Fertilization occurs once both the ova and the sperm reach the genital chamber. Females lay fertilized eggs often in clutches, on a soft substrate suitable for larval development. After embryogenesis, the larvae hatch from the egg and start immediately feeding on the soft substrate. The larvae transition through three larval instars, followed by pupation. The developmental time varies with environmental factors such as temperature and humidity.

Under optimal laboratory conditions, *D. melanogaster* require about 10 days (at 25°C) to develop from egg to adult. This is among the shortest developmental periods known for any drosophilid species (Markow and O'Grady, 2006). Generally, adults and larvae feed on the yeasts and bacteria growing on the substrate. Once adults eclose they fly off to search for food and mates. Females tend to have a greater requirement than do males for proteins (to develop eggs), while males require energy in the form of carbohydrates to search for mates and perform courtship (Powell, 1997).

Sexual conflict in *Drosophila melanogaster:*

A fundamental pre-requisite for sexual conflict in *D. melanogaster* is that females are promiscuous and can mate with more than one male within a reproductive cycle. As a result, sperm of different males come in contact in the female reproductive system, where they have to compete for fertilization of the ova (Imhof *et al.*, 1998). In males, selection is generally thought to have favoured an increase in gamete number at a cost of the contribution to per individual zygote resources (i.e. resulting in many small gametes). In females, selection generally favours the production of much larger ova size, which limits the maximum number of gametes (Trivers, 1972). These differing strategies, and the consequences that flow from them, can result in conflicts of interest. A strategy that is favourable for one sex may inflict costs in the other, leading to an evolutionary contest between the two sexes, whereby males seek to maximize their progeny output despite any cost this might incur in females, and females may therefore resist mating costs inflicted upon them by males (Rice, 1996). Male harassment may also be costly and harmful to females, contributing to sexual conflict. In *D. melanogaster*, males have also evolved reproductive strategies based on the effects of seminal protein transfer, the side effects of which can impose costs on females (Chapman *et al.*, 1995). Ultimately, this could lead to the evolution of reduced re-mating rates, which might benefit males but which could also reduce the indirect genetic benefits for females accrued via increased genetic variation in offspring.

1) Inter-sexual Selection

When males and females encounter each other, there may be some degree of choice about whether or not to mate. In *D. melanogaster,* selection is likely to be stronger among females to show discrimination in mate choice. This is because the female's contribution to ova exceeds male investment in sperm. Therefore, selective mating is likely to be exercised principally by female *D. melanogaster*, accepting males with which they wish to mate and repelling those of poor perceived quality. Males, on the other hand, do not appear to exert strong mating preferences (Merrell, 1949). This "selective mating" within *Drosophila* females acts both to minimise hybridization and to influence population genetic structure.

Male Behavior: Male *Drosophila* do not attempt to mate with a female without first performing a preliminary courtship display. This consists of a sequence of fixed-action patterns: first a mature male detects the presence of a female, then orients himself toward her, then follows her, taps with his forelegs, sings a species-specific courtship song by extending and vibrating one wing for few seconds and then continues the courtship behavior by licking her genitalia. Depending on whether the female accepts or rejects the male, mounting and copulation may (or may not) take place (Sturtevant, 1915; Yamamoto *et al.*, 1997).

In order to locate females and properly direct courtship, a male requires all of his visual, olfactory and auditory senses. Any mutation which alters these senses could affect quantitative and qualitative aspects of male courtship (Markow and Manning, 1980). For example, males with visual defects show markedly reduced competitive courtship success (Markow and Manning, 1980). One of the strongest stimuli given by the male comes from wing vibration, producing an acoustic signal (or love song), which diverts a current of air over the female antennae. In the case of blind mutant male *NorpA* (no receptor potential), an "inappropriate courtship" is displayed; these males performed the wing vibration oriented, not toward the female but to the location where she had been moments earlier. In addition, olfactory cues are also important for courtship, as male mutant *sbl* (*smellblind*) exhibit a delay in courtship and they frequently lick and attempt to copulate with the female's head instead of her posterior abdomen (Markow, 1987).

Female Behavior: female mating behavior in *D. melanogaster* comprises fewer complex elements. If receptive, female spread her wings and allows copulation to occur. If unreceptive, the female will refuse to mate and will give several rejection signals. These rejection behaviours differ between virgin and fertilized females. Virgins usually escape from courting males by decamping and exhibiting fending off behaviours, whereas the fertilized female typically extrudes her ovipositor, which physically prevents genital contact. Immature virgins also produce a rejection sound by flicking both wings (Yamamoto *et al*., 1997).

Sexual receptivity is influenced by the release of juvenile hormone in the haemolymph. In virgin females, sexual receptivity peaks 48 hours after eclosion, and starts to decline after 8 day (Manning, 1967). Female receptivity is reduced after copulation due to three factors: the **"copulation effect",** the "**sperm effect"** and the **"seminal fluid effect"** (Spieth and Ringo, 1983). **Copulation effects** refer to a decrease in receptivity due to copulation (Manning, 1967). The **seminal fluid effect** involves rejection of males by fertilised females triggered by receipt of seminal fluid proteins including Sex Peptide, which are transferred by males during copulation (Chapman *et al.*, 1995; Liu and Kubli, 2003). Finally, the **"Sperm effect"** refers to the inhibition of mating in *D. melanogaster* due to the presence of sperm in the seminal receptacles (Manning, 1967).

2) Intra-sexual Selection

Intra-sexual selection and harmful mating strategies are observed in species with greater last male mating advantage, such as is found in *D. melanogaster* (Lefevre and Jonsson, 1962). Male *D. melanogaster* compete with one another both at the pre-copulatory stage, when they attempt to mate with a female, and at the post-copulatory stage, when sperm compete within the female for fertilization of the ova. The different types of pre and post copulation strategies are described below:

- **Coercion:** The occurrence of coercive mating in *D. melanogaster* may account for at least some of the observed sperm competition in *Drosophila* (Manning, 1962). After their first mating, female *D. melanogaster* typically become unreceptive and tend to reject courting males for at least 24h (Manning, 1967). However, at least in the laboratory, males can often overcome this female rejection behavior and apparently drive females into re-

mating (Manning, 1962). In addition, sexually mature male *D. melanogaster* may also be able to make newly emerged females copulate with them, as such females appear incapable of performing rejection behaviours (Markow, 2000). In many cases, such coercive copulations appear to occur in the absence of courtship.

- **Contest competition:** Intense aggressive behavior has been detected between male *D. melanogaster* in situations in which access to females is limited. Male aggressive behavior consists of several offensive and defensive actions (Chen *et al.*, 2002). Male aggressive behavior patterns consist of an initial wing threat to their conspecific rivals, achieved by spreading, raising and twisting their wings, just before a very quick charge in which the aggressor usually rises on their hindlegs shortly before impact is made, followed by boxing, which compromises several variations of vigorous slashing and tapping with the front legs, often while both males have risen up on their hindlegs (Dow and Schilcher, 1975). Evidence suggests that the more successful a male *D. melanogaster* is in a fight, the fitter he is in terms of mating success (Dow and Schilcher, 1975).

- **Scramble**: Scramble competition among male *D. melanogaster* is important in determining mating success. Larger males have higher mating success, because they are more active and can move faster, and therefore encounter more receptive females, while also being more effective at tracking a moving female during courtship (Partridge *et al*., 1987).

- **Sperm competition:** The first observations of multiple paternity in insects were those of Nonidez (1920) who noted that, when female *D. melanogaster* mated with two males in close succession, they produced offspring that were sired by both males. Sperm competition is defined as "the competition within a single female between the sperm from two or more males for the fertilization of the ova" (Parker, 1970). This is due mainly to the capacity of females to store many hundreds of sperm and because of high rates of multiple inseminations. *D. melanogaster* males have adopted different strategies to increase their reproductive success then under the threat of sperm competition:

1. **Sperm quantity:** male *D. melanogaster* increase the number of sperms in their ejaculate when the perceived risk of rivalry is high. When males copulate in the presence of rivals,

the copulating males also increase their sperm allocation by 20% (Garbaczewska *et al*., 2013) and prolong their mating duration to increase their paternity share (Bretman *et al*., 2009; Kim *et al*., 2013). Males detect the presence of rivals using multiple senses: through hearing, smelling and touch (Bretman *et al.*, 2011). In addition, male *D. melanogaster* adjust their ejaculate sizes with respect to female mating status, female body size and female age. Male *D. melanogaster* deliver significantly more sperm when mated respectively to large, young or previously mated females compared with small, old or virgin females (Lüpold *et al.*, 2011).

2. **Sperm viability:** The greater the longevity of the sperm within the female, the higher the chances of fathering offspring. Sperm viability in *D. melanogaster* is influenced mainly by sperm storage effects influenced by the female (Snook and Hosken, 2004). *D. melanogaster* sperm have a lifespan of several weeks at ambient temperatures and can live even up to 3 months at 10°C in the female sperm storage organs (Muller and Settles, 1927; Parker, 1970).

3. **Mating plug**: To reduce the probability that their ejaculates will be displaced during a subsequent mating, male *D. melanogaster* deploy mating plugs. Mating plugs start to form via coagulation in the distal part of the female's reproductive tract as soon as ejaculate transfer occurs (Lung and Wolfner, 2001). The plug acts to facilitate sperm movement into storage, prevent a second insemination or prevent sperm loss. Hence the evolution of such mating plugs may also be shaped by natural selection, since males able to ensure minimum sperm leakage would have a selective advantage over others. The *D. melanogaster* mating plug is separable into two parts, the posterior region containing proteins “PEB-me, PEBII and PEBIII” that are secreted by the male ejaculatory bulb, and the anterior region composed of proteins, including Acp36DE, from the accessory glands (Lung and Wolfner, 2001; Chapman and Davies, 2004; Ram and Wolfner, 2005; Avila *et al.*, 2011). The plug may start to form before sperm transfer, so that initially the sperm may be located more caudally than the plug, passing through or around it (Lung and Wolfner, 2001). Shortly after its formation, the mating plug also starts to harden within the female reproductive tract, preventing sperm from subsequently flowing back down the female reproductive tract (Avila *et al.*, 2015).

4. **Sperm displacement/ sperm precedence:** One strategy used by males in sperm competition is to displace any previously stored sperm from within their new mates (Lefrve and Jonssom, 1962). In *D. melanogaster*, when females re-mate, the last male sires a majority of the offspring, generally out-competing the sperm from any previous mates (Boorman and Parker, 1976; Ellen *et al.*, 1984). This phenomenon is called last male sperm precedence, in which males compete by displacing and/ or incapacitating previously stored sperm (Price *et al.*, 1999). The mechanism behind sperm displacement appears to be physical, with incoming second-male sperm somehow physically displacing the resident sperm from the seminal receptacle and the spermathecae (Manier *et al.*, 2010). In *D. melanogaster* the proportion of offspring sired by the second male (P2) is estimated at between 0.86 and 0.96 (Gromko *et al.*, 1984).

5. **Pheromones:** The sexual attractiveness and receptivity of mature female *D. melanogaster* is often signalled via the release of pheromones (Jallon, 1984). In *D. melanogaster,* several pheromones are transferred by males to females during copulation that can influence the female's subsequent mating behavior (Ferveur, 2005). Some of these pheromones are known to act as an anti-aphrodisiac, reducing a female's attractiveness to other males, and therefore increasing a male's reproductive success. **1)** The **Cuticular hydrocarbon (7-tricosene)** is a major pheromonal component used by male *D. melanogaster* and it is usually absent from virgin females. During mating, male *D. melanogaster* transfer 7-tricosene externally to the female's cuticle. This pheromone reduces female attractiveness to any subsequent males for 3-4 hours after mating (Tompkins and Hall, 1981; Scott, 1986). However, females begin to generate and emit their own 7-tricosene 6 hours after mating, suggesting that the deposition of this anti-aphrodisiac by males reduces female attractiveness until the point at which she can synthesize her own pheromone (Scott, 1986; Scott and Jackson, 1988). **2)** It has been suggested that female attractiveness might also be regulated by the male-specific lipid **11-*cis*-vaccenyl acetate (cVA).** cVA is an acetate ester that is synthesized in the male ejaculatory bulb and transferred along with sperm during mating (Brieger and Butterworth, 1970). Once it is transferred into the female tract, the enzyme esterase 6 metabolizes cVA to produce the anti-aphrodisiac *cis-vaccenyl* alcohol. Both the alcohol

and the acetate reduce female attractiveness and the probability of further matings (Mane *et al*., 1983). However, subsequent experiments showed that the loss of cVAc from mated female is independent of Esterase 6, and that the transfer of cVAc to cis-vaccenol (cVOH) does not occur *in vivo* (Vander-Meer *et al*., 1986). Moreover, recent work suggests that cVA can elicit different behaviours in the two sexes. In males cVA inhibits courtship of other males, and in females there is an activation of receptivity to other males (Kurtovic *et al*., 2007). Additionally, cVA is shown to be a potent aggregation pheromone, attracting both males and females to substrates for feeding, mating and egg laying (Wertheim *et al*., 2001; Wertheim *et al*., 2006; Billeter and Levine, 2015; Billeter and Wolfner, 2018).

6. **Seminal fluids:** In *D. melanogaster*, the seminal fluids that are transferred from males to females during mating cause significant changes in female post mating behaviour, physiology and gene expression (Wolfner, 1997, 2007; Chapman, 2001). These seminal products can help to ensure reproductive success for both sexes and may include adaptations that have been shaped by sexual selection as well as sexual conflict. The functions of seminal fluid proteins are important cornerstone to this thesis and are described in greater detail in the following section.

Male Accessory Glands:

An early transplantation attempt of male *D. melanogaster* accessory glands into the abdomen of virgin females induced changes in female receptivity and oviposition (Merle, 1968). Such operations confirmed that male accessory glands induce characteristic behavioral and physiological changes in mated females. The male accessory glands form an important secretory tissue within the male reproductive system. They produce and secrete a complex mixture of accessory gland secretions (Chen, 1984). These secretions are transferred along with sperm during copulation. The seminal fluid components of the accessory glands induce specific behavioral and physiological responses in the female after mating (Chen *et al*., 1988;

Monsma and Wolfner, 1988), while others have been suggested to play a role in sperm storage (Tram and Wolfner, 1999) and sperm nutrition (Fowler, 1973).

In *D. melanogaster,* the non-sperm part of the ejaculate is composed of molecules synthesized by the secretory cells of the accessory glands, and by the secretory cells of the ejaculatory duct and bulb. The male accessory gland in *D. melanogaster* is composed of two morphologically and biochemically distinct secretory cell types surrounded by a muscular sheath: the binucleate "main" cells and the interspersed "secondary" cells (Figure 3), each of which expresses some shared but some distinct sets of genes (Bertram *et al.*, 1992). In mature pupae at about 1-2 days prior to adult emergence small secretory granules are detectable in the accessory gland cells. At emergence, both "main" and "secondary" cells are fully differentiated and initiate molecule synthesis. As the fly matures, the gland lumen accumulates a large quantity of secretions (Chen, 1984). The major proportion of the accessory gland epithelium is composed of a single layer of secretory main cells (96%), and the secondary cells account for only about 5-10% of the total epithelial cell population. The main cells are flat, hexagonal, binucleate cells that secrete their products into the gland lumen. The main-cell secretions were originally thought to be responsible for most of the changes in female behavior and physiology (Kalb *et al,* 1993) but an increasing role for the secondary cells is now becoming apparent. The secondary cells are located at the distal tip of each gland and are composed of several large vesicles containing filamentous bodies of bundles of tubules wrapped around a homogeneous proteinaceous core. It has been suggested that these tubules are involved in sperm storage (Chen, 1984; Monsma *et al*., 1990). The muscle cells that encase each accessory gland presumably act to squeeze the gland contents into the ejaculatory duct, where the semional fluid proteins mix with sperm and with other ejaculatory duct products before being transferred to females (Bertram *et al.*, 1992). Male accessory gland secretory activity is primarily regulated by the sesquiterpenoid juvenile hormone (Chen, 1984).

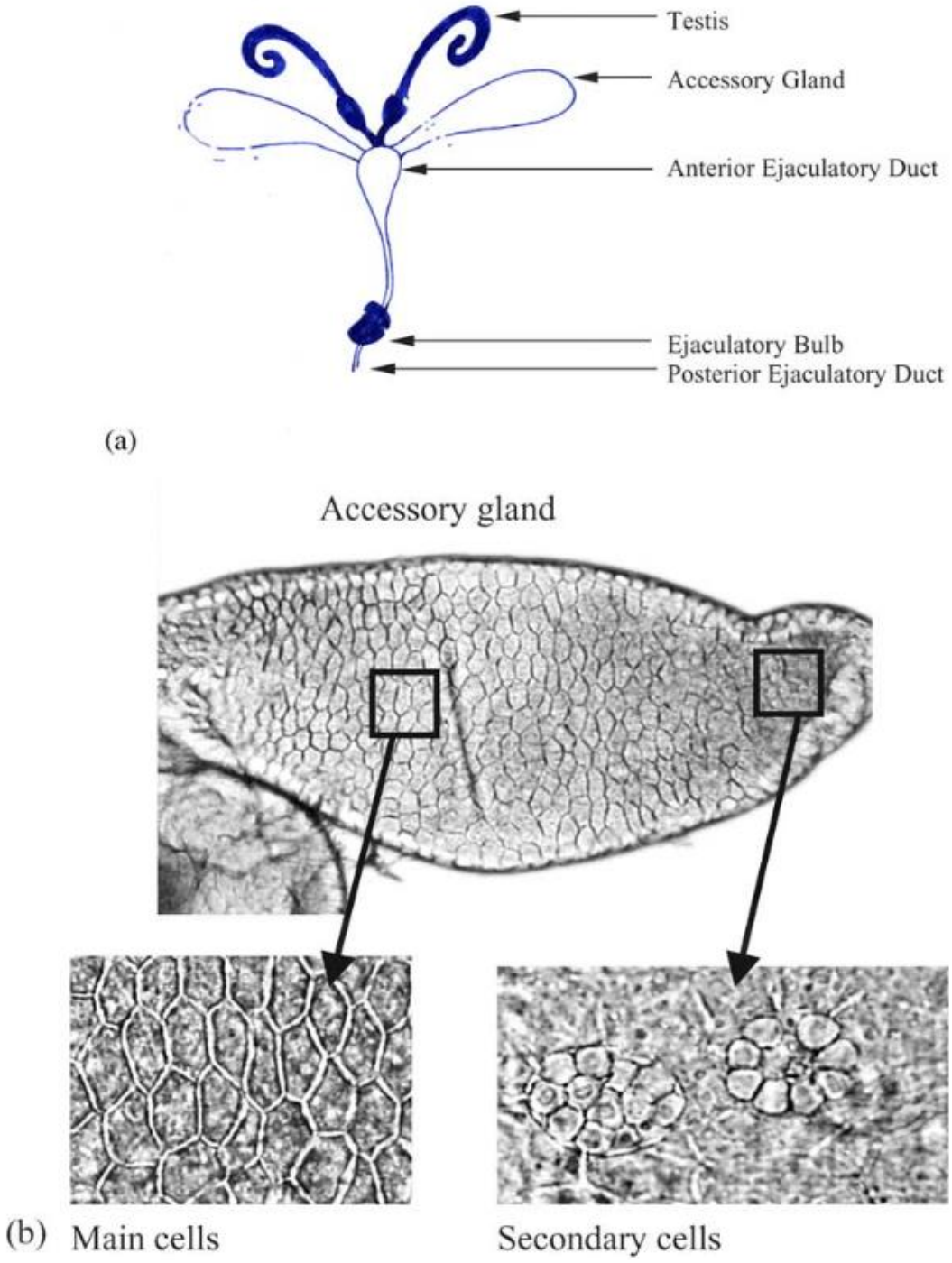


Figure 3: (a) Diagram of internal male *Drosophila melanogaster* reproductive system. (b) Male accessory gland, showing "main" cells and vacuolated "secondary" cells. Glands were dissected into phosphate buffered saline and images captured using a video camera at 100×magnification. After Chapman & Davies 2004.

Seminal fluid Proteins:

In *D. melanogaster,* sperm are transferred within the seminal fluid, which itself comprises of a variety of secretory products originating from the male accessory glands, the ejaculatory duct and ejaculatory bulb. The biological changes induced by the transfger of accessory gland proteins include decreasing receptivity in females to re-mating (Manning, 1962, 1967) increasing egg laying (Chen, 1984), increasing ovulation (Chen *et al.*, 1988), decreasing female lifespan (Chapman *et al.*, 1995), changing female feeding behavior (Carvalho *et al.*, 2006) and increasing the post-mating aggression exhibited by females (Bath *et al.*, 2017). The accessory gland proteins also contribute to mating plug formation (Lung *et al.*, 2001). The well-known genetic tools available for *D. melanogaster*, combined with its amenability for biochemical, physiological and genomic analysis, has made this species the most extensively studied with respect to accessory gland function.

Ejaculation of seminal fluids and sperm appear to be temporally separated events in *D. melanogaster,* within a copulation that normally lasts for approximately 20 min (Gromko *et al.*, 1984). The seminal fluid molecules from the accessory glands, ejaculatory duct and bulb can be detected in the females about 5 to 10 min after the start of copulation (Monsma and Wolfner, 1988). A gelatinous mating plug is formed within 7 min of the start of mating (Lung, Kuo and Wolfner, 2001). The first sperm do not appear to arrive in the female reproductive tract until 9-10 min after the start of mating (Fowler, 1973) and must, as noted earlier, migrate through or around the nascent mating plug. The sperm can be detected in the spermathecae 12 min after the start of mating but complete sperm storage can take up to 9 hours (Gromko *et al.*, 1984). During a single mating, up to the third of the stored accessory gland secretion may be transferred (Baumann and Angst, 1975).

It has been estimated that *D. melanogaster* possess approximately 163 seminal fluid proteins (Sfps) that are transferred from males to females during copulation, and many of these Sfps induce post-mating effects on females (Avila *et al.*, 2011; Sepil *et al.*, 2018). The functions of many Sfps is not yet known. However, those that have been characterized have marked effects on the reproductive success of males and females (McGraw *et al.*, 2004). The Sfps are named according to their cytological location on chromosomes. They are diverse and can range from small peptides to prohormone-like polypeptides and to large glycoproteins (Gillott, 2003;

Chapman and Davies, 2004). Table 1 contains a summary of the currently known Sfps and their functions, with further details of specific Sfps given in the following section. One of the best studied Sfps, on which I focus in this thesis, is Acp70A, also known as Sex Peptide.

Table 1: Summary of some of the known Accessory gland proteins and their functions

Trait	Acps	References
Simulating egg laying	Acp70A Acp26Aa CG33943 DUP99B	Herndon and Wolfner, 1995; Heifetz *et al.*, 2000; Chapman, 2001b; Chapman *et al.*, 2001; Saudan *et al.*, 2002; Chapman *et al.*, 2003; Ding *et al.*, 2003; Kubli, 2003; Liu and Kubli, 2003; Ram and Wolfner, 2007
Simulating ovulation	Acp26A	Herndon and Wolfner, 1995; Heifetz *et al.*, 2000
Reducing female sexual receptivity	Acp70A DUP99B	Chen *et al.*, 1988; Aigaki *et al.*, 1991; Saudan *et al.*, 2002; Chapman *et al.*, 2003; Ding *et al.*, 2003; Liu and Kubli, 2003; Chapman and Davies, 2004
Increasing sperm storage and sperm retention	Acp36DE	Bertram *et al.*, 1996; Neubaum and Wolfner, 1999; Chapman *et al.*, 2000
Reducing longevity	Acp70A Acp62F CG8137 CG10433	Lung *et al,* 2001; Wigby and Chapman, 2005; Mueller *et al.,* 2007
Increasing success in sperm competition	Acp26Aa Acp29AB Acp36DE Acp53Ea Acp62F	Clark *et al.*, 1995; Lung and Wolfner, 2001; Lung *et al*., 2001; Mueller *et al.*, 2008; Avila and Wolfner, 2009
Formation of the mating plug	Acp62F Acp76A CG6289 CG9334	Wolfner *et al.*, 1997; Ram and Wolfner, 2005
Changes to immune molecules	Acp70A	Peng *et al*., 2005; Domanitskaya *et al.*, 2007
Protection against proteolysis	Acp76A Acp62F CG8137 CG6289 CG9334 CG14560 CG10433	Coleman *et al.*, 1995; Ram and Wolfner, 2005

Sex Peptide:

In *Drosophila melanogaster,* Sex Peptide is encoded by the *CG17673* gene (FBgn0003034 or *Acp70A*), located on chromosome 3L. *Acp70A* is a single copy gene with 2 exons and a small intron (Cirera and Aguade, 1997; Gramates *et al.*, 2017). Sex Peptide is synthesized in the main cells of the male accessory glands as a 55 amino-acid precursor, which includes a hydrophobic 19 amino-acid signal sequence. The signal is cleaved off as the Sex Peptide is released into the accessory gland lumen, leaving the 36 amino acid mature form of the Sex Peptide (Chen *et al.*, 1988). Details surrounding Sex Peptide transfer are still unknown. However, at least some Sex Peptide appears to be transferred to females while bound to lipid rafts (Clive Wilson, pers. Comm.). Once in the female reproductive tract, Sex Peptide can be found free in the seminal fluid, or it can bind to sperm tails via its N-terminal end (amino acids 1 to 7). Sex Peptide free in the seminal fluid may be responsible for short-term post mating responses. The C-terminal end of Sex Peptide that is bound to sperm can subsequently be released by a cleavage that occurs between Sex Peptide amino acid sites 7 (Arginine) and 8 (Lysine), via an as yet unidentified trypsin (Pilpel *et al.*, 2008; LaFlamme *et al.*, 2012). Sex Peptide is then released from sperm and, together with free Sex Peptide, may enter the female circulatory system by crossing the posterior vaginal wall (Lung and Wolfner, 1999). The N-terminal part of Sex Peptide has been shown to activate Juvenile Hormone biosynthesis (Figure 4). The C-terminus of Sex Peptide is highly conserved, containing two cysteines that form a disulfide bridge, and is considered to play a major role in additional post-mating responses (Schmidt *et al.*, 1993). Once the C-terminal part of Sex Peptide enters the female's haemolymph, it activates specific Sex Peptide receptors located in a small subset of sensory neurons that innervate the female uterus and that project into the central nervous system, to elicit at least some of the varied post-mating responses to Sex Peptide receipt in females (Peng *et al.*, 2005; Yapici *et al.*, 2008).

The binding of Sex Peptide to sperm tails is interesting and it has been suggested that this may avoid degradation by proteases. In addition, longer sperm tails could, in theory, also retain more Sex Peptide, increasing male reproductive fitness. It is possible that a linkage between sperm tail length and Sex Peptide transfer could contribute to the extreme sperm elongation observed in some *Drosophila* species (Liu and Kubli, 2003).

The molecular interactions between males and females at mating significantly alter post-mating gene expression in females (McGraw *et al.*, 2008). In *D. melanogaster,* Sex Peptide is proposed to represent a master regulator. Once it is transferred to the female, it affects the expression of many genes, both in the head, thorax and abdomen of mated females (Domanitskaya *et al.*, 2007; Gioti *et al.*, 2012). In the head, Sex Peptide regulates genes coding for proteins involved in neurological process, behavior, metabolism, proteolysis, signal transduction, transcription and transport (Kubli, 2003; Carvalho *et al.*, 2006; Isaac *et al.*, 2010; Gioti *et al.*, 2012). In the abdomen, Sex Peptide is reported to alter the expression of antimicrobial peptide genes in the Toll and IMD pathways, as well as ovary development and signal transduction genes (Peng *et al.*, 2005).

Mating behavior specific to males and females is set during development and is determined by the sex-specific transcripts of the *fruitless* (*fru*) gene (Manoli *et al.*, 2005). However, another behavioral switch occurs in female *Drosophila* after mating and is triggered by Sex Peptide. Sex Peptide activates a specific receptor, the sex peptide receptor (SPR), which is expressed in the female reproductive tract and nervous system (Häsemeyer *et al.*, 2009). How Sex Peptide precisely regulates female post-mating behavior is still unknown. Sex Peptide receptor is a G-coupled-protein receptor (aka *CG16752*), which is required in neurons that express *fruitless, doublesex* and *pickpocket* (Haussmann *et al.*, 2013). Once activated, the Sex Peptide receptor triggers a post-mating behavioral switch in females (Yapici *et al.*, 2008; Häsemeyer *et al.*, 2009; Yang *et al.*, 2009; Kim *et al.*, 2010). This post-mating switch consists of increased egg production (Chapman *et al.*, 2003; Liu and Kubli, 2003), increased feeding, altered food preferences (Carvalho *et al.*, 2006; Ribeiro and Dickson, 2010), loss of sleep (Isaac *et al.*, 2014), decreased receptivity (Chapman *et al.*, 2003; Liu and Kubli, 2003) and stimulation of the immune system (Domanitskaya *et al.*, 2007). All these changes enhance current reproductive efforts but may also simultaneously impose mating costs on females (Wigby & Chapman 2005) presumably due to increased energy costs. The behavioral modifications induced by Sex Peptide typically last for approximately two weeks, which matches the approximate duration of maximal sperm storage for a male in a female from an initial mating (Avil *et al.*, 2015).

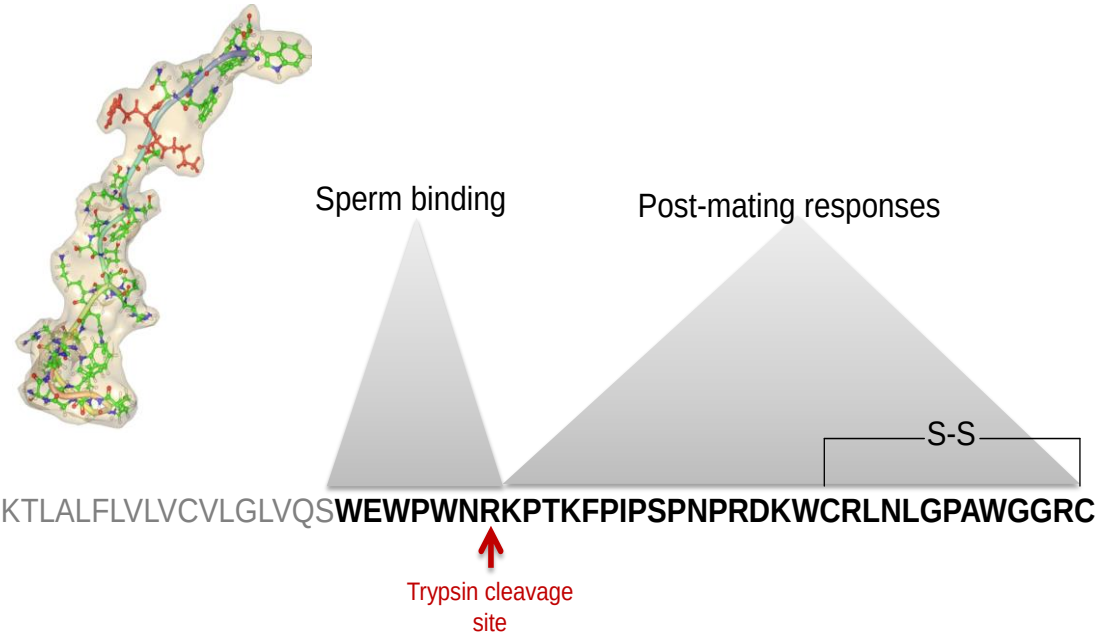


Figure 14: 3D representation and Amino Acid sequence of mature Sex-peptide. The amino acid sequence of mature Sex-peptide of 36 aa (in bold black), and the precursor of Sex-peptide of 19aa (Grey). After Domanitskaya et al., 2007.

Functions of Sex Peptide:

1. **Egg laying:** Sex Peptide stimulates egg laying in females for 7-10 days after mating. The juvenile hormone III-bisepoxide (JHB3) plays an important role in regulating oogenesis and is also involved in sexual maturation of virgin females (Kubli, 2003). When Sex Peptide is transferred to females during copulation, the N-terminal part stimulates the release of juvenile hormone III-bisepoxide (JHB3) from the *corpora allata*, which in turn stimulates the progression of the oocyte within the female ovary (Moshitzky *et al.*, 1996; Soller *et al.*, 1997, 1999).

2. **Receptivity:** Sex Peptide plays a major role in inducing changes in female receptivity. When females are injected with purified Sex Peptide in the abdominal cavity, or when Sex Peptide is ectopically expressed in females, there is a decrease in receptivity to mating that lasts 1-3 days. Normally mated females show a decrease in receptivity up to 7-9 days after mating (Chen *et al.*, 1988; Aigaki *et al.*, 1991). The longer persistence of receptivity reduction in mated females can be explained by the binding of Sex Peptide to sperm, in order to avoid degradation by proteolysis. This suggests that sperm are a necessary substrate for the slow release of Sex Peptide, prolonging its effects (Liu and Kubli, 2003; Chapman and Davies, 2004).

3. **Sleep behavior:** Sex Peptide also affects the sleep behavior of mated females. Both males and virgin females show periods of quiescence during both the light and dark phases of a 24 h light dark cycle. During a sleep period, they spend time conserving their energy by staying still and minimizing activity. In contrast, mated females show an increase in foraging and egg laying activity for 8 to 10 days after copulation and only display periods of quiescence during the night-time. This effect of wakefulness is not detected in females inseminated by Sex Peptide null (SP^0) males. This suggests that Sex Peptide induces lack of daytime sleep in mated females. It is also possible that SP-induced loss of sleep could be accompanied by direct stress related to the reduction in lifespan of mated females (Isaac *et al.*, 2010). It should be noted, however, in tests of sleep behaviour to date, females have typically been maintained under conditions that lack an oviposition substrate. Hence the increased wakefulness of females mated with SP^+ males may instead reflect their high egg load (and motivation to seek out oviposition sites) in comparison to females mated to SP^0 males, rather than a direct effect of Sex Peptide on sleeping behaviour itself.

4. **Sperm competition:** When the risk of sperm competition is perceived to be high (e.g., when other rival males are present in the environment or mating arena), mating males transfer more Sex Peptide to females during mating (Wigby *et al.*, 2009; Fricke *et al.,* 2010). Once in the female, Sex Peptide decreases female receptivity and stimulates egg production. Hence, by transferring more Sex Peptide, males ensure lower sperm competition, higher investment in current production of offspring and hence higher reproductive success (Wigby and Chapman, 2005).

5. **Feeding behavior:** Newly mated flies increase their food intake 2.3 times over that of virgins. This effect has also been attributed to Sex Peptide transfer during copulation (Carvalho *et al.*, 2006). However, this increase in feeding could also be indirectly related to the nutritional demands associated with increased egg production (Barnes *et al.*, 2008). Interestingly, Sex Peptide also triggers changes in the food preferences of mated females, as following copulation and Sex peptide transfer, females consume significantly more protein-rich food, such as yeast (Ribeiro and Dickson, 2010).

6. **Longevity and fitness:** Sex Peptide mediates a cost of mating in females. When female *D. melanogaster* are continually exposed to wild-type males throughout their lifetimes, they show significantly lower fitness and lower reproductive success compared to females exposed to Sex Peptide lacking males. Thus, continual receipt of Sex Peptide throughout life can decrease female fitness and lifespan (Wigby and Chapman, 2005; Mueller *et al.*, 2007). However, the mechanism(s) by which Sex Peptide decreases female longevity are still unknown as are whether lower exposure to Sex Peptide is less costly or even beneficial to females.

7. **Immune response:** Sex Peptide induces a post-mating immune response. It is suggested that Sex Peptide does this by chemical mimicry of sugar components of the bacterial cell wall. Thus, Sex Peptide may induce the immune system via pattern recognition receptors, which in turn activate the transcription of antimicrobial peptide genes in the epithelial tissue of the female abdomen (Peng *et al*., 2005; Domanitskaya *et al.*, 2007). The interaction between Sex Peptide and the immune response is discussed in more detail in chapter 3.

8. **Aggressiveness:** Sex peptide transfer also induces female aggression directed toward other females. Thus, Sex Peptide influences the female social competitive environment, with potentially important fitness consequence for females and their offspring (Bath *et al.*, 2017).

Having described the reproductive arena in which Sex Peptide has its effects, I now describe the broader evolutionary processes that may shape the evolution of Sex Peptide and its functional effects.

Genetic Variation:

Various biotic and abiotic factors can result in evolutionary change, leading to variation in allele frequencies within and between populations. In natural populations, this genetic variation is influenced by many factors such as mutation, selection, genetic drift, gene flow and genetic shuffling. The main source of genetic variation is mutations such as **in**sertions or **del**etions (INDELs) of DNA bases, or single nucleotide polymorphisms (SNPs). Mutations are any

"heritable change in genetic material, which can be a change in nucleotide sequence as well as the formation of a chromosome rearrangement" (Hartl and Clark, 2007). Thus, mutations create new alleles that contribute to genetic variation within the gene pool. This genetic variation provides the raw material for natural and sexual selection. Mutations can be harmful, neutral or beneficial. Most SNPs have minor effects, in contrast to the larger changes brought about by INDELs. Several models explain the maintenance of the genetic variation by means of selection among populations. The ***classical hypothesis*** asserts that genetic variation consists mostly of harmful mutant alleles, hence natural selection on populations exerts predominantly purifying or negative selection, leading to an overall reduction in genetic variation. The ***balancing hypothesis*** holds the view that genetic variation is abundant and that many beneficial mutant alleles are maintained in the population by positive or balancing selection. Both schools are consistent with Darwin's **natural selection theory**, whereby beneficial mutations affect the organism's fitness by enhancing survival and reproduction, the progressive genetic improvement increases gradually from generation to generation, which constitutes the process of **evolutionary adaptation**. In opposition, genetic variation in a population in the absence offitness effects is not subject to selection, yet it is preserved, and the populations evolve under **random genetic drift.** The ***neutral theory*** proposed that selectively neutral genetic variation is maintained by a balance between the rate of neutral mutations and random genetic drift. In this case genetic variation has no significant effect on the ability of an organism to survive and reproduce (Hartl and Clark, 2007).

The gap in our understanding for the relationship between genotype and phenotype partly results from the very complex interactions that occur between genes and the environment. With the advent of next generation molecular sequencing methods, it is now possible to study both the genotype and phenotype, and to characterize the genetic variation that occurs within a population. With the availability of full genome sequencing data, genome-wide associations can be used to search the whole genome for mutations or other genomic features that have effects on phenotypes. This process is termed "adaptation genomics" (Hartl and Clark, 2007). To determine if the genetic variation present in a population is due to adaptive evolution and not genetic drift, it is common to screen for positive selection in protein coding regions. This is often done by measuring the rate of change in terms of sequence substitutions. Substitutions can occur at the genome or protein level. Some substitutions at the genome level (nucleotides)

result in alterations to protein sequences (amino acid changes): these are **non-synonymous** substitutions. Those that do not alter the protein sequence are known as **synonymous** (or silent) substitutions. The evolutionary rate of change can be estimated by comparing them (Zuckerkandl, 1976; Kimura, 1980). Synonymous substitutions (*dS*) give the number of silent substitutions per synonymous site, thus providing an estimate of substitutions under neutral evolution (genetic random substitution). Non-synonymous substitutions (*dN*) give the number of amino-acid substitutions per non-synonymous site. The parameter $\omega = dN/dS$ thus measures the rate of protein evolution. ω has been defined to describe the type of selection on the protein-coding sequences: $\omega=1$ indicates neutrality, $0 \leq \omega<1$ indicates stabilizing selection and $\omega>1$ indicates directional selection (Larracuente *et al.*, 2008).

In *D. melanogaster*, genes such as those encoding seminal fluid proteins that are involved in reproductive processes, can evolve extremely rapidly. This could be the result of sexually antagonistic co-evolution between the sexes, but also due to ecological conditions. Interestingly, in *D. melanogaster*, the genes encoding male accessory gland proteins are among the most rapidly evolving genes known in the melanogaster genome (Swanson et al 2001). The evolutionary rate of change of non-secreted and non-reproductive proteins is significantly slower in comparison to that of secreted accessory gland proteins and proteins involved in reproduction in general (Kern *et al.*, 2004). Several studies have demonstrated that the accessory gland proteins and reproductive proteins have high levels of variation and display non-neutral patterns of evolution (Civetta and Singh, 1995; Swanson and Vacquier, 2002). In many cases, the high levels of variation are driven by adaptive evolution (positive Darwinian selection), which may suggest that genetic diversification is beneficial for reproduction. This could be due to within- and between-sex interactions within sexual selection. For example, a study by Chow et al (2010) demonstrated that the third chromosome (which contains the *Sex Peptide* gene) and the X chromosome (containing the *Sex Peptide Receptor* gene), both have a large effect on fertility and female receptivity, and both harbour high levels of genetic variation. Furthermore, Sex Peptide also shows variation in expression level that correlates with female post-mating behaviours such as egg laying and receptivity (Smith *et al.*, 2012). It is suggested that this variation in expression level could be maintained by regulatory mechanisms (such as transcription factors) (Smith *et al.*, 2009). However, further studies are essential to reveal the

whole Sex Peptide cascade, before we will fully understand the evolution of this important system.

In *D. melanogaster,* the genes involved in the immune response also show significant genetic variation, driven by adaptation to local differences in pathogen exposure, environmental conditions and demographic factors (Tinsley *et al*, 2006). This genetic variation is maintained by temporal and spatial variation in the costs and benefits of pathogen defense. In mated female *D. melanogaster,* the resistance to costs mediated by males is also considered to be an important component of female fitness, both because males transfer accessory gland proteins (e.g. Sex Peptide) that affect female survival, and because mating alters female immune responses (Short and Lazzaro, 2013). Once Sex Peptide is transferred to females, it triggers a nonspecific immune response by activating the transcription of several antimicrobial peptides. However, exactly how Sex Peptide induces the transcription of antimicrobial peptides is still unknown and requires further investigation.

To investigate the functional biology of genomic features that are likely to be involved in adaptive evolution, here of the reproductive system, functional genetic approaches can be used. **Functional genomics** refers to the relatively new field that focuses on understanding how genomic variation can affect ecological success and the evolutionary fitness of natural populations. In these approaches, gene expression assays have been used to identify genetic variation underlying specific phenotypic traits (Feder and Mitchell-Olds, 2003).

Aims of the thesis:

Through the deployment of seminal fluid proteins such as Sex Peptide, *D. melanogaster* males have a direct and global influence on the physiology, behaviour, reproductive and immune systems of the female after copulation. This ultimate control supports the idea of Sex Peptide as a "master regulator" of female post-mating responses. In most studies to date, genetic variation underpinning the Sex Peptide pathways in females has been minimized in order to clearly delineate Sex Peptide function. However, in this thesis, I used a genomics approach to investigate the extent and nature of the genetic variation in Sex Peptide influenced traits in mated females as well as the in genetic variation for Sex Peptide transfer by males. In doing this,

I aimed to shed light on the potential evolutionary changes in female post-mating behaviours such as immune, egg laying, receptivity and starvation lifespan responses.

By comparing fully sequenced lines from the Drosophila Genetic Reference Panel (DGRP), with respect to male release of, and female responses to, Sex Peptide, I was able to map these phenotypes to genomic variation and to functionally annotate the genomic variation identified.

In **chapter 2**, I characterized genomic variation in the male release of Sex Peptide. To do this, I developed a novel protein quantification method that combined both ELISA and Q-PCR methods. This technique showed high sensitivity and I was able to accurately quantify and detect significant variation in Sex Peptide transfer among males from the DGRP lines tested. This phenotypic variation was fed into a Genome Wide Association Study (GWAS), which revealed a number of polymorphisms in genes that are candidates for direct or indirect links to Sex Peptide development and transfer.

In **chapter 3**, I measured genetic variation in female immune changes mediated by Sex Peptide. Previous studies have shown that Sex Peptide induces an immune response in females following mating, reflected in the expression of anti-microbial peptides (AMPs). I measured the variation in AMP loci, using Q-PCR, in virgin females and in females mated to males with or without Sex Peptide. The GWAS, performed on the AMP variation when females were mated to males with Sex Peptide, revealed a number of candidate genes involved in the Sex Peptide immune response.

In **Chapter 4**, I characterized genomic variation in female phenotypic responses to Sex Peptide. More specifically, I tested the effect of Sex Peptide on female egg laying, receptivity and starvation lifespan. A GWAS was then performed on all these phenotypic traits, which revealed a set of candidate genes for influencing female behavioral changes following mating and Sex Peptide receipt.

In the final **chapter 5**, I compared the candidate genes that potentially influence male release of Sex Peptide versus female responses to it. I synthesized the results of my research by combining all the genes predicted by the different GWAS analyses to look for commonalities and hence to determine whether the genome-wide basis of Sex Peptide responses differs

between different traits and across the two sexes.

Altogether, this thesis provides significant new insight into the genome-wide signatures associated with the effects of Sex Peptide and highlights candidate genes of interest for further exploration.

Statement of contribution

All work detailed in the thesis was conducted and written by myself under the supervision of Professor Tracey Chapman and Professor Bregje Wertheim. The bioinformatics statistical analysis in Chapter 4 was conducted with the help of Dr Wayne Rostant.

A novel immuno-Quantitative PCR assay for quantifying Sex Peptidein Drosophila melanogaster

Jessy Rouhana, Bregje Wertheim, Tracey Chapman

Abstract

The primary function of copulation is successful fertilization, and this process can be aided by the effects of seminal fluid proteins (Sfps) transferred from males during mating. However, intriguingly, Sfps can also favor the interests of males whilst generating costs in females, resulting in **sexual conflict**. This manipulation of one sex by the other through molecular interactions has been well studied in the fruit fly ***Drosophila melanogaster.*** One enigmatic Sfp known as **"Sex Peptide"** generates strikingly diverse changes in the behavior, reproductive and immune system of females. Collectively, these effects can benefit males and/or females, but sometimes they also cause costs in females due to 'over-investment'. Hence Sex Peptide is a potential mediator of sexual conflict. Sexual conflict is predicted to fuel the generation of genetic variation. This predicts the existence of significant genetic variation in natural population among males in the ability to synthesize and release potential agents of sexual conflict, such as Sex Peptide. To test this idea of existing variation in the wild, we screened for variation among males in the quantity of Sex Peptide released during mating into females, in 31 inbred, genome-sequenced lines of the Drosophila Genetic Reference Panel (DGRP). We did this by developing and then applying a novel molecular technique, known as **Immuno-Quantitative PCR**, which combines the use of antibodies with quantification by real-time PCR. The use of the DGRP lines also allowed us to perform a genome wide association (GWAS) analysis on the quantity of Sex Peptide released into females during mating. We found significant variation in Sex Peptide release and significant associations in the GWAS. Gene ontology analysis of GWAS candidates highlighted significant over-representation of genes involved in *Drosophila melanogaster* reproduction. Several of the top-ranking genes from the GWAS are involved in development and reproduction, specifically in the development of germ cells. Other top associated genes were of unknown function and represent promising candidate genes for further study of Sex Peptide synthesis and transfer. The results of this study suggest that there is significant variation in male Sex Peptide release and highlights potential candidate genes involved.

Keywords

Sex Peptide, Immuno-Q-PCR, *Drosophila melanogaster*, DGRP, GWAS, Sexual conflict

Introduction

Successful reproduction requires cooperation of both sexes. However, males and females have adopted different strategies to maximise their reproductive success. This has set the scene for potential battles between the interests of males and females that may have significant evolutionary impacts. The agents of sexual conflict include seminal fluid proteins transferred along with sperm during mating. These proteins not only help nourish sperm during transfer, but also play role in female reproductive behaviour as well as having effects on offspring performance (Martan and Shepherd, 1976; Robertson *et al.*, 2014; Sirot *et al.*, 2015). Males of different species transfer a large number of seminal fluid proteins to females during mating: varying between 50 and 160 in insects (Baer *et al.*, 2009; Rogers *et al.*, 2009; Sirot *et al.*, 2011; Sepil *et al.*, 2018), 766 proteins in mice (Dean *et al.*, 2009) and 923 proteins in humans (Pilch and Mann, 2006). Interestingly, many of the seminal fluid proteins described across different taxa tend to feature the same types of proteins such as proteases and protease inhibitors, sugar-binding lectins, cysteine-rich secretory proteins, antimicrobial, and coagulation proteins such as transglutaminases, small peptides (Sirot *et al.*, 2015). Seminal fluid proteins have been extensively studied in just a few species and for most of these seminal fluids their full functions and potential involvement in sexual selection and sexual conflict is yet to be discovered.

The fruit fly *Drosophila melanogaster* is a powerful model system for study, in which the cryptic battles between the actions of seminal fluid proteins and their resulting responses in females have been well researched. These proteins have long lasting effects on females after mating, including on fertility, immunity, libido, eating and sleep (Gillott, 2003; Liu and Kubli, 2003; Wigby and Chapman, 2005; Ram and Wolfner, 2007; Avila *et al.*, 2011). Accompanying these striking changes is the activation of diverse sets of genes in response to the receipt of seminal fluid components that accompany sperm (Gioti *et al.*, 2012). Collectively, these effects often favour the interests of males, increasing the number of offspring that are produced, whilst sometimes simultaneously generating costs in females in terms of their lifetime reproductive success and survival, resulting in sexual conflict (Chapman *et al.*, 2003). This can generate a tug-of-war, in which males employ seminal fluid proteins as a mechanism to ensure that females increase their investment in the current brood or withhold from re-mating, even if this would not suit the longer term interests of females (Bretman *et al*., 2009).

In *D. melanogaster,* one key player in this sexual conflict is Sex Peptide, which is transferred into females, with some Sex Peptide then binding to sperm tails and some remaining free along with other seminal fluid proteins (Peng *et al.*, 2005). Once in the females, the free Sex Peptide appears to be responsible for short-term post-mating responses, while the sperm-bound Sex Peptide is stored along with sperm in the spermathecae and seminal receptacle. This source may prolong the persistence of Sex Peptide effects in females (Ram and Wolfner, 2009; Findlay *et al.*, 2014). Locally, Sex Peptide influences the rate of sperm release from storage. When it is cleaved and released from the sperm tail into the haemolymph Sex Peptide generates a wider range of post-mating responses (Pilpel *et al.*, 2008; Yapici *et al.*, 2008). These include an increase in egg laying, increase in feeding behaviour, altered immunity, decreased female receptivity to re-mating, decreased sleeping behaviour and increased post-mating aggression in females (Manning, 1967; Chen *et al.*, 1988; Liu and Kubli, 2003; Carvalho *et al.*, 2006; Barnes *et al.*, 2008; Wigby *et al.*, 2008; Isaac *et al.*, 2010; Avila *et al.*, 2011; Kubli and Bopp, 2012; Isaac *et al.*, 2014; Bath *et al.*, 2017) This suggests that Sex Peptide is a 'master regulator' of female reproduction. Hence males effectively have a mechanism by which they can direct and globally influence the behaviour, reproductive and immune system of the female.

The genes encoding semen proteins often evolve rapidly and there is significant genetic variation in the Sex Peptide gene (Cirera, 1997) and in Sex Peptide-mediated traits (Wigby *et al.*, 2009; Short and Lazzaro, 2010). This is consistent with the idea that Sex peptide is involved in mediating sexual conflict and sexually antagonistic coevolution between the sexes (Rice, 1996). Therefore, the genetic variation observed in these traits may also, at least in part, be shaped by sexually antagonistic coevolution. In most studies of molecular mechanisms of Sex Peptide action, genetic variation has been experimentally minimised in order to clearly delineate function. However, to understand the evolutionary processes and dynamics that characterise Sex Peptide mediated interactions between males and females, the next key step is to quantify the amount of Sex Peptide that males produce, transfer to females during mating. This will help detect variation in Sex Peptide effects in detail.

With the latest advances in DNA technology and genome sequencing it is now possible to trace the influence of Sex Peptide on the expression and intensity of sexual conflict with ever-greater resolution. High-throughput sequencing technologies have provided a resource of

>200 fully genome-sequenced *D. melanogaster* lines (MacKay *et al.*, 2012). These lines and their genome sequences are publicly available and are a formidable resource for Genome Wide Association Studies (GWAS) to link gene variation to phenotypic variation. Here we used 31 DGRP lines of the DGRP core set, which captures most of the genetic variation existing in these strains, to identify alleles associated with observed Sex Peptide phenotypes (Chow et al., 2013). To understand the impact of the sexual conflict on the evolution of the fruit fly genome it is important to understand the role of genetic variation in the production and release of Sex Peptide. When the allocation of Sex Peptide to females varies between different lines, this may select females to mount different levels of resistance to Sex Peptide in order to minimise or regulate their post mating responses.

Our specific focus here was in quantifying variation in Sex Peptide release from males and associating this genetic variation in Sex Peptide transfer to allelic variations in the genome, using lines of the *Drosophila* genomic reference panel (DGRP) (MacKay *et al.*, 2012). For this we developed a novel application of a new technique, namely immuno-Q-PCR, giving high sensitivity to quantify Sex Peptide. The technique uses the specificity of antibodies for detecting the presence of Sex Peptide combined with the unparalleled sensitivity of quantitative PCR for accuracy of quantification of conjugated oligonucleotides. The immuno-Q-PCR method achieved a rapid, sensitive and accurate quantification of Sex Peptide in females immediately after mating. The quantified Sex Peptide was then used to assess the variation among males in Sex Peptide transfer during mating.

Material and Methods

Stocks and fly culturing

Male *D. melanogaster* used in this experiment were from the core set of the *Drosophila* genomic reference panel (DGRP). These are isogenic lines that were set up from a natural population from North Carolina, USA (MacKay *et al.*, 2012). The DGRP lines and their genome sequences are publicly available and therefore represent a great resource for identifying the underlying genomic basis of phenotypic variation. The females used in these assays were from the wild type Dahomey strain. The flies were reared on a standard yeast agar medium (100g yeast, 50g sucrose, 15g agar, 30ml Nipagin solution and 3ml Propionic Acid) and maintained at 25°C and 50% humidity on a 12Light: 12Dark cycle.

Within 8h of eclosion, flies were separated by sex (on ice). Virgin female Dahomey were pooled in groups of 10, while the male DGRP flies were kept in individual vials. They were maintained for 4-5 days on standard yeast agar medium. The samples needed for this experiment were generated by individually crossing males from each of the 31 different DGRP lines to individual virgin wild type Dahomey females. All mating assays were done on the same day to avoid potentially confounding environmental effects. Time of introduction of the females until start of mating (latency) and time of start and end of mating (duration) was recorded for each of the pairs. Only matings that lasted at least 5 min were scored as successful and pairs that did not mate within 4 hours were discarded. We tested for line variation in mating latency and mating duration, as these could contribute to variation in transfer of Sex Peptide. Immediately after the end of mating females were separated from the males and were flash frozen in liquid nitrogen and stored in -80 °C until protein extraction.

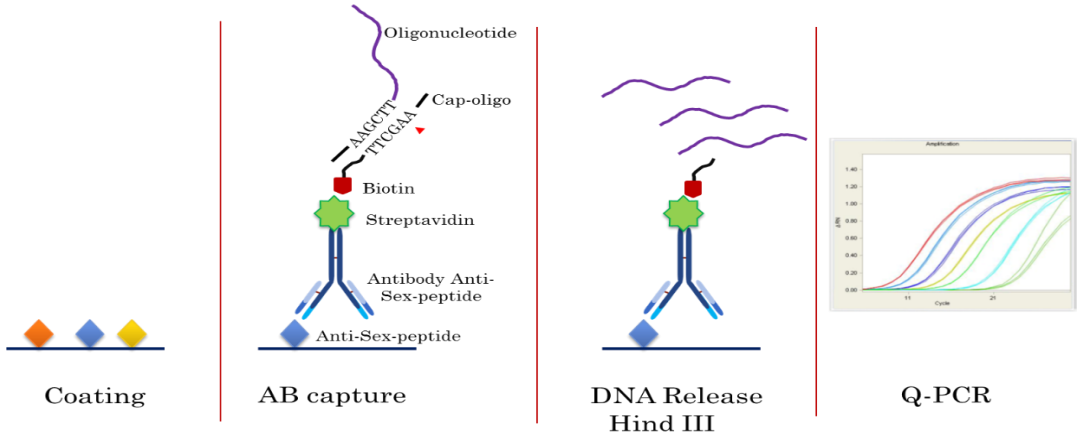
Protein extraction

To reduce sample variation, we pooled sets of 3 Dahomey females that had been mated to males from the same DGRP line. The frozen female flies were then homogenized using an electric pestle (VWR International, Inc., Bridgeport, NJ) for 30sec at 4°C in 200ul of filtered homogenising buffer consisting of 5% Dulbecco's phosphate buffering solution (DPBS; 14mM NaCl; 0.2mM KCl; 0.1mM KH2PO4; 0.7mM Na2HPO4) and protease inhibitors (PI; Roche Complete protease inhibitor cocktail tablets). Once homogenised another 200µl of the homogenising buffer was added (final sample volume 400µl). The samples were then

centrifuged for 2min 12000rpm at 4°C through a Costar column (Corning® Costar® Spin-X® centrifuge tube filters) to purify the sample from debris, such as membranes, wings and cuticles. All the protein samples were extracted on the same day and stored at -20°C until absolute quantification.

Quantification of sex peptide release by immuno-Q-PCR assay

A diagram of the principles and major steps of the immuno-Q-PCR assay is shown in Figure 1. Briefly, each well of the ELISA plate (BD flacon 35327) is coated with a sample of the fly protein extract. After washing with block solution (1%BSA, 1xDPBS, Tween 0.05%), the protein samples are incubated with an anti Sex Peptide antibody tagged with a quantification oligonucleotide. After a washing step to clean the wells of any unbound construct, the quantification oligonucleotide is then released by HindIII restriction enzyme digestion and quantified by Quantitative-PCR. The amount of the quantification oligonucleotide is proportional the amount of Sex Peptide in each of the samples in the wells.



<u>Figure 1</u>: Schematic diagram summarising the main steps of the immuno-Q-PCR used to quantify Sex Peptide release. ELISA plates were first coated with the fly protein extract. In the next stage an oligonucleotide labelled antibody Anti-Sex Peptide complex is applied to the plate to recognise and bind to the Sex Peptide. The oligonucleotide is then released from the antibody complex using a Hind III restriction enzyme digest. In the final step, the released oligonucleotide is quantified by using Q-PCR.

Antibodies, peptide, oligonucleotides and primers

The anti Sex Peptide polyclonal antibody and synthetic Sex Peptide were generated by a commercial provider (Eurogentec, Seraing, Belgium). A Sex Peptide specific region H-CKPTKFPIPSPNPRD-NH2 was synthetized and an anti-Sex Peptide polyclonal antibody was

generated against this region in rabbit. Quality control of the produced antibody was ensured by using an ELISA assay (Eurogentec, Seraing, Belgium). We designed a single-stranded synthetic oligonucleotide (74bp) for quantification, avoiding matches with any known sequence of any living organism, to avoid amplification of any contaminants. The oligonucleotide was designed to share a 13bp complementary sequence with the capture oligonucleotide (22bp) that was conjugated to the antibody, and a HindIII restriction enzyme site, to allow its release. The capture oligonucleotide was biotinylated at 3' end by the manufacturer. All the probes and the antibodies were synthesised by Eurogentec, Seraing, Belgium (Table1). For the qPCR reaction, a primer set was designated that amplified the quantification oligonucleotide.

Table1: Peptides, oligonucleotide and primer sequences for the immuno-qPCR assay. Underlined is the complementary sequence between the capture oligonucleotide and the oligonucleotide.

Synthetic Sex Peptide	15aa	H-CKPTKFPIPSPNPRD-NH2
Capture oligonucleotide	22bp	5'-TGG ATC CTA AGC TTG AGC ATT T-3'*Biotin
Quantification oligonucleotide	74bp	5'-TGC TCA AGC TTA GGA TCC ATA GCG TGT ACC ATG TAA ACC TTA TAA CTT ACC TCA GAC TAG TTG GAA GTG TGG C-3'
Forward Primer	23bp	5'-CCA TAG CGT GTA CCA TGT AAA CC-3'
Reverse Primer	21bp	5'- GCC ACA CTT CCA ACT AGT CTG-3'

Streptavidin conjugation to the anti Sex Peptide antibody

The anti-Sex Peptide antibody was conjugated to streptavidin using the Lightning-Link Streptavidin Conjugation Kit (Innova BioSciences Ltd., Cambridge, UK) following the manufacturer's instructions. 100ml of antibody (1.075mg/ml) was mixed with 10ml of LL-Modifier reagent and then the mixture was added to a vial containing 100mg of Lyophilized LL-streptavidin. After 3h incubation at room temperature (RT), 10ml of LL-quencher reagent was added. After 30 min of incubation at RT the conjugate was stored at -18°C.

Binding the capture oligonucleotide to the quantification oligonucleotide

A 10μl of biotinylated capture oligonucleotide (10μM, $5.6x10^{15}$ copies/μl) was incubated for 15 min at 48°C, with 100μl of the quantification oligonucleotide (10μM, $5.75x10^{14}$ copies/μl). The complex was then diluted 1/10 (final concentration approximately of $5.7x10^{13}$ copies/μl) with DNAse RNAse free water and was hybridised to the antibody. The binding of the capture

oligonucleotide to the quantification oligonucleotide was tested by electrophoresis on a 2% agarose gel.

Binding the antibody to the oligonucleotides complex

The oligonucleotide complex was then linked to the antibody against Sex Peptide by using the chemical interaction of streptavidin-biotin. The antibody, at a concentration of 1.34µg/µl, was incubated at room temperature for 1h with the oligonucleotide complex 0.0224µg/µl ($5.7x10^{13}$ copies/µl). After diluting the antibody-oligonucleotides complex 1/800 in a filtered block solution (1%BSA, 1xDPBS, Tween 0.05%) it was used in the immuno-Q-PCR. The binding of the antibody to a higher concentration of the oligonucleotide ($5.7x10^{14}$ copies/µl) was tested by electrophoresis on a 2% agarose gel.

Standard curve of the synthetic Sex Peptide

A standard curve for the immuno-Q-PCR was constructed from a series of dilutions of purified synthetic Sex Peptide (Table 1), as well as a no template control (NTC). Synthetic Sex Peptide at 2mM, 1mM, 0.5mM, 0.05 mM and 0.0005mM was run in parallel to the biological samples on every immuno-Q-PCR plate as a reference, to generate the standard curve for the absolute quantification of Sex Peptide. Each dilution was tested in triplicate on each plate. Several Immuno-Q-PCR experiments were required to optimise the assay and the slope of the standard curve. The reproducibility of the assay was tested by the repeatability of the C_t values of the standard curve, on each of the ELISA/Q-PCR plates. The threshold cycle (C_t) was calculated automatically by the ABI7300 software and represents the cycle number at which the fluorescence passes a set threshold. As part of the optimisation process, we tested the for repeatability by replicating the same biological samples on different ELISA plates, followed by Q-PCR, (supplementary data, Figure 1).

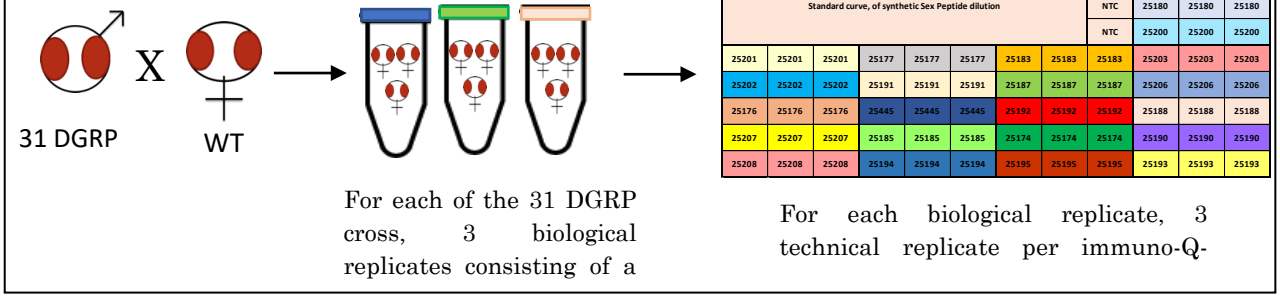


Figure 2: **Schematic diagram summarising the experimental setup.** Males from 31 of the core DGRP set were mated to wild type (WT) females and x h after the end of mating, females were frozen and subjected to the immuno-qPCR, with the qPCR plate design shown on the right (numbers indicated DGRP lines tested).

Immuno-Q-PCR protocol

After optimisation of the conditions of each step, the immuno-Q-PCR was performed according to the following detailed protocol:

(i) Coating

Microtitre ELISA plates were coated with 50µl of sample protein extract or synthetic Sex Peptide. The plates were covered and incubated at 4°C overnight on a shaker. After removing the effluent, the wells were washed with 100µl of wash solution (1xDPBS, Tween 0.05%). The bound samples were then blocked with 100µl of block solution (1%BSA, 1xDPBS, Tween 0.05%) and incubated overnight on a shaker at 4°C. Afterwards the block solution was removed via a washing step with wash solution. Then 50µl of the complex antibody oligonucleotide was added and incubated for another hour at RT. Lastly, the antibody-oligonucleotide complex was removed and the wells washed 3 times with 150µl of wash solution. For each sample, 3 technical replicates were tested on the same Immuno-Q-PCR plate and 3 biological replicates were tested on different immuno-Q-PCR plates (Figure 2). A total of 4 plates were required and each plate included a standard curve in triplicate.

Hind III restriction

The oligonucleotide was released from the complex prior to Q-PCR by a HindIII restriction enzyme digestion. After washing the wells, the oligonucleotide was digested with 1U of Hind III (Promega, Madison, USA) for 1 hour at room temperature. The enzyme was then inactivated by incubating 15 min at 65°C. The eluted material was collected and stored at -18°C until needed.

Quantitative Polymerase Chain reaction

The Q-PCR was performed on 2µl of the eluted oligonucleotide using an Applied Biosystems 7300 machine. The Q-PCR was conducted in a total volume of 20µl, with 1µl of forward and reverse primer (Table 1) and 0.5X Sybr Green TM (BioRad Laboratories Inc). The Q-PCR thermal cycles consisted of an initial activation at 95°C for 5min followed by 30 cycles of: 10sec of denaturation at 95°C, 30sec of hybridization at 50°C, 30sec of elongation at 72°C. The fluorescence data were collected after each cycle.

Immuno-Q-PCR data analysis

Sex Peptide in the samples was quantified by using absolute quantification via the standard curve method. The ABI 7300 software measures amplification of Sex Peptide in samples and in a standard dilution series. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates the absolute quantity of Sex Peptide in the biological samples.

Box 1: Key steps in optimising the immuno-QPCR

- Test all primers to be used in the immuno-QPCR assay first with standard PCR, to ensure there are no primer dimers.
- The capacity of detection of the antigen by the antibody varies between samples. Therefore, different concentrations of the antibody and oligonucleotide need to be tested with a series of dilutions, in order to arrive at the optimal dilution to use.
- Avoid the use of milk as a blocking solution to avoid non-specific conjugation/ binding reactions
- A sterile working environment and frequent tip and glove changes is essential, to avoid contamination.
- The washing steps are key to the success of the assays.
- A long incubation for the blocking step is essential to obtain consistent results and avoid non-specific binding of the antibody-oligonucleotide complex to the plate.

Statistical analysis

The statistical analysis of the variation in mating latency, mating duration and Sex Peptide release by males was performed using RStudio (Version 0.99.903). In all tests, variation was considered significant at a *P* value threshold of $P<0.05$. The mating latency was log-transformed to improve normality. Both mating latency and mating duration analyses were performed using Gaussian general linear model (GLMs). For the Sex Peptide release, the analysis was performed on the median of the technical replicates, for the 3 biological replicates of each line. Statistical analyses were performed using the "Glmer" function on the "lem4" package (Bates *et al.*, 2014). The significance of factors was determined by step-wise model reduction from the maximal model via likelihood ratio tests (LRT). The significance of the deviance (-2 times the difference between the log likelihood of the reduced model and the log likelihood of the full model) was tested by comparison with the *Chisq* distribution. The maximal model included the DGRP lines and ELISA plates as random factors, and in the reduced model, the random effect of lines was omitted. We performed this analysis to detect genetic variation among the lines that contributed to the Sex Peptide release variation.

Genome wide association and network analysis

In order to identify polymorphisms that might be associated with variation in Sex Peptide release across the 31 DGRP inbred lines, a Genome-wide association Study (GWAS) was performed. As input data, we used the median Sex Peptide values of the biological replicates derived from the immuno-Q-PCR. The DGRP webserver (MacKay *et al.*, 2012) (dgrp2.gnets.ncsu.edu) was used to generate the GWAS. The GWAS analyses accounted for effects of *Wolbachia* infection, cryptic relatedness due to major inversions, and residual polygenic relatedness. Based on the GWAS results, only the top candidate genes associated with median relative Sex Peptide transfer levels were considered for subsequent network mapping and gene ontology enrichment analysis. Top candidate are genes with p values smaller than 1e-5 from simple regression (SinglePval) or mixed effects model (SingleMixedPval) provided by the DGRP webserver. The network mapping was done using Cytoscape 3.4.0 with the GeneMANIA plugin (Data Version:13/07/2017) (Shannon *et al.*, 2003; Montojo *et al.*, 2010). GeneMANIA is a multiple association network integration algorithm for predicting gene function, not affected with redundant and irrelevant networks (Mostafavi *et al.*, 2008). The geneMANIA server predicts a functional network by associating

genes based on gene information drawn from various existing databases: i) co-expression patterns, when expression levels of two genes are similar across different conditions, ii) genetic interactions, when functional associations between genes are already known, e.g. one gene modifies a second, etc, iii) co-localisation, in which genes are expressed in the same tissue or where proteins are found in the same cellular location, iv) predicted networks, when a functional relationship between genes is found in orthologous proteins in different organisms, v) shared protein domains, when gene products have the same protein domain, and vi) physical interactions, when genes have been found to interact in a protein-protein interaction assay (Montojo *et al.*, 2010). All the genes detected in composite networks were then subjected to functional enrichment analysis using DAVID Bioinformatics Resources 6.8, NIAID/NIH (Huang *et al.*, 2009). This identified over- or under-represented functions present among the candidate genes associated with Sex Peptide release and transfer. A set of 12 additional candidate genes highlighted by the analysis and with putative direct links to Sex Peptide, or with associated reproductive functions, were further explored (Table 2). The localisation of gene expression patterns for these genes were derived from adult expression data in the Flyatlas database (Chintapalli, Wang and Dow, 2007). For genes with no Flyatlas entry, tissue-specific expression was instead derived from the modENCODE data (Roy *et al.*, 2010) and from literature reviews.

RESULTS

Mating latency and duration

There was no significant variation in mating latency across the 31 DGRP lines (GLM, *Df*=333,332, *P*=0.5425) (Figure 3a). There was also no significant variation observed in mating duration between the 31 different DGRP lines (GLM, *Df*=308,308, *P*=0.4174). Therefore, mating latency and mating durations were not included as covariates in the analysis for variation in Sex Peptide transfer. Mating latency was recorded within a 4-hour time period. Mating durations lasting less than 5 min are reported not to transfer sperm (Gilchrist and Partridge, 2000) or accessory gland proteins (Monsma and Wolfner, 1988) and were considered unsuccessful matings and removed from the analysis of mating duration. Copulations of more than 45 min were also excluded from the data as they represent rare occurrences where individuals failed to separate following mating.

Binding the antibody to the oligonucleotides complex

The binding of the capture oligonucleotide to the quantification oligonucleotide and antibody was tested by electrophoresis on a 2% agarose gel. Figure 3 shows that the single strand synthetic oligonucleotide migrated as a single band of approximately 50bp (lane 1). A same size band of ~50bp was detected when mixing the antibody and the oligonucleotide (without the conjugation step) (lane 2). After incubating the quantification and capture oligonucleotides for binding and running the complex on the gel, 2 DNA fragments were detected, one at ~50bp and one at 70bp (lane 3). When the antibody was incubated with the quantification and capture oligonucleotide complex, 3 bands were detected, one at ~50bp, one at 70bp and a third at 250-300bp (lane 4). In the presence of the antibody only, no DNA fragment was observed (lane 5). The fragment at 250-300bp suggests that the antibody is binding to the capture/quantification oligonucleotide complex and, due to its structure, is unable to migrate through the agarose gel to the same extent as for the oligonucleotide alone. At this concentration of the single stranded quantification oligonucleotide ($5.7x10^{14}$ copies/µl) not all of it seems to bind to the antibody (as indicated by the presence of the 50bp fragment). However, at least some does bind to the quantification oligonucleotide, as shown by the fragment at 70bp.

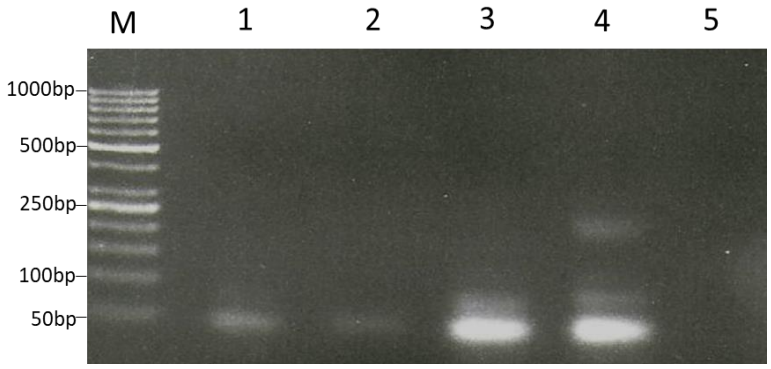


Figure 3: **Agarose electrophoresis gel of the migration of different elements of the oligonucleotide / antibody binding complex in immuno-Q-PCR assay**. **Lane M**: 50bp DNA ladder, **lane 1:** Oligonucleotide (1µM), **lane 2:** Oligonucleotide (1µM) + anti Sex Peptide Antibody, **lane 3:** Quantification oligonucleotide (10µM) + capture oligonucleotide incubated for 15 min at 48 °C and, **lane 4:** Quantification oligonucleotide (10µM) + capture oligonucleotide (10µM) + Antibody incubated 1h at room temperature and **lane 5:** Antibody alone.

Expression level of Sex Peptide

The repeatability tests for the immuno-Q-PCR method showed that there was a highly significant correlation between the same samples tested in two independent immuno-Q-PCR assays and run on different plates (P= 5.948e-11) (supplementary data Figure 1). The assay development was technically challenging and required several rounds of optimisation, but ultimately I obtained a clear negative control and a high repeatability of 91.23%. The analysis of the immuno-Q-PCR data revealed that there was significant variation in Sex Peptide release in males from the 31 different DGRP lines (GLMER, $Chisq$=7.7701, P=0.005312**) (Figure 4). The amount of Sex Peptide that the males transferred to females during mating ranged from a median of 0.01 mM to 0.4 mM, as estimated from the quantification using a standard curve of known Sex Peptide concentrations. The no template control (NTC) showed a very low concentration (0.0007 mM) of Sex Peptide in comparison to the DGRP lines. That it showed a detectable signal, in the absence of Sex Peptide, could be due to technical issues such as non-specific binding, which cannot be entirely eliminated. A total of 10 outliers within the lines, with concentration values of Sex Peptide higher than 1mM (values that were beyond the standard curve) were not included in the data analysis, as this indicated the presence of non-specific binding.

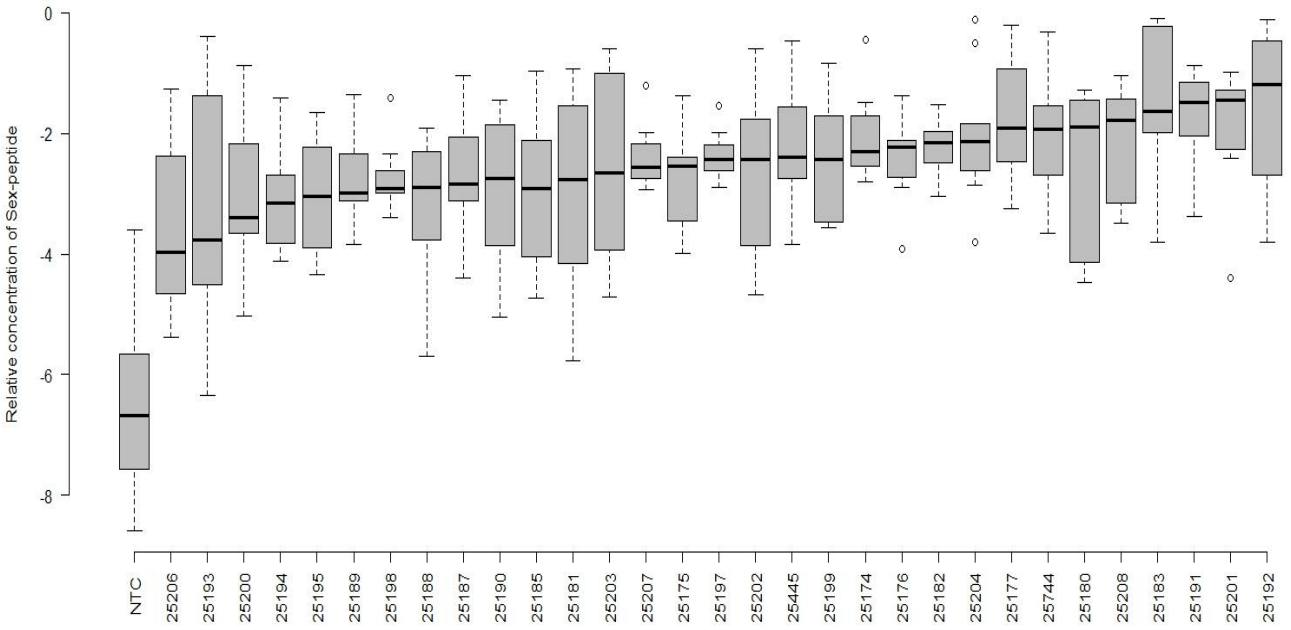


Figure 4: Sex Peptide release from males of 31 DGRP lines into wild type Dahomey females. Boxplot ranked by median Sex Peptide values derived from all technical replicates resulting from the immuno-Q-PCR on Sex Peptide release from males of the different DGRP lines, plus no template control (NTC). Box plots show the median (horizontal line within the box), the box representing the interquartile range, the whiskers the highest and lowest values and outliers represented by points.

GWAS analysis

Genomic variants, such as single nucleotide polymorphism (SNPs), insertions and deletions, associated with the variation in Sex Peptide release were identified, using a genome wide association study (GWAS) (Figure 5). The GWAS was carried out on the median of the Sex Peptide data from 31 different DGRP lines. SNPs, deletions or insertions exceeding a threshold of $P<10^{-5}$ were considered as significant candidates to follow up further in this study. A total of 157 significant polymorphisms were identified in or near 54 known genes (supplementary data Table 1) and 18 polymorphisms were found in intergenic regions that did not code for any protein coding genes within 1kb downstream or upstream of their location (data not shown). Most variation in annotated genes was found in the form of SNPs, including 4 deletions and 6 insertions. 46 of these polymorphisms were located in a single long non-coding RNA gene, FBn0052111 (CR32111 in the archive Flybase database). This gene, as well as 5 others among the candidates identified, has yet no known molecular and biological function.

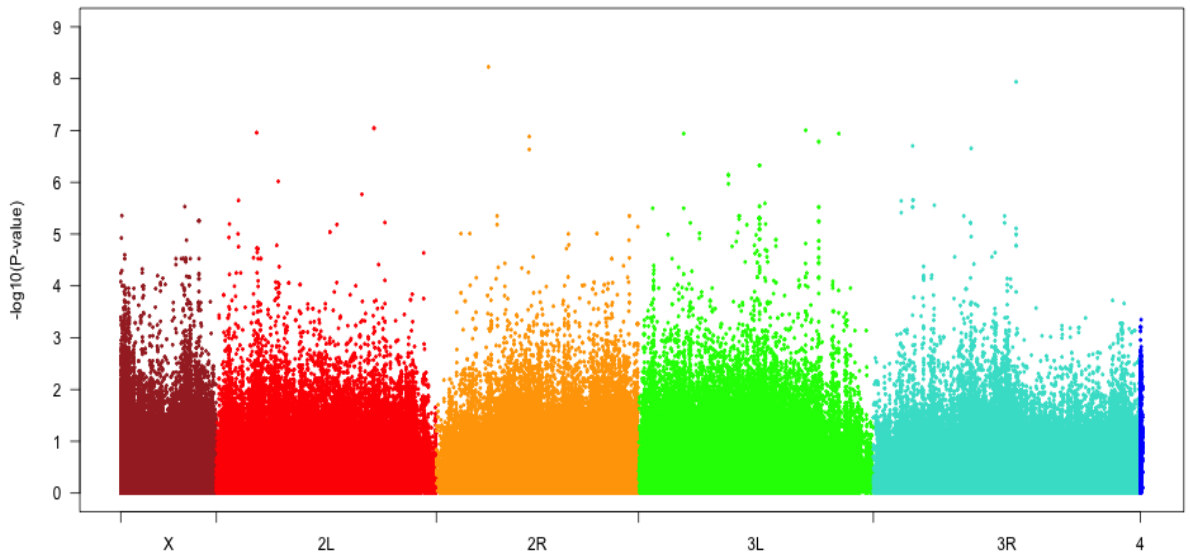


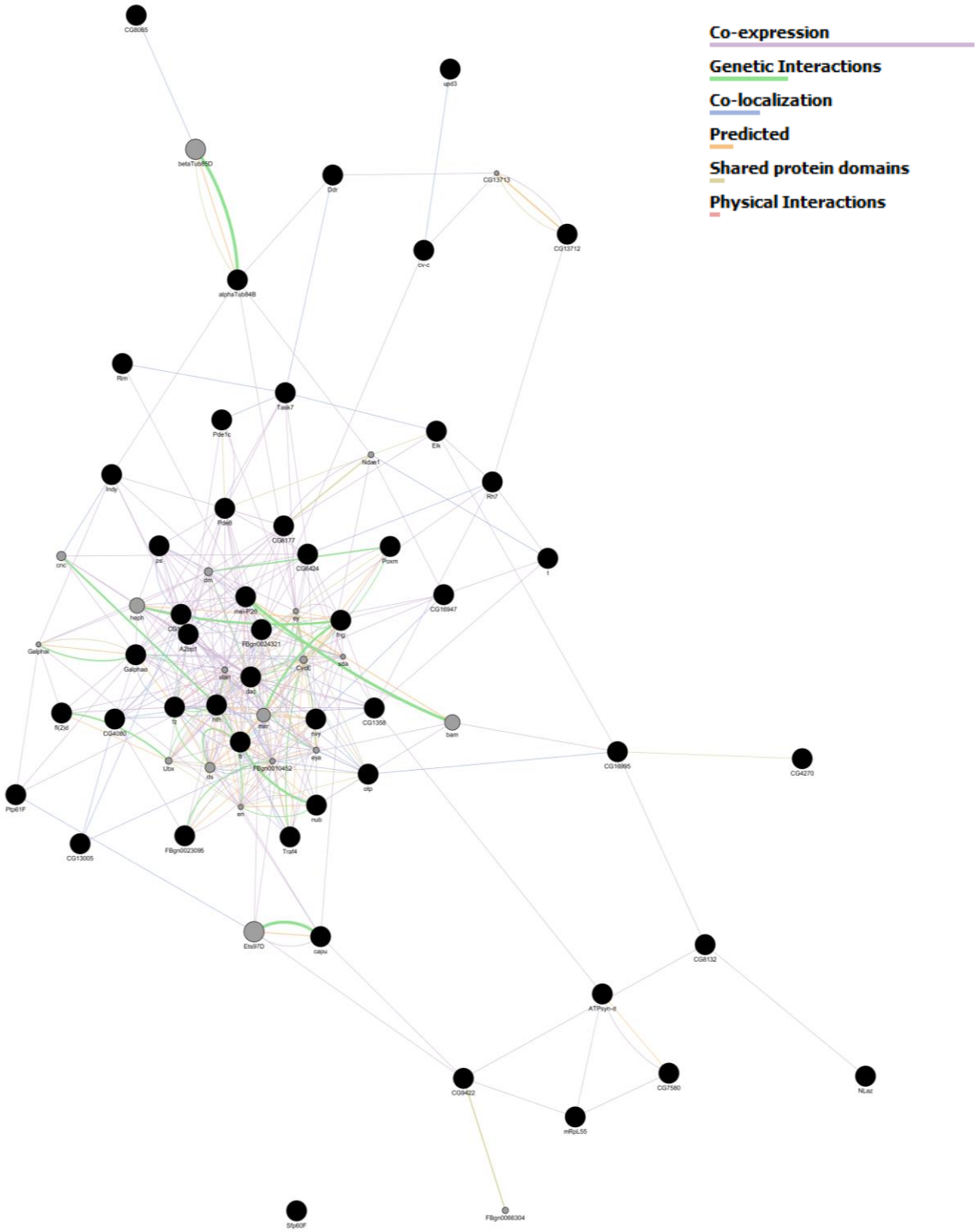
Figure 5: Manhattan plot (−log10[P] genome-wide association plot) of a genome wide association study on Sex Peptide transfer to females by males from the core set of 31 DGRP lines.

The functional gene network mapping was performed using the geneMANIA app in Cytoscape (Mostafavi *et al.*, 2008). Of the 54 candidate genes, only 49 are shown in the network mapping: FBgn0052027, FBn0052111, FBgn00527773, FBgn0263768 and FBn0265415 were not recognised by geneMANIA and were excluded from downstream analyses. Of the remaining 49 candidate genes, gene network mapping showed: 72.05% of the genes form a co-expression network; 12.79% of the genes have genetic interactions; 7.89% co-localise in the same tissue or cell compartment; 3.73% form a predicted network; 2.22% shared protein domains and 1.41% have physical protein-protein interactions (Figure 6). The functional enrichment analysis by GeneMANIA displayed a set of over-representation of gene ontology annotations (supplementary data Table 2) and identified several genes involved in development, maturation and protein processing. A set of genes of specific interest to this study (7/269) are those involved in germ cell development (q-value=0.045). Sex Peptide becomes bound to sperm in females, hence sperm (and thus germ cell development) and Sex Peptide might be expected to co-vary.

Gene enrichment analysis on the top candidate genes was performed using the DAVID Bioinformatics Resource 6.8 programme. The results for the biological processes (supplementary data Table 3) revealed a number of *D. melanogaster* developmental protein

genes (*P*=7.50E-04) as well as membrane (*P*=1.2E-2) and reproduction proteins (*P*=7.90E-02). All the genes were then clustered based on functional annotation in DAVID, GeneMANIA and Flybase (Gramates *et al.*, 2017) (supplementary data Table 4). These databases revealed four main functional annotation groups. The first is reported to be involved in organism development and maturation (e.g. development of imaginal discs, homeobox transcription factors). The second set is involved in RNA and protein processing. The third set of proteins is associated with membrane adhesion molecules, membrane potential and transmembrane transport. The fourth group comprised reproduction proteins. All protein groups are summarised in the Venn diagram shown in Figure7.

Based on the gene ontology and the network mapping, a set of 12 candidate genes with putative direct links with Sex Peptide, or with reproductive functions, were further explored (summarised in Table 2). According to the Flyatlas and modENCODE data, most of the 12 selected candidate genes were expressed in male testis or accessory glands and sometimes in both. Several of these genes were male-specific and represent accessory gland and seminal fluid protein genes (i.e. *CG1995, Sfp60F, Pde8* and *NLaz*) (Chintapalli *et al*., 2007; Ram and Wolfner, 2007; Findlay *et al.*, 2008; Ruiz *et al.*, 2011; Avila *et al.*, 2015; Kubrak *et al.*, 2016). Others were involved in germ cell development in either males or females (*A2bp1, capu, mei-P26* and *fng*) (Emmons *et al.*, 1995; Page *et al.*, 2000; Terry *et al.*, 2006; Chintapalli *et al.,* 2007; Tastan *et al.*, 2010; Insco *et al.*, 2012; Yang *et al.*, 2013; Yoo *et al.*, 2015). The *fl(2)d* gene is involved in sex determination (Penalva *et al.*, 2000). One gene in particular, *Pde1C*, seems to have a direct connection to male mating behaviour and female sperm storage (Morton *et al.,* 2010). Additional genes were highly or uniquely expressed in male reproductive organs, although their molecular and biological functions are still unknown (*capu, CG1358 and CG8065*) (Swiss-Prot Project Members, 2004; Chintapalli *et al*, 2007).



<u>Figure 6:</u> Interaction networks of genes involved in Sex Peptide release. Black nodes depict genes containing significant SNPs from the DGRP analysis (Query genes). Grey nodes are genes involved in the mapping network in *Drosophila melanogaster*.

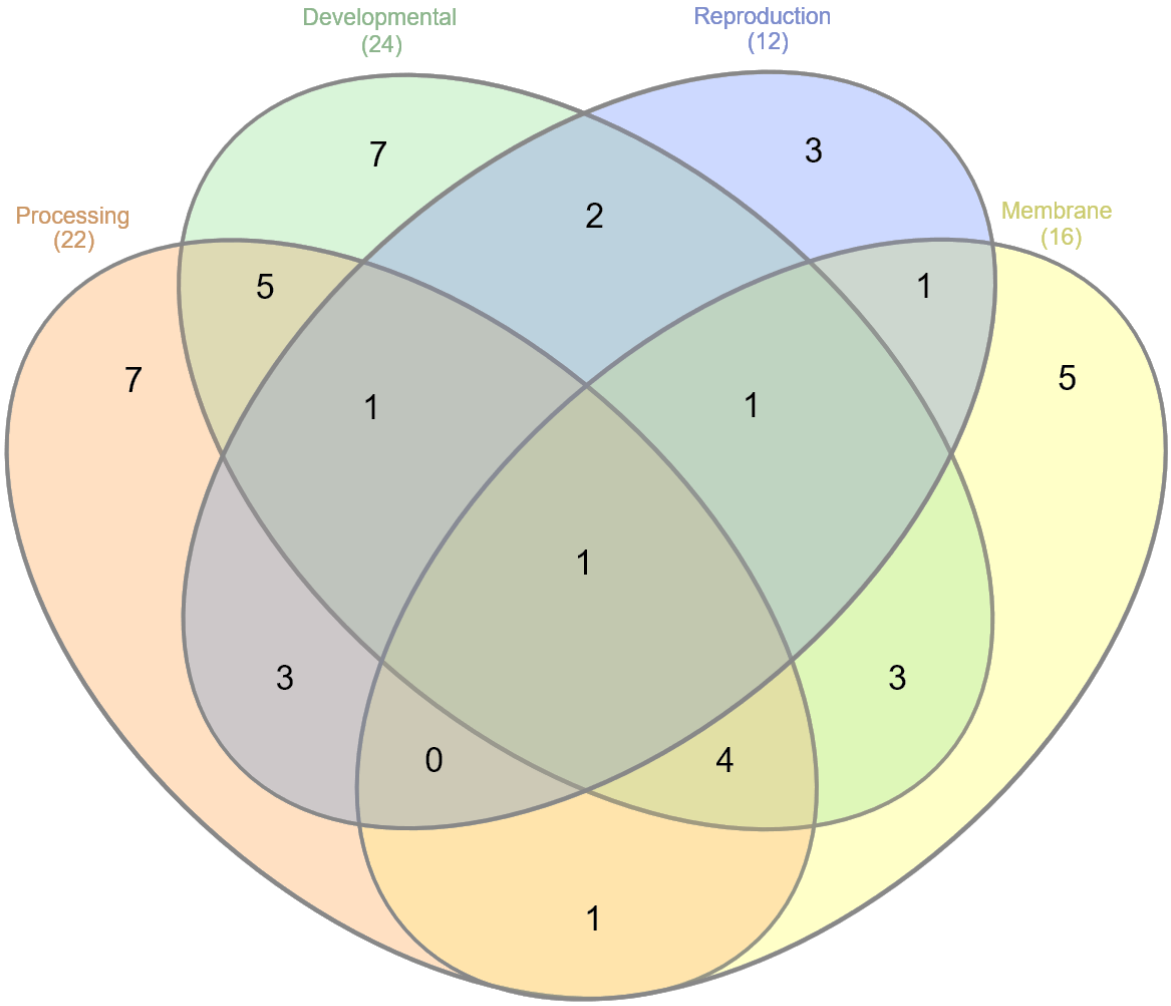


Figure 7: Venn diagram (Heberle *et al.*, 2015) of gene clusters, based on functional annotation of the top candidate genes associated with male Sex Peptide release from the GWAS analysis. The functional annotation tables were generated by using DAVID, Flybase, and GeneMANIA. Most of these candidate genes can be divided into four clusters: RNA and protein processing (15 genes), reproduction protein (11), developmental protein (18) and membrane proteins (19). Some of these genes fall in more than one of these functional clusters.

Table 2: Summary of tissue-specific gene expression (from FlyAtlas and ModENCODE) of the 12 candidates 'sexual reproduction' genes identified from the GWAS analysis of sex peptide release. The scale of expression level was derived from the information on Flybase.

Gene	Annotation	Sexual reproduction function	Male testis	Male accessory gland	Female spermathecae	Female ovary	Brain& neural system	Digestive tissue	Heart and circulation	Head and eyes	Caracas	Salivary gland	References
CG16995	FBgn0031412	Accessory gland protein transferred to females during mating.											Ram and Wolfner, 2007
capu	FBgn0000256	In females: determinant of polarity in the development of oocyte. In males: highly expressed in adult testis, function unknown.											Emmons *et al.*, 1995; Chintapalli., *et al* 2007; Yoo *et al.*, 2015
fl(2)d	FBgn0000662	Has a non-sex-specific function in males and in females. In females it is also Involved in sex determination and dosage compensation, as well as germline development.											Penalva *et al.*, 2000
Pde1c	FBgn0264815	Required for male fertility and mating behaviour, transferred to female during mating, important for sperm storage in female reproductive tract.											Morton *et al.*, 2010
Pde8	FBgn0266377	Expression in male accessory gland and central nervous system.											Chintapalli *et al.*, 2007
A2bp1	FBgn0052062	Regulation of ovarian germ cell development and differentiation.											Tastan *et al.*, 2010; Carreira-rosario *et al.*, 2016
fng	FBgn0011591	In males: expression in the testis might involved in germline development. In females: involved in maintaining the germline stem cell niche.											Terry *et al.*, 2006; Yang *et al.*, 2013
NLaz	FBgn0053126	Seminal fluid protein involved in lipid metabolism, regulated by JNK signalling contributes to longevity, and is highly expressed during male reproductive dormancy.											Findlay *et al.*, 2009; Ruiz *et al.*, 2011; Kubrak *et al.*, 2016
mei-P26	FBgn0026206	Regulates the proliferation and differentiation of early male germ cells.											Page *et al.*, 2000; Insco *et al.*, 2012
Sfp60F	FBgn0259968	Seminal fluid protein transferred to female, one of the seminal fluid proteins that forms the mating plug.											Findlay *et al.*, 2008; Findlay *et al.*, 2009; Avila *et al.*, 2015
CG8065	FBgn0036075	Unknown function. Peak expression observed at stages throughout the pupal period and in adult male stages.											Chintapalli *et al* ., 2007
CG1358	FBgn0033196	High level of expression in male accessory gland and the central nervous system, involved in transmembrane transport.											Swiss-Prot Project Members, 2004; Chintapalli el al., 2007

Expression level	
No expression	
Low expression	
Moderate expression	
High expression	
Very high expression	

Discussion

Results summary

We developed and successfully employed a novel quantification method for detection of Sex Peptide release: the immuno-Q-PCR assay. Using this, we detected significant variation among 31 DGRP lines in Sex Peptide release by males into wild type Dahomey females during mating. Our study showed no significant variation in mating latency or mating duration between males from 31 DGRP lines, indicating this variation in Sex Peptide transfer is unlikely to be mediated by differences in mating behaviour among the lines. To search for genetic variation associated with variation in Sex Peptide release, we conducted a GWAS. This analysis yielded significant associations between Sex Peptide release and a set of 54 candidate genes. An extensive gene ontology search revealed that these top candidate genes clustered within the following functional categories: development, membrane, protein and RNA processing and reproduction. A literature search on the reproductive gene cluster showed that four of these genes were seminal fluid proteins. Some have yet unidentified functions; two are cyclic nucleotide phosphodiesterase that seem to be involved in male fertility and female mating behaviour; some are involved in germ cell development in males and/or in females; and others are uniquely expressed in male testis and/or accessory gland protein but have unknown molecular and biological functions. The significant variation detected in Sex Peptide transfer could exacerbate the exprression of sexual conflict and this would be interesting to test further. Our study also highlighted new candidate genes not previously detected by other analysis methods, which might be associated with Sex Peptide function, reproduction and post-mating gene expression in females.

Immuno-Q-PCR

The immuno-Q-PCR approach was highly sensitive, repeatable and had greater resolution in comparison to conventional immunoassays (Zhou *et al*., 1994; Niemeyer *et al*., 2007). It has been widely used in the biomedical and immunological research field for detecting the presence of bacterial infection in humans (McKie *et al*., 2002; Halpern *et al*., 2014; Mehta *et al*., 2017). To our knowledge, this is the first time this method has been used in the study of protein quantification method in an insect. To better understand the processes that contribute to variation in Sex Peptide transfer during mating, we developed the immuno-Q-PCR to achieve rapid, sensitive and accurate quantification. This required significant optimisation to achieve

high reproducibility and the required resolution to detect variation in Sex Peptide transfer. We tested the specificity of the primers with PCR, to ensure there were no primer dimers. The optimal primer concentration was determined by performing several PCRs with different primer dilutions. Similarly, to determine the correct working concentrations of the antibody against Sex Peptide and capture oligonucleotide, a series of dilutions was tested by PCR and Q-PCR. The agarose gel electrophoresis was an efficient and effective way of detecting the existence of the different component parts of the antibody / oligonucleotide complex as they bound together. Different blocking solutions were also tested, as some had limited blocking abilities and were revealed to be a source of contamination or non-specific binding. The concentration of the synthetic Sex Peptide was also optimised to obtain a good slope for the standard curve. Standard curve slopes ranged between 1.90 to 2.27 and the R^2 values from 0.92 to 0.297. These were the best amplification efficiencies obtained from this immuno-Q-PCR. The assay was also highly repeatable, as shown by assays of the same samples conducted across different plates. Background signal was present at low levels and could have resulted from non-specific binding of the antibody, binding of oligonucleotide to the ELISA plate or other off-target protein, the efficiency of the blocking, Hind III restriction digest or wash steps. Although present, the background was minimised and was in line with typical measurements for this kind of technique (Halpern *et al.*, 2014; Chang *et al.,* 2016). We ultimately managed to accurately quantify significant variation in Sex Peptide release in males from 31 different DGRP lines. We conclude that the immuno-Q-PCR is an efficient technique that enabled us to detect and quantify protein variation at the nano- and pico-molar level for the first time in insects in this manner.

GWAS

To understand the genes and pathways that were associated with variation in Sex Peptide release, we used an unbiased GWAS on a core set of 31 out of the total of 205 DGRP lines (MacKay *et al.*, 2012; Mackay and Huang, 2018). We detected significant genetic variation among the lines. However, it is important to be aware of the potential for false positives arising from the use of a relatively modest number of lines in comparison to the full set (Mackay and Huang, 2018). As a result of the GWAS, we found that a total of 157 polymorphisms, spread across all chromosomes, were significantly associated with the Sex Peptide release trait. Eighteen of these polymorphisms (17 SNPs and 1 insertion) with significant associations did not code for any known protein coding gene within 1kb up or downstream of their location. The 139 remaining polymorphisms showing significant associations were linked with 54 protein coding genes. Six of these had an unidentified function and could be important novel candidates for further investigation with respect to Sex Peptide synthesis and release. They may also have yet undescribed roles in post-mating responses in females.

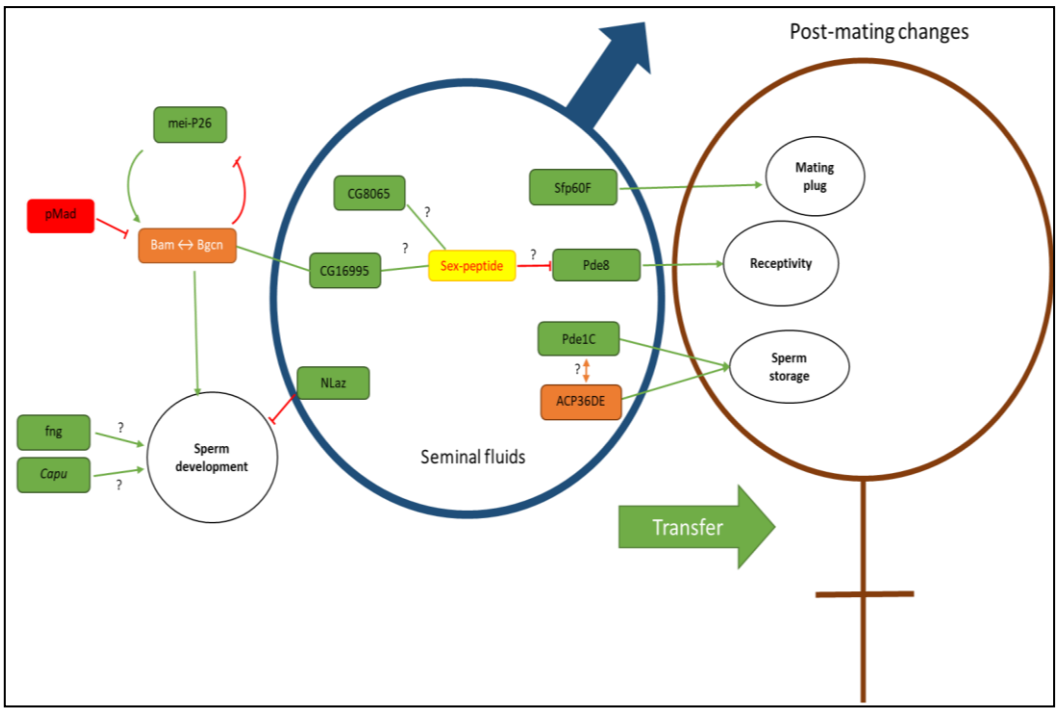


Figure 8: Pathway summary based on 9 of the 12 candidate 'sexual reproduction' genes identified in the GWAS analysis of sex peptide release.

Networks and functional enrichment

The functional enrichment analysis with GeneMANIA and DAVID arranged the 54 top candidate genes into 4 main functional clusters: development, membrane, protein and RNA processing and reproduction. Within these, we found homeobox genes that encode for homeodomain transcription factor proteins (*otp, hth*, *nub, nk7.1* and *poxm*), which are responsible for regulation of *Drosophila* anatomical development (Hayashi and Scott, 1990; Gehring, 1992). For example, the homeodomain protein *otp* is required for normal brain development (Walldorf *et al.*, 2000). *hth* is a homeobox gene necessary for embryo development and is associated with muscle (Bryantsev *et al.*, 2012), brain (Nagao *et al.*, 2000) and eye development (Singh *et al.*, 2011) as well as 23 other biological functions. Other associations with genes for ubiquitin-proteins and transferase activity may show roles for recognition and degradation by the proteasome (Finley, 2009), as well as metal and zinc binding proteins (CG4080, *Galphao, fng, mei-P26, Rim, nvy* and *pde1C*). Another set of gene association were with RNA slicing and processing (*fl(2)d, ps, CG4080* and *CG11486*). Moreover, the functional enrichment analysis clustered a set of genes that encode membrane proteins *(Task7, fz, Ptp61F, Rh7, ft, Elk, Caps* and *fng*). *Task7* is involved in potassium ion transmembrane transport (Döring *et al.*, 2006) and *fz* is an integral membrane protein required for both the intercellular transmission and the intracellular transduction of tissue polarity (Park, Liu and Adler, 1994). This could indicate that Sex Peptide release also involves transmembrane transport from the main cells into the accessory gland.

According to FlyAtlas and modEMCODE, some of the genes showing significant associations with Sex Peptide release, e.g. *ft, ptp61F, Galphao* and CG4080, are moderately to highly expressed in the testis and accessory glands. This is consistent with the genes being part of the 'reproductive gene' cluster. The development of accessory gland proteins, and more specifically the synthesis and the release of Sex Peptide, is a very complex process that requires the interaction of several proteins, with potentially different functions, including developmental, membrane and RNA and protein processing genes.

Candidate 'sexual reproduction' genes

We explored in more detail 12 candidate genes showing significant associations with reproductive functions. The known pathways of some of these genes and their possible relationship with Sex Peptide is summarised in Figure 8. Of these 'sexual reproduction' genes, 3 code for seminal fluid proteins transferred along with sperm. *Sfp60F* is a seminal protein that is transferred during mating and is one of the proteins that constitute the mating plug (Avila, Cohen, *et al.*, 2015), though the exact biological function of this protein is still unknown. *NLaz* is an additional seminal fluid protein candidate (Findlay *et al.,* 2009) identified by this GWAS, which, when over expressed, represses growth, promotes stress tolerance and extends lifespan (Hull-Thompson *et al.*, 2009). *NLaz* is also up-regulated in reproductively dormant males, in which spermatogenesis and the development of seminal vesicles and accessory glands is arrested (Kubrak *et al.*, 2016). The role of the accessory gland protein candidate *CG16995* is not yet known (Ram and Wolfner, 2007), making it a potentially interesting novel candidate to test in terms of Sex Peptide release.

Among the 'sexual reproduction' candidate genes, two (*Pde1C* and *Pde8*) of the six genes that code for cyclic nucleotide phosphodiesterases (PDEs, with important roles in the cAMP and cGMP signalling cascade (Day *et al.*, 2005)) were already implicated in determining male reproductive success. *Pde1c* is required for male fertility and mating behaviour in *D. melanogaster* and males lacking *Pde1c* have lower mating rates and longer copulation latencies. It has also been suggested that *Pde1c* is transferred during mating and is necessary for sperm storage in the female spermatheca and the seminal receptacle (Morton *et al.*, 2010). There is as yet no evidence to link *Pde1c* to seminal fluid proteins such as *ACP36DE* whose important roles in the process of sperm storage have already been described (Neubaum and Wolfner, 1999; Tram and Wolfner, 1999; Chapman *et al.*, 2000). However, it is possible that, owing to the complexity of sperm storage (Qazi and Wolfner, 2003; Schnakenberg *et al.*, 2012) important facets, which *Pde1c* could potentially influence, still remain to be discovered. This makes *Pde1c* a particularly interesting gene as its function in sperm storage could represent an important aspect of male's reproductive strategy.

Pde1c is required for sperm storage and Sex Peptide is required for sperm release (Avila *et al.*, 2015 (2)). Both of these processes are important for reproductive success and these gene products could act in concert or synergise. Further studies are required to test these possibilities

and whether the combined functions of these two genes lead to higher fertilization success overall. *Pde8* has recently been shown to reduce post-mating receptivity when silenced (Gorter, 2018). As Sex Peptide also decreases female receptivity (Chapman *et al.*, 2003: Liu & Kubli 2003) this could suggest that Sex Peptide could inhibit the expression of *Pde8* in females, leading to a reduction in female receptivity. More experiments are needed to validate this hypothesis.

Candidates *Capu, fng*, *mei-P26* and *A2bp1* are all involved in germ cell development in one or both sexes (Emmons et al., 1995; Insco *et al.*, 2012; Yang *et al.*, 2013; Carreira-rosario *et al.*, 2016). Similarly, *Fl(2)d* is required for the development of the female germline and sex determination, with females developing into males in loss-of-function mutants (Penalva *et al.*, 2000). Since Sex Peptide binds to the transferred sperm tail (Peng *et al.*, 2005), this could suggest that any gene that influences the germ cell development and transfer could have an indirect effect on the transferred Sex Peptide. This could explain the variation detected in the transferred Sex Peptide of the 31 DGRP lines – that it is in fact indicating variation in sperm release. Again, further work is needed to confirm this theory. *CG8065* is uniquely expressed in males and *CG1358* is highly expressed in the male accessory gland – neither has yet been studied and little to no information is available about their biological or molecular function. None of the genes described above has previously been shown to have a direct link with Sex Peptide. However, the 'reproductive candidate genes' generated by the GWAS highlights new findings. Moreover, these genes are all part of a co-expression network, suggesting that they potentially function together to produce significant variation in Sex Peptide release.

Conclusion

The immuno-Q-PCR method was shown to be a powerful quantification technique. It detected significant variation in Sex Peptide release, which was then analysed in a GWAS that yielded new data on genes with previously limited or even unknown functions. These new candidate genes could represent missing pieces of the Sex Peptide functional network. Further experiments are required to investigate whether these candidate genes aid Sex Peptide in generating the full suite of post-mating responses in female.

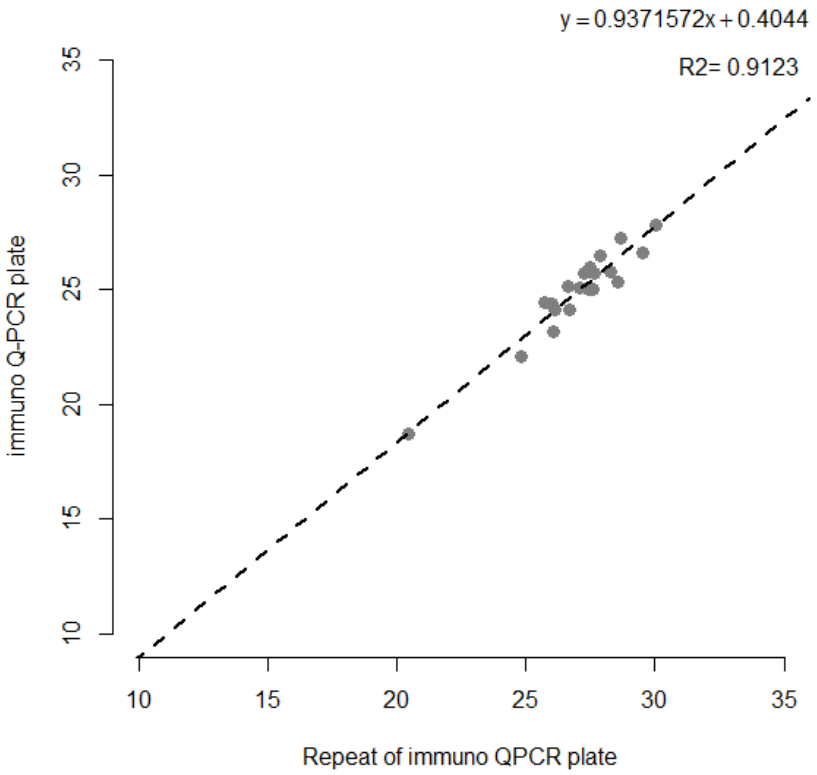
Acknowledgement

Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. We are grateful to Wayne Rostant for his advice with the statistical analysis of the data, Damian Smith for sharing his expertise on the ELISA and Emily Fowler, Janet Mason and Elizabeth Duxbury who helped to perform the mating assays. This project was funded by Ubbo Emmius scholarship, the UEA School of Biological Sciences, John and Pamela Salter Charitable Trust and The Leverhulme Trust.

Authors and Contributors

JR, TC and BW conceived the study, JR conducted the research, JR and BW analysed the data, JR wrote the chapter and JR, BW and TC revised the chapter.

Supplementary data



<u>Figure 1:</u> Repeatbility of the Immuno-Q-PCR. Correlation of repeat samples tested independently by two immuno-Q-PCR rections across different plates**.** Scatter plot shows a significant correlation (*P*= 5.948e-11) with an R^2 value of 0.91.

Table 1: Functional annotation of top candidate genes from the GWAS, according to the functional annotation analysis, using the DAVID functional enrichment programme (Huang *et al*., 2009).

Gene	Annotation	Gene ontology
capu	FBgn0000256	Actin filament organization, chorion-containing eggshell formation, pole plasm assembly, pole plasm RNA localization, protein transport, actin filament-based process, actin nucleation, pole plasm oskar mRNA localization, oogenesis
fl(2)d	FBgn0000662	RNA splicing, via transesterification reactions, regulation of alternative mRNA splicing, via spliceosome, mRNA processing, sex determination, primary sex determination, soma, female germ-line sex determination, lateral inhibition, compound eye development, mRNA methylation
ft	FBgn0001075	Cell morphogenesis involved in differentiation, establishment of planar polarity, establishment of imaginal disc-derived wing hair orientation, homophilic cell adhesion via plasma membrane adhesion molecules, heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules, establishment of tissue polarity, imaginal disc growth, imaginal disc pattern formation, imaginal disc-derived wing morphogenesis, cell proliferation, negative regulation of cell proliferation, tissue development, negative regulation of gene expression, ommatidial rotation, establishment or maintenance of polarity of larval imaginal disc epithelium, single organismal cell-cell adhesion, calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules, peptide cross-linking, negative regulation of Wnt signaling pathway, regulation of protein localization, regulation of tube length, open tracheal system, pupal development, wing disc development, hippo signaling, regulation of growth, establishment of ommatidial planar polarity, cell-cell adhesion mediated by cadherin, establishment of epithelial cell apical/basal polarity, equator specification, regulation of imaginal disc growth, negative regulation of imaginal disc growth, negative regulation of growth, regulation of organ growth, establishment of body hair planar orientation, microtubule cytoskeleton organization involved in establishment of planar polarity
fz	FBgn0001085	Establishment of planar polarity, establishment of imaginal disc-derived wing hair orientation, morphogenesis of a polarized epithelium, compound eye morphogenesis, homophilic cell adhesion via plasma membrane adhesion molecules, establishment or maintenance of cell polarity, establishment of tissue polarity, signal transduction, G-protein coupled receptor signaling pathway, multicellular organism development, border follicle cell migration, salivary gland morphogenesis, R3/R4 cell fate commitment, imaginal disc-derived wing morphogenesis, heart development, protein localization, asymmetric protein localization, asymmetric cell division, Wnt signaling pathway, ommatidial rotation, sensory organ precursor cell fate determination, sensory perception of pain, establishment of cell polarity, regulation of actin filament bundle assembly, regulation of tube length, open tracheal system, regulation of hemocyte proliferation, imaginal disc-derived wing hair organization, imaginal disc-derived wing hair site selection, non-canonical Wnt signaling pathway, establishment of ommatidial planar polarity, establishment of epithelial cell apical/basal polarity, negative regulation of Notch signaling pathway, positive regulation of axon extension, axon extension, canonical Wnt signaling pathway, positive regulation of axon guidance
Galphao	FBgn0001122	Eestablishment of imaginal disc-derived wing hair orientation, G-protein coupled receptor signaling pathway, adenylate cyclase-modulating G-protein coupled receptor signaling pathway, ventral cord development, heart development, asymmetric cell division, establishment of endothelial blood-brain barrier, Wnt signaling pathway, calcium-mediated signaling, septate junction assembly, cortical actin cytoskeleton organization, axon ensheathment in central nervous system, behavioral response to starvation, negative regulation of synaptic growth at neuromuscular junction, negative regulation of dendrite morphogenesis, sensory perception of sweet taste, detection of temperature stimulus involved in sensory perception of pain, establishment of glial blood-brain barrier, cell adhesion involved in heart morphogenesis,
hth	FBgn0001235	Protein import into nucleus, translocation, eye development, compound eye photoreceptor fate commitment, regulation of transcription from RNA polymerase II promoter, specification of segmental identity, head, specification of segmental identity, antennal segment, brain development, peripheral nervous system development, salivary gland boundary specification, imaginal disc-derived wing morphogenesis, leg disc proximal/distal pattern formation, imaginal disc-derived leg morphogenesis, somatic muscle development, proximal/distal pattern formation, specification of organ identity, protein localization to nucleus, segmentation, regulation of cell fate specification, regulation of neuron differentiation, positive regulation of transcription from RNA polymerase II promoter, haltere morphogenesis, compound eye development, head morphogenesis, Malpighian tubule development, positive regulation of decapentaplegic signaling pathway
Poxm	FBgn0003129	Transcription, DNA-templated, regulation of transcription, DNA-templated, larval somatic muscle development, dendrite morphogenesis
Ptp61F	FBgn0267487	Protein-tyrosine phosphatase, active site; Protein-tyrosine phosphatase, receptor/non-receptor type; Protein-tyrosine/Dual specificity phosphatase.
alphaTub84B	FBgn0003884	Microtubule-based process, mitotic spindle assembly checkpoint, antimicrobial humoral response
nvy	FBgn0005636	Axon guidance, muscle organ development, chaeta morphogenesis, regulation of glucose metabolic process, negative regulation of transcription, DNA-templated, dendrite morphogenesis
dac	FBgn0005677	Transcription, DNA-templated, regulation of transcription, DNA-templated, axon guidance, leg disc proximal/distal pattern formation, genital disc morphogenesis, negative regulation of gene expression, mushroom body development, neuron differentiation, spermathecum morphogenesis, genital disc development, genital disc sexually dimorphic development, compound eye photoreceptor development, photoreceptor cell fate specification, antennal joint development, compound eye development
Elk	FBgn0011589	Phosphorelay signal transduction system, potassium ion transport, regulation of glucose metabolic process, regulation of membrane potential, transmembrane transport
fng	FBgn0011591	Cell fate specification, compound eye morphogenesis, fucose metabolic process, protein O-linked glycosylation, Notch signaling pathway, germarium-derived egg chamber formation, dorsal/ventral pattern formation, imaginal disc, dorsal/ventral lineage restriction, imaginal disc, imaginal disc-derived wing morphogenesis, imaginal disc-derived wing margin morphogenesis, regulation of Notch signaling pathway, cuticle pattern formation, imaginal disc-derived leg segmentation, female germ-line stem cell population maintenance, negative regulation of Notch signaling pathway, wing disc dorsal/ventral pattern formation, oogenesis, compound eye development
otp	FBgn0015524	Regulation of transcription, DNA-templated, multicellular organism development, lateral inhibition
ATPsyn-d	FBgn0016120	Response to oxidative stress, determination of adult lifespan, ATP synthesis coupled proton transport, proton transport, negative regulation of TOR signaling, ATP metabolic process, regulation of mitochondrial membrane potential, negative regulation of ERK1 and ERK2 cascade

caps	FBgn0023095	Cell adhesion, homophilic cell adhesion via plasma membrane adhesion molecules, axon guidance, synapse assembly, larval salivary gland morphogenesis, imaginal disc-derived wing morphogenesis, motor neuron axon guidance, cell migration, branch fusion, open tracheal system, lateral inhibition, photoreceptor cell axon guidance
NK7.1	FBgn0024321	Regulation of transcription, DNA-templated
mei-P26	FBgn0026206	Meiotic nuclear division, gamete generation, germ cell development, protein ubiquitination
Traf4	FBgn0026319	Eye development, apical constriction involved in gastrulation, phagocytosis, activation of NF-kappaB-inducing kinase activity, ventral furrow formation, dorsal closure, protein ubiquitination, adherens junction organization, salivary gland cell autophagic cell death, positive regulation of apoptotic process, asymmetric protein localization involved in cell fate determination, positive regulation of JNK cascade, imaginal disc fusion, thorax closure, autophagic cell death, defense response to Gram-negative bacterium
CG6424	FBgn0028494	Coiled coil, Complete proteome, Reference proteome
CG13005	FBgn0030794	Complete proteome, Proteomics identification, Reference proteome
CG4270	FBgn0031407	Extracellular region
CG16995	FBgn0031412	Multicellular organism reproduction
CG16947	FBgn0031816	Protein ubiquitination
CG9422	FBgn0033092	Cellular response to starvation, neurogenesis
CG1358	FBgn0033196	Transmembrane transport
Pde8	FBgn0266377	Signal transduction, mesoderm development, sensory perception of pain, cAMP metabolic process
CG11486	FBgn0035397	Nuclear-transcribed mRNA poly(A) tail shortening, deadenylation-dependent decapping of nuclear-transcribed mRNA, mRNA processing, protein phosphorylation, positive regulation of cytoplasmic mRNA processing body assembly, RNA phosphodiester bond hydrolysis, exonucleolytic
CG13712	FBgn0035570	Regulation of localization
CG4080	FBgn0035983	RNA processing and modification
CG8177	FBgn0036043	Anion transport, regulation of intracellular pH, chloride transmembrane transport
CG8065	FBgn0036075	Complete proteome, Reference proteome
Rh7	FBgn0036260	G-protein coupled receptor signaling pathway, G-protein coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger, neuropeptide signaling pathway, visual perception, phototransduction, protein-chromophore linkage
UQCR-Q	FBgn0036728	Microtubule-based process, mitotic spindle assembly checkpoint, antimicrobial humoral response
Indy	FBgn0036816	Sodium ion transport, pyruvate transport, determination of adult lifespan, regulation of sequestering of triglyceride, succinate transport, citrate transport, transmembrane transport
CG8132	FBgn0037687	General function prediction only
Task7	FBgn0037690	Stabilization of membrane potential, potassium ion transmembrane transport
mRpL55	FBgn0038678	Translation, multicellular organism development, mitochondrial translation
CR32027	FBgn0052027	Unknown genes
A2bp1	FBgn0052062	Regulation of alternative mRNA splicing, via spliceosome, nervous system development, imaginal disc-derived wing vein specification, memory,negative regulation of translation, germarium-derived oocyte differentiation, positive regulation of transcription, DNA-templated, oogenesis
CR32111	FBgn0052111	Unknown genes
CR32773	FBgn0052773	Unknown genes
NLaz	FBgn0053126	Response to oxidative stress, determination of adult lifespan, multicellular organism reproduction, carbohydrate homeostasis, triglyceride homeostasis
Ddr	FBgn0053531	Protein phosphorylation
upd3	FBgn0053542	Embryonic development via the syncytial blastoderm, immune response, JAK-STAT cascade, positive regulation of cell proliferation, antimicrobial humoral response, intestinal stem cell homeostasis, paracrine signaling, regulation of imaginal disc-derived wing size, regulation of JAK-STAT cascade, positive regulation of JAK-STAT cascade, oogenesis, intestinal epithelial structure maintenance
Rim	FBgn0053547	Intracellular protein transport, neurotransmitter secretion, neuromuscular synaptic transmission, synaptic vesicle exocytosis, vesicle-mediated transport, calcium ion regulated exocytosis, regulation of membrane potential, regulation of synaptic plasticity, calcium ion-regulated exocytosis of neurotransmitter, clustering of voltage-gated calcium channels, regulation of synaptic vesicle exocytosis
nub	FBgn0085424	Negative regulation of antibacterial peptide biosynthetic process, transcription, DNA-templated, regulation of transcription from RNA polymerase II promoter, pattern specification process, ganglion mother cell fate determination, ventral cord development, wing disc development, limb joint orphogenesis, dendrite morphogenesis
t	FBgn0086367	Histamine metabolic process, histamine biosynthetic process, visual perception, flight behavior, dopamine biosynthetic process, cuticle pigmentation, adult chitin-containing cuticle pigmentation
cv-c	FBgn0086901	Establishment of mitotic spindle orientation, cell morphogenesis, assembly of actomyosin apparatus involved in cytokinesis, mitotic spindle organization, metaphase/anaphase transition of mitotic cell cycle, epidermal growth factor receptor signaling pathway, Rho protein signal transduction, neuromuscular synaptic transmission, dorsal closure, open tracheal system development, epithelial cell migration, open tracheal system, Malpighian tubule morphogenesis, midgut development, head involution, imaginal disc-derived wing vein morphogenesis, cortical actin cytoskeleton organization, negative regulation of Rho protein signal transduction, maintenance of epithelial integrity, open tracheal system, spiracle morphogenesis, open tracheal system, regulation of dendrite morphogenesis, spindle localization
Sfp60F	FBgn0259968	Multicellular organism reproduction
ps	FBgn0261552	mRNA splicing, via spliceosome
CR43685	FBgn0263768	Unknown genes
Pde1c	FBgn0264815	Signal transduction, sensory perception of pain, multicellular organism reproduction, cAMP metabolic process, cGMP metabolic process, male mating behavior
CR44327	FBgn0265415	Unknown genes

Table 2: Functional enrichment of candidate genes networks, as analysed by GeneMANIA (Montojo *et al.*, 2010; Warde-Farley *et al.*, 2010). Coverage is the ratio of the number of annotated genes in the displayed network vs the number of genes with that annotation in the genome. The *q*-value is estimated using the Benjamini-Hochberg procedure. Categories are displayed up to a *q*-value cut-off of 0.1

GO id	Description	q-value	Coverage
GO:0007417	Central nervous system development	1.13E-05	12/225
GO:0035282	Segmentation	6.80E-05	11/227
GO:0001708	Cell fate specification	6.80E-05	8/92
GO:0001745	Compound eye morphogenesis	5.65E-04	10/230
GO:0001709	Cell fate determination	6.47E-04	8/134
GO:0048592	Eye morphogenesis	6.47E-04	10/242
GO:0009880	Embryonic pattern specification	0.0010143	9/199
GO:0048663	Neuron fate commitment	0.0015095	6/67
GO:0007420	Brain development	0.0030754	7/121
GO:0007163	Establishment or maintenance of cell polarity	0.0030754	8/176
GO:0007350	Blastoderm segmentation	0.0037407	8/183
GO:0035159	Regulation of tube length, open tracheal system	0.0043868	4/24
GO:0002009	Morphogenesis of an epithelium	0.0043868	9/273
GO:0007525	Somatic muscle development	0.0043868	5/52
GO:0016318	Ommatidial rotation	0.0043868	4/25
GO:0046552	Photoreceptor cell fate commitment	0.0043868	5/53
GO:0007156	Homophilic cell adhesion	0.0043868	4/23
GO:0048859	Formation of anatomical boundary	0.0043868	5/52
GO:0045595	Regulation of cell differentiation	0.0043868	9/258
GO:0007164	Establishment of tissue polarity	0.0043868	6/95
GO:0001736	Establishment of planar polarity	0.0043868	6/95
GO:0001738	Morphogenesis of a polarized epithelium	0.0043868	6/93
GO:0042067	Establishment of ommatidial planar polarity	0.0045886	5/56
GO:0035218	Leg disc development	0.0046107	6/99
GO:0007447	Imaginal disc pattern formation	0.0046107	6/99
GO:0001737	Establishment of imaginal disc-derived wing hair orientation	0.0046107	4/27
GO:0061564	Axon development	0.0054966	9/288
GO:0007409	Axonogenesis	0.0056023	9/290
GO:0035161	Imaginal disc lineage restriction	0.0059912	3/10
GO:0035089	Establishment of apical/basal cell polarity	0.0059912	3/10
GO:0007411	Axon guidance	0.0076958	8/233
GO:0035216	Haltere disc development	0.0076958	3/11
GO:0007419	Ventral cord development	0.0080549	4/33
GO:0008356	Asymmetric cell division	0.0080934	5/68
GO:0030010	Establishment of cell polarity	0.0085754	4/34
GO:0097485	Neuron projection guidance	0.0085768	8/241
GO:0045893	Positive regulation of transcription, DNA-templated	0.0086221	8/243
GO:0090162	Establishment of epithelial cell polarity	0.0086221	3/12
GO:0035152	Regulation of tube architecture, open tracheal system	0.008713	5/71
GO:0061339	Establishment or maintenance of monopolar cell polarity	0.0094451	3/13
GO:1902680	Positive regulation of RNA biosynthetic process	0.0094451	8/250
GO:0061162	Establishment of monopolar cell polarity	0.0094451	3/13
GO:0006935	Chemotaxis	0.0094451	8/252
GO:0010628	Positive regulation of gene expression	0.0094451	8/250
GO:0045746	Negative regulation of Notch signaling pathway	0.0094451	4/37
GO:0051254	Positive regulation of RNA metabolic process	0.0100532	8/255
GO:0007480	Imaginal disc-derived leg morphogenesis	0.0136975	5/81
GO:0045935	Positive regulation of nucleobase-containing compound metabolic process	0.0137101	8/268
GO:0007478	Leg disc morphogenesis	0.0138714	5/82
GO:0010557	Positive regulation of macromolecule biosynthetic process	0.0138714	8/270
GO:0048469	Cell maturation	0.0142985	6/136
GO:0007424	Open tracheal system development	0.0147559	7/201
GO:0007526	Larval somatic muscle development	0.0152069	3/16
GO:0008283	Cell proliferation	0.0152069	8/276
GO:0030855	Epithelial cell differentiation	0.0156113	5/86
GO:0035317	Imaginal disc-derived wing hair organization	0.0162417	4/45
GO:0035151	Regulation of tube size, open tracheal system	0.0162417	4/45
GO:0060541	Respiratory system development	0.0164214	7/208
GO:0035316	Non-sensory hair organization	0.0165607	4/46
GO:0035315	Hair cell differentiation	0.0165607	4/46
GO:0007479	Leg disc proximal/distal pattern formation	0.0165607	3/17
GO:0090066	Regulation of anatomical structure size	0.0181324	6/147
GO:0051173	Positive regulation of nitrogen compound metabolic process	0.0190677	8/292
GO:0035150	Regulation of tube size	0.019956	4/49
GO:0045451	Pole plasm oskar mRNA localization	0.019956	4/49
GO:0030029	Actin filament-based process	0.0206757	7/221
GO:0035223	Leg disc pattern formation	0.0206757	3/19
GO:0007315	Pole plasm assembly	0.0206757	4/50

GO:0009913	Epidermal cell differentiation	0.0220094	4/51
GO:0048665	Neuron fate specification	0.0235462	3/20
GO:0009952	Anterior/posterior pattern specification	0.0239109	6/159
GO:0035272	Exocrine system development	0.0239109	6/160
GO:0021700	Developmental maturation	0.0239109	6/160
GO:0007431	Salivary gland development	0.0239109	6/160
GO:0019094	Pole plasm mRNA localization	0.0239109	4/53
GO:0007316	Pole plasm RNA localization	0.0250086	4/54
GO:0002164	Larval development	0.0275022	5/104
GO:0007449	Proximal/distal pattern formation, imaginal disc	0.0283449	3/22
GO:0060811	Intracellular mRNA localization involved in anterior/posterior axis specification	0.0289852	4/57
GO:0060810	Intracellular mRNA localization involved in pattern specification process	0.0289852	4/57
GO:0007309	Oocyte axis specification	0.0289852	5/106
GO:0051093	Negative regulation of developmental process	0.0294881	6/169
GO:0007028	Cytoplasm organization	0.0301617	4/58
GO:0016339	Calcium-dependent cell-cell adhesion	0.0301617	3/23
GO:0007308	Oocyte construction	0.0324119	5/110
GO:0046530	Photoreceptor cell differentiation	0.0329404	6/174
GO:0030866	Cortical actin cytoskeleton organization	0.0331646	3/24
GO:0005938	Cell cortex	0.0340565	5/112
GO:0048863	Stem cell differentiation	0.0365709	5/114
GO:0007155	Cell adhesion	0.0385271	6/181
GO:0048732	Gland development	0.0385271	6/181
GO:0043588	Skin development	0.038741	4/64
GO:0009954	Proximal/distal pattern formation	0.038741	3/26
GO:0007400	Neuroblast fate determination	0.038741	3/26
GO:0045197	Establishment or maintenance of epithelial cell apical/basal polarity	0.038741	3/26
GO:0050839	Cell adhesion molecule binding	0.0429778	3/27
GO:0044087	Regulation of cellular component biogenesis	0.0438686	6/188
GO:0007219	Notch signaling pathway	0.0438686	5/121
GO:0008544	Epidermis development	0.0439039	4/67
GO:0035222	Wing disc pattern formation	0.0439039	4/67
GO:0060322	Head development	0.0452362	3/28
GO:0007314	Oocyte anterior/posterior axis specification	0.0452362	4/68
GO:0007281	Germ cell development	0.0452362	7/269
GO:0022610	Biological adhesion	0.0475689	6/193
GO:0030865	Cortical cytoskeleton organization	0.0478314	3/29
GO:0014017	Neuroblast fate commitment	0.0478314	3/29
GO:0048867	Stem cell fate determination	0.0478314	3/29
GO:0008358	Maternal determination of anterior/posterior axis, embryo	0.0496686	4/71
GO:0048813	Dendrite morphogenesis	0.0496686	6/196
GO:0008298	Intracellular mRNA localization	0.0496686	4/71
GO:0009968	Negative regulation of signal transduction	0.0496686	6/197
GO:0048599	Oocyte development	0.0496686	5/128
GO:0042659	Regulation of cell fate specification	0.0501471	3/30
GO:0016358	Dendrite development	0.0510814	6/199
GO:1902578	Single-organism localization	0.0514939	5/130
GO:1902580	Single-organism cellular localization	0.0514939	5/130
GO:0010453	Regulation of cell fate commitment	0.0587536	3/32
GO:0022612	Gland morphogenesis	0.0596579	5/135
GO:0007435	Salivary gland morphogenesis	0.0596579	5/135
GO:0061061	Muscle structure development	0.0603274	6/208
GO:0023057	Negative regulation of signaling	0.0603274	6/208
GO:0030036	Actin cytoskeleton organization	0.0603274	6/208
GO:0009994	Oocyte differentiation	0.0617216	5/137
GO:0010648	Negative regulation of cell communication	0.0640062	6/211
GO:0048865	Stem cell fate commitment	0.0718044	3/35
GO:0001751	Compound eye photoreceptor cell differentiation	0.0755541	5/144
GO:0035088	Establishment or maintenance of apical/basal cell polarity	0.0820212	3/37
GO:0061245	Establishment or maintenance of bipolar cell polarity	0.0820212	3/37
GO:0001754	Eye photoreceptor cell differentiation	0.0820212	5/147
GO:0008105	Asymmetric protein localization	0.0873841	3/38
GO:0014016	Neuroblast differentiation	0.0873841	3/38
GO:0008593	Regulation of Notch signaling pathway	0.093645	4/88

Table 3: Functional enrichment table derived from DAVID algorithm for the candidate genes generated by the GWAS. % is defined as the percentage of total genes assigned to the given term; Count is the number of genes assigned to each functional term.

Category	Term	Count	%	*P*-Value	Benjamini
UP_KEYWORDS	Developmental protein	9	16.7	8.60E-04	5.70E-02
INTERPRO	Homeodomain-like	5	9.3	2.40E-03	2.80E-01
GOTERM_BP_DIRECT	Establishment of imaginal disc-derived wing hair orientation	3	5.6	4.40E-03	7.30E-01
UP_KEYWORDS	Homeobox	4	7.4	5.50E-03	1.70E-01
INTERPRO	Homeodomain	4	7.4	6.90E-03	3.70E-01
GOTERM_BP_DIRECT	Homophilic cell adhesion via plasma membrane adhesion molecules	3	5.6	8.10E-03	6.90E-01
GOTERM_BP_DIRECT	Imaginal disc-derived wing morphogenesis	5	9.3	1.10E-02	6.50E-01
GOTERM_BP_DIRECT	cAMP metabolic process	2	3.7	1.20E-02	5.80E-01
GOTERM_CC_DIRECT	Integral component of plasma membrane	7	13	1.40E-02	5.20E-01
GOTERM_BP_DIRECT	Compound eye development	4	7.4	1.40E-02	5.60E-01
SMART	HOX	4	7.4	1.50E-02	4.10E-01
INTERPRO	Allergen V5/Tpx-1-related, conserved site	2	3.7	1.50E-02	5.00E-01
GOTERM_MF_DIRECT	3',5'-cyclic-AMP phosphodiesterase activity	2	3.7	1.70E-02	7.40E-01
UP_KEYWORDS	Coiled coil	12	22.2	1.80E-02	3.30E-01
INTERPRO	3'5'-cyclic nucleotide phosphodiesterase	2	3.7	2.30E-02	5.40E-01
INTERPRO	3'5'-cyclic nucleotide phosphodiesterase, catalytic domain	2	3.7	2.30E-02	5.40E-01
INTERPRO	HD/PDEase domain	2	3.7	2.60E-02	5.20E-01
GOTERM_BP_DIRECT	Establishment of tissue polarity	2	3.7	3.20E-02	7.90E-01
KEGG_PATHWAY	Hippo signaling pathway - fly	3	5.6	3.40E-02	3.80E-01
UP_KEYWORDS	Ion transport	4	7.4	3.50E-02	4.50E-01
SMART	HDc	2	3.7	3.60E-02	4.70E-01
GOTERM_BP_DIRECT	Oogenesis	5	9.3	3.70E-02	7.90E-01
GOTERM_BP_DIRECT	Axon guidance	4	7.4	3.70E-02	7.50E-01
GOTERM_MF_DIRECT	Ubiquitin-protein transferase activity	4	7.4	3.90E-02	7.90E-01
UP_SEQ_FEATURE	splice variant	7	13	4.00E-02	9.70E-01
UP_KEYWORDS	Zinc-finger	5	9.3	4.30E-02	4.50E-01
GOTERM_BP_DIRECT	Establishment of epithelial cell apical/basal polarity	2	3.7	4.30E-02	7.60E-01
UP_KEYWORDS	Transport	6	11.1	4.40E-02	4.00E-01
INTERPRO	Zinc finger, RING/FYVE/PHD-type	4	7.4	4.50E-02	6.50E-01
INTERPRO	Homeobox, conserved site	3	5.6	4.80E-02	6.20E-01
GOTERM_BP_DIRECT	Wing disc development	3	5.6	4.80E-02	7.60E-01
GOTERM_MF_DIRECT	Protein binding	7	13	5.10E-02	7.50E-01
GOTERM_BP_DIRECT	Eye development	2	3.7	5.50E-02	7.70E-01
UP_KEYWORDS	Metal-binding	8	14.8	5.60E-02	4.30E-01
INTERPRO	PAS domain	2	3.7	5.90E-02	6.50E-01
GOTERM_CC_DIRECT	Cell cortex	3	5.6	6.00E-02	8.00E-01
GOTERM_BP_DIRECT	Regulation of protein localization	2	3.7	6.20E-02	7.90E-01
UP_KEYWORDS	Receptor	5	9.3	6.40E-02	4.30E-01
UP_KEYWORDS	Alternative splicing	6	11.1	6.50E-02	4.00E-01
GOTERM_BP_DIRECT	Leg disc proximal/distal pattern formation	2	3.7	6.60E-02	7.80E-01
UP_KEYWORDS	Membrane	17	31.5	7.80E-02	4.20E-01
UP_SEQ_FEATURE	Compositionally biased region:Poly-Ala	3	5.6	8.00E-02	9.70E-01
GOTERM_BP_DIRECT	Multicellular organism reproduction	4	7.4	8.40E-02	8.40E-01
GOTERM_BP_DIRECT	Asymmetric cell division	2	3.7	9.90E-02	8.70E-01
GOTERM_BP_DIRECT	Cortical actin cytoskeleton organization	2	3.7	9.90E-02	8.70E-01

Table 4: The clustering of the GWAS candidate genes based on geneMANIA and DAVID functional enrichment analyses. The clusters were used to generate the Venn Diagram shown in Figure 7.

RNA and protein processing	Developmental protein	Reproduction protein	Membrane protein	Others	Unrecognised
FBgn0024321	FBgn0003129	FBgn0031412	FBgn0016120	FBgn0031407	FBgn0052027
FBgn0003129	FBgn0001235	FBgn0000256	FBgn0036043	FBgn0033092	FBgn0052111
FBgn0267487	FBgn0015524	FBgn0000662	FBgn0011589	FBgn0035570	FBgn0052773
FBgn0000662	FBgn0024321	FBgn0264815	FBgn0036816	FBgn0036728	FBgn0263768
FBgn0001235	FBgn0085424	FBgn0266377	FBgn0037690	FBgn0037687	FBgn0265415
FBgn0085424	FBgn0005677	FBgn0052062	FBgn0000256		
FBgn0015524	FBgn0267487	FBgn0011591	FBgn0036260		
FBgn0001122	FBgn0000662	FBgn0053126	FBgn0001075		
FBgn0036816	FBgn0052062	FBgn0026206	FBgn0001085		
FBgn0035397	FBgn0003884	FBgn0259968	FBgn0023095		
FBgn0026319	FBgn0005636	FBgn0036075	FBgn0033196		
FBgn0001085	FBgn0266377	FBgn0033196	FBgn0035983		
FBgn0031816	FBgn0000256		FBgn0267487		
FBgn0035983	FBgn0011591		FBgn0005636		
FBgn0026206	FBgn0001085		FBgn0053542		
FBgn0005636	FBgn0038678		FBgn0011591		
FBgn0053547	FBgn0023095				
FBgn0264815	FBgn0001075				
FBgn0011591	FBgn0036816				
FBgn0036075	FBgn0036260				
FBgn0053531	FBgn0028494				
FBgn0261552	FBgn0030794				
	FBgn0086901				
	FBgn0086367				

Genetic variation in immune responses to Sex Peptide in Drosophila melanogaster

Jessy Rouhana, Tracey Chapman, Bregje Wertheim

3

Abstract

Sexual conflict can lead to an "arms race" between males and females, where males optimise their reproductive fitness in a way that results in costs to females, and females evolve counter-adaptations to resist males. In *Drosophila melanogaster* one route by which sexual conflict can be manifested is via the transfer of seminal fluid proteins (Sfps), such as Sex Peptide transferred along with the sperm. These proteins are responsible for initiating several important post-mating responses (PMRs) in the female. Sex Peptide can be considered a 'master regulator' as it is responsible for several different PMRs, including alterations to immune gene expression, which was studied here. Females can evolve resistance to mitigate the costs arising from the expression of sexual conflict, but also to tailor their responses according to local ecological conditions such as nutritional status. Both of these effects can lead to the maintenance of genetic variation in female PMRs. In most studies this genetic variation is experimentally minimised to clearly delineate Sex Peptide function. However, to understand the evolutionary processes and dynamics that characterise Sex Peptide -mediated interactions, a key step is to identify and study this genetic variation. To investigate immune gene responses to Sex Peptide, we screened for variation in expression of 6 antimicrobial peptides (AMPs) upon receipt of Sex Peptide in females from 31 genome-sequenced lines of *D. melanogaster*. This showed significant variation in immune response to Sex Peptide. A Genome Wide Association Study (GWAS) was then conducted and revealed a set of candidate genes putatively involved in modulation of the Sex Peptide post-mating immune response in females. We discuss the function of these genes and their involvement in the immune pathways. Overall the study implicated several genes involved in negative regulation of the Imd immune pathway, and a number of immunoglobulin genes with possible immune functions.

Keywords

Sex-peptide, Immunity, *Drosophila melanogaster*, DGRP, GWAS, Sexual selection

Introduction

Mating is a complex interaction between males and females, and both sexes may have contrasting strategies to optimise their reproductive fitness. While the sexes need to co-operate overall to reproduce successfully, the strategies they employ often also have conflictual elements. For example, while males can increase their fitness by mating with multiple females, they suffer a reduced fitness when the females re-mate with other males (Gage, 2004). Furthermore, males may benefit from a large investment made by their mates in current reproduction, even if that is at the cost of the female's subsequent lifespan and/or future reproduction (Fowler and Partridge, 1989; Arnqvist and Nilsson, 2000; Franklin and Stuart-Fox, 2017). Thus, males may evolve antagonistic strategies to manipulate the female after mating, while females may evolve counter-adaptations to defend themselves against these manipulations.

Mating initiates a series of events within female *Drosophila melanogaster* in addition to sperm storage and ovulation. Females receive at least 163 different seminal fluid proteins (Sfps) that are transferred along with the sperm and these molecules trigger diverse responses in female physiology and behavior (Findlay *et al.*, 2008; Sepil *et al.*, 2018). These remarkable post mating responses (PMRs) include, among others, altered ovulation, receptivity, sleeping and eating behavior (Chen *et al.*, 1988; Chapman *et al.*, 2000; Heifetz *et al.*, 2000; Lung *et al.*, 2002; Ram *et al.*, 2005; Carvalho *et al.*, 2006; Avila and Wolfner, 2009; Isaac *et al.*, 2010). Interestingly, these Sfps promote fertilization and can favour the interests of males whilst also generating costs in females (Chapman *et al.*, 2003). This sexual conflict can lead to an arms race, where females evolve counter-adaptations to mitigate the costs and manipulations mediated by the transfer of Sfps by males. These counter-adaptations may, in part, consist of mechanisms to tailor the sensitivity or regulatory control of Sfps (Sirot *et al.*, 2015).

In the fruit fly *D. melanogaster,* one Sfp in particular, known as ACP70A or 'Sex Peptide' has been widely studied. Once it is transferred into females it generates a wide range of responses, including increased egg laying and feeding behavior, induction of immune gene expression, decreased sleep and decreased sexual receptivity (Manning, 1967; Chen, 1984; Chen *et al.*, 1988; Chapman *et al.*, 1995; Peng, Zipperlen and Kubli, 2005;

Carvalho *et al.*, 2006; Domanitskaya *et al.*, 2007; Ribeiro and Dickson, 2010; Isaac *et al.*,, 2014). Sex Peptide adheres to sperm tails once inside the females (Clive Wilson, pers. Comm, Peng *et al.*, 2005 b), and can influence the expression of a diverse array of genes in females over a period of at least several hours and in different parts of the body (Gioti *et al.*, 2012). There are significant alterations in expression of genes linked to egg development, early embryogenesis, immunity, nutrient sensing and behavior (Gioti *et al.*, 2012). This widespread reprogramming of female gene expression suggests that Sex Peptide is a 'master regulator' of female reproduction.

One of the many PMRs induced by mating is the activation of the immune responses (Peng, Zipperlen and Kubli, 2005; Innocenti and Morrow, 2009; Barribeau and Schmid-Hempel, 2017). Insects possess a sensitive and sophisticated innate immune system (Gillespie, Kanost and Trenczek, 1997; Schmid-Hempel, 2005). This is activated upon attack by pathogens (bacteria, fungi, viruses), parasites (protists, nematodes), parasitoids or non-self-molecules (Gillespie *et al*, 1997; Beckage, 2008). In bacterial infections the innate immune system produces antimicrobial peptides (AMPs) to combat infection, once the epithelial barrier has been breached. Three main immune pathways activate the transcription of AMPs, the Toll, immune deficiency (Imd) and the Janus kinase/signal transducer pathways (JAK/STAT). Different pathways are specifically induced in response to the type of pathogen or immune challenge. For example, the Toll pathway controls the resistance to fungal and gram-positive bacterial infection and the Imd pathway is responsible for the defense against Gram-negative bacteria (Lemaitre and Hoffmann, 2007; Obbard *et al.*, 2009). The JAK/STAT pathway is suggested to be involved in several biological processes, including the repair of tissue damage, regulation of haemocyte proliferation, stress responses, and resistance against parasitoids and viruses (Lemaitre and Hoffmann, 2007; Myllymäki and Rämet, 2014). The immune response induced after mating in *D. melanogaster* could be a response to wounding and tissue damage during mating (females are grasped by the males using claspers; Wigby *et al.*, 2008), and/or to the introduction of the foreign substances in the reproductive tract activating the epithelial localized immunity (Tzou *et al.*, 2000).

One of the mechanisms by which the immune response can be triggered during mating is through receipt of Sex Peptide itself. It is suggested that Sex Peptide chemically mimics

the sugar components of the bacterial cell wall, eliciting factors of the innate immune response (Domanitskaya *et al.*, 2007). If this hypothesis is correct, then Sex Peptide circulating in the haemolymph may be detected by the same pattern recognition receptors that normally detect pathogens. After these receptors are activated by direct contact with Sex Peptide, they would trigger the humoral immune response via both the Toll and Imd pathways, which in turn will activate the expression of different AMPs (Peng, Zipperlen and Kubli, 2005; Domanitskaya *et al.*, 2007; Gioti *et al.*, 2012). It is not clear what the adaptive consequences of Sex Peptide eliciting an immune response might be. The activation of AMPs might be a side-effect, or might be a direct effect imposed by males to manipulate female physiology to induce an "immune-like" response (Morrow and Innocenti, 2012). An alternative hypothesis is that the immune response may assist in protecting the sperm from infection and thus ensure a long term protective effect for offspring, which may increase reproductive success overall (Lung *et al*., 2001). The final idea is that immune induction following mating may protect the female from sexually transmitted diseases or pathogens (Knell and Webberley, 2004; Miest and Bloch-Qazi, 2008).

It appears that the effect of Sex Peptide on the immune response may vary significantly between different *D. melanogaster* populations. According to previous studies, Sex Peptide induces the expression of seven AMPs from both the Toll and Imd pathways. This upregulation was clear in Canton S and Oregon R wild-type lines (McGraw *et al.*, 2004; Peng, Zipperlen and Kubli, 2005; Wigby *et al.*, 2008), but not in the Dahomey wild-type (Gioti *et al.*, 2012). This phenotypic variation in immune gene expression in response to receipt of Sex Peptide suggests the presence of underlying genetic variation between these different populations. This variation could be driven by adaptation to local differences in exposure to different environmental conditions and / or demographic factors (Tinsley *et al*., 2006). Alternatively, this could be the result of sexually antagonistic co-evolution between the sexes, where females have evolved resistance to males' reproductive fitness adaptations (Rice, 1996).

The PMR effects induced by Sex Peptide receipt have been well studied, but little is known about the underpinning genetic variation in, or evolution of, female PMRs. To better understand the pace, dynamics and trajectory of co-evolution arising from the potential

manipulation of gene expression in one sex by the other, it is necessary to understand the molecular interactions between males and females. With the availability of fully genome sequenced lines for *D. melanogaster*, genome-wide association studies (GWAS) can be used to search the whole genome for polymorphisms or other genomic features that influence specific phenotypes. This makes it now possible to study the complex interactions between genotype and phenotype, by studying the genetic variation that occurs within a population and linking it to individual phenotypic variation.

In this study, we investigated the genetic variation in females of *D. melanogaster* of immune responses to mating and receipt of Sex Peptide. We used a panel of isofemale lines to investigate the natural genetic variation in AMP expression after mating with wild-type (SP^+) or Sex Peptide null-mutant males (SP^0). The transcription of several AMPs, regulated through the Toll and the Imd immune pathways, was quantified in females before and after mating to SP^0 and SP^+ males in isofemale lines from two different populations. In the first assay, we tested 6 AMPs in lines from 2 genetic backgrounds to examine the generality of the changes of expression in AMPs in response to Sex Peptide. In a second assay, we selected 3 immune genes that were representative of the three immune response pathways, and we combined the phenotypic assessment with a Genome-Wide Association Study (GWAS) to identify which polymorphisms may be involved in the phenotypic variation. This second assay was done in a single genetic background, to avoid the confounding effects of the 2 genetic backgrounds used in the first assay upon the GWAS analysis.

Material and Methods:

Fly Lines:

Female Isolines

Two sets of inbred, genome-sequenced lines of *D. melanogaster* were used in this study, one originating from the *D. melanogaster* Drosophila Genome Reference Panel (DGRP) (Mackay et al., 2012) and one from a set of French lines (Verspoor and Haddrill, 2011). The DGRP lines were collected from the wild in North Carolina, USA (Mackay et al., 2012) and the French lines from Montpelier, France (Verspoor and Haddrill, 2011), respectively. The genome sequences of all these lines are publicly available (Bergman & Haddrill 2015; Mackay et al., 2012). Both lines were tested in the initial mating assay, but only the DGRP lines were used for the second assay and the GWAS analysis.

Sex Peptide knockout line

The Sex Peptide-lacking males were derived from mutant Control (SP^+) and Sex Peptide null (SP^0) lines (Liu and Kubli, 2003). The SP^0 (*SP^0/Δ130*) males bear a non-functional Sex Peptide gene. SP^0 males were generated from a cross between (*SP^0/TM3 Sb ry*) males in which the Sex Peptide gene is knocked out, to SPΔ2-7 females (*Δ130/TM3 Sb ry*) in which Δ130 is a deletion of amino acid 2 to 7 in the N-terminal region of the Sex Peptide gene. The SP^+ (*SP^0, SP^+/Δ130*) control line contains the SP^0 knock out and the wild-type Sex Peptide genes in tandem. These were obtained by crossing SP^0, *SP^+/TM3 Sb ry* males to *Δ130/TM3 Sb ry* females. The deletion and Sex peptide mutant fly stocks were previously back-crossed into the Dahomey wild-type genetic background, to increase the vigour of the males and to introduce a wild-type genetic background for both SP^+ and SP^0 males (Fricke *et al.,* 2010).

Dahomey wild type

Dahomey wild-type *D. melanogaster* were kept in large population cages with overlapping generations on Sugar Yeast Agar (SYA) medium (100g yeast, 50g sucrose, 15g agar, 30ml Nipagin solution and 3ml Propionic Acid) at 25°C in a 50% humidified room on a 12L:12D cycle.

Rearing, collection and mating

Mating assay 1

In the first mating assay, flies were reared in glass 70ml bottles on a sugar-rich (SR) medium (17g agar, 26g dry yeast, 54g sugar and 13ml Nipagin (10% w/v) solution per litre) at 20°C in a 50% humidified room under 12L:12D cycle. A total of 13 French lines and 17 DGRP lines were used in this assay. The flies were collected immediately from stocks as virgins under CO_2 anaesthesia and were held in groups of 2 (for males) and 1 (for females) in 10ml SR vials, until they were used in experiments, at 3-5 days old. The mating assays were conducted by placing two males (to increase competition and ensure one male mated with the female) and one female together in a vial with SR medium. The extra male was removed once the mating started, and the mated male was removed from the vial immediately after the pair separated. Mated females were flash frozen in liquid nitrogen 4 hours after the start of mating (mating duration is usually 10-15 min). To collect virgin females, females were kept in vials containing a single female, and these were also flash frozen 4 hours after the start of experiment, such that they were of the same age at death and collected at the same time of day. Samples were then stored at –80°C until RNA extraction.

Mating assay 2

All flies for this assay were kept on a SYA medium (100g yeast, 50g sucrose, 15g agar, 30ml Nipagin solution and 3ml Propionic Acid) at 25°C with 12L:12D cycle and 50% humidity. To standardize the rearing environment and to synchronise adult hatching time of virgin females, 5 sets of vials with 5 males and 5 females for each of the 31 different DGRP lines and the Dahomey wild type were placed in SYA vials for 24 hours before being discarded. The resulting offspring were raised in these vials until the collection of the virgin females. To generate the SP-lacking and control males, 50 females (genotype *Δ130/TM3 Sb ry*) were placed with 50 males of either genotype (SP^0, *SP^+/TM3 Sb ry*), (*SP^0/TM3 Sb ry*) in bottles containing 70ml SYA medium each, and were transferred daily into new food. This generated a synchronised cohort of males and standardised larval density. When hatched, both the DGRP females and SP^0 and SP^+ males were collected on ice and were held individually (for females) or in groups of 10 (for males) for 4-5 days until mating. The mating assay for all 31 DGRP lines was split

over two days and Dahomey females were tested on both days to serve as a reference for random day-to-day variation. Each female was mated once with either a SP^0 or SP^+ male. Immediately after mating the males were discarded. For each DGRP line, 9 females mated to each type of male were collected individually. Females were then flash-frozen in liquid N_2 at 4 hours after the end of mating. The virgin females for each DGRP line were kept separately without males and were also flash frozen 4 hours after the start of the mating experiment. Samples were then stored at -80°C until RNA extraction.

RNA extraction, cDNA conversion and Q-PCR quantification

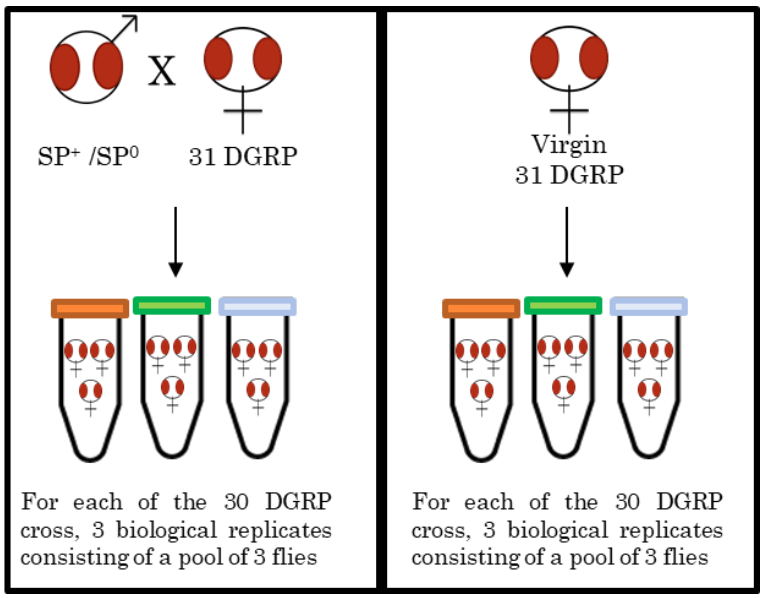


Figure 1: Schematic diagram summarising the experimental setup.

RNA was extracted from pools comprising three adult female flies. The RNA extraction was performed on three biological replicates for each line and mating treatment (SP0, SP+, virgin) (Figure 1). Total RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer's instructions and each extraction was DNase treated using DNase (Ambion) to remove any contaminating DNA. For each sample, complementary DNA (cDNA) was synthesized from 0.5-1µg total RNA using the RevertAid H Minus First Standard cDNA Synthesis kit (Thermo scientific). The cDNA was then diluted 100 times before preparing the quantitative Real-Time PCR (qPCR). The qPCR was performed using SYBR green (Quanta Biosciences) with ROX as the internal passive reference, and 2µl of diluted cDNA was used for each reaction of 20µl

total volume, containing the forward and reverse primers at the final concentration of 400nM and 10µl of SYBR green/ROX buffer solution. Two technical replicates for each biological replicate were performed to correct for pipetting errors while performing the qPCR. The repeatability was tested by calculating the correlation between the technical replicates (Supplementary data Figure 1). This showed that the expression levels of the technical replicates were highly repeatable (P< 2.2e-16). In total 4 outliers (>2sd) were discarded due to excessive varibility. The mean of both technical replicates was then used in the data analysis and comprised the datapoint for each of the three biological replicates for each sample. Reactions were run using the Abi7300 machine with the following qPCR profile: 5min of activation phase at 95°C, 35 cycles of 10sec at 95°C, 30sec at 56°C and 30sec at 72°C. The primers used are listed in Table 1.

To compare the expression of immune response genes between the different lines, when mated to SP^+, SP^0 males or remaining virgin, a relative quantification was performed on the expression of 6 different genes with known function as anti-microbial peptide or as activator of the immunity signal transduction cascade (mating assay 1) or a subset of 3 of these (mating assay 2), as well as 1 housekeeping gene (*Actin 5C*) (Table 1). Expression data were first analysed with LinRegPCR to obtain the starting concentrations of the 7 gene transcripts for each sample (Ramakers *et al.*, 2003; Ruijter *et al.*, 2009) and then relative expression levels were calculated by normalizing the 6 AMPs against the gene expression data of the housekeeping gene *Actin 5C*.

Table 1: qPCR primers of the different genes used in this study.

Gene name	Annotation	Pathway	Primers Forward	Primers Reverse	Size
Mtk	**CG8175**	Toll & Imd	5'-AACTTAATCTTGGAGCGA-3'	5'-CGGTCTTGGTTGGTTAG-3'	140bp
Dro	**CG10816**	Imd	5'-CCATCGTTTTCCTGCT-3'	5'- CTTGAGTCAGGTGATCC-3'	150bp
IM1	**CG18108**	Toll	5'-TCCACTGTCGCCCGATCC-3'	5'- CTTGGGTTGAAACTTCCT-3'	92pb
Dpt-A	**CG12763**	Imd	5'-GCTGCGCAATCGCTTCTACT-3'	5'-TGGTGGAGTGGGCTTCATG-3'	68bp
Dpt-B	**CG10794**	Imd	5'-GAACCACTGGCATATGCTCC-3'	5'-GCTCAGATCGAATCCTTGCT-3'	110bp
PGRP-SB1	**CG9681**	Imd	5'-TTTGCTGCTTAGCTCTATCC-3'	5'-TGATGGATGATCACATAGTCG-3'	120bp
Act5C	**CG4027**	Housekeeping gene	5'-CCGACCGTATGCAGAAGGAG-3'	5'-TGGAAGGTGGACAGCGAAG-3'	130bp

Statistical analysis

All statistical analyses were conducted using RStudio (Version 0.99.903) (RStudio, 2016). For the first mating assay a two-way analysis of variance (ANOVA) was used to test for differences between the lines in response to the three mating treatments (SP^0, SP^+ and virgins). The ANOVA was carried out on the log-transformed relative expression level of the different anti-microbial peptide for each line and each type of cross. The residuals of these analysis conformed to normality. In addition, model simplifications were carried out with ANOVA to test for the separate effects of mating and of Sex Peptide on the expression of AMP genes (Crawley, 2013). For this, we analysed the effect of mating (pooling the treatments SP^+ and SP0), in comparison to virgin females, on AMP gene expression. We compared whether each of these simplified models differed in Akaike Information Criteria (AIC) from the more complex models. AIC estimates the quality of each model, the less information a model loses, the higher the quality of that model. We sought in each case the model with the smallest AIC, which indicated the best model fit (Bertrand *et al.*, 2006).

For the second mating assay, the statistical analyses were performed using the "Glmer" function on the "lme4" package (Bates *et al.*, 2014). The significance of factors was determined by step-wise model reduction from the maximal model via likelihood ratio tests (LRT), whereby the deviance (D) is the difference between the log likelihood of the reduced model and the log likelihood of the full model, using the Chi-square test for assigning significance. The maximal model included the cross (mating treatment) as a fixed effect, and the DGRP lines and qPCR plates as random factors. In the simplified model, the random effect of DGRP lines was omitted to test for significant genetic variation in gene expression. Linear regression models were used to test for correlation of AMP gene expression levels between the different mating treatments (Chambers, 1992).

Genome-wide association study

In order to identify single nucleotide polymorphisms (SNPs) associated with variation in the immune response to different types of mating for the 31 DGRP inbred lines, a Genome-Wide Association Study (GWAS) was performed on the qPCR results of assay 2,

using SNPs and indels with minor allele frequencies ≥ 0.05 on the DGRP webserver (dgrp2.gnets.ncsu.edu)(Mackay et al., 2012). These GWAS analyses accounted for effects of *Wolbachia* infection, cryptic relatedness due to major inversions, and residual polygenic relatedness (Mackay et al., 2012). The analyses were performed separately on the mean relative expression of three AMPs after mating to SP^+ or SP^0 males: the *IM1* gene, the *Dpt-B* gene and *Mtk* gene. These 3 genes were selected based on the results from assay 1, and because they each represent regulation through separate immune pathways (Toll or Imd) and the combined Toll and Imd pathway (Table 1).

All of the top candidate genes from the GWAS with a *P*-value < 10^{-5} were then used for functional enrichment analysis using DAVID bioinformatic resources 6.8, NIAID/NIH (Huang, Lempicki and Sherman, 2009) to identify over-represented functional annotations among the genes associated with responses to mating with SP^+ males.

Network mapping using geneMANIA Cytoscape 3.4.0 plugging (Data Version: 13/07/2017) (Shannon *et al.*, 2003; Montojo *et al.*, 2010; Warde-Farley *et al.*, 2010) was also performed on the top candidate genes generated by the GWAS when females mated to SP^+ males for all three AMP genes tested. The geneMANIA server predicts a functional network for genes in Cytoscape (Montojo *et al.*, 2010; Warde-Farley *et al.*, 2010). Network mapping by GeneMANIA is based on several databases, including 1) gene co-expression, where genes are linked when their expression level is similar across the same conditions; 2) genetic interactions, with two genes being functionally associated if the effects of perturbing one are modified by perturbations to a second, 3) physical interactions, where the proteins are linked if they were found to interact in a protein-protein interaction study, 4) co-localisation, where two genes are linked if they are both expressed in the same tissue or if their gene products are both identified in the same cellular location, 5) shared protein domains, where genes are linked if they have the same protein domain, and 6) the predicted network specifies a functional relationship between genes, often protein interactions, that have orthologues in different organisms. It is designed to account for random network associations as explained in the previous chapter, p54 (Mostafavi *et al.*, 2008).

Results:

Mating assay 1

In the first mating assay, we screened for changes in expression in several AMPs in response to Sex Peptide receipt across 30 genotypes originated from French and American isofemale lines. The analysis of variance (ANOVA) of the relative expression data showed a significant variation in AMP expression across lines, across the different mating treatments (SP^0, SP^+ or Virgins) and an interaction between lines and mating treatments. This occurred in AMPs for both major immune pathways tested (Toll and Imd) (Table 2). The initial analysis showed a significant effect of mating overall on the expression of all 6 tested AMPs, irrespective of whether the males were SP^+ or SP^0. However, for *Dpt-B* and *Mtk* the effect of mating with either SP^+ or SP^0 males differed, as indicated by a significant effect of SP^+ or SP^0, with some lines expressing a higher expression of *Mtk* (*P*= 0.008266**, *Df*=58, 157) and *Dpt-B* (*P* =0.01955*, *Df*=58, 158) in SP^+ mating than in SP^0 mating. For all the other AMPs, there was no significant difference in expression following mating to either SP^+ or SP^0 males (Table 2).

Together the data show that there was significant genetic variation of AMP gene expression in response to mating and to the receipt of Sex Peptide. However, different lines showed very different responses. The effects occurred in both the Toll and Imd pathways. Based on this assay a selection of 3 representative AMPs from both pathways that showed a good level of expression were selected for a more detailed investigation of 31 DGRP lines, as described below.

Mating assay 2

To measure the immune activation in the tested AMPs of the Toll and Imd pathways, the transcription of *Mtk, Dpt-B* and *IM1* was studied in more detail in an assay of a core set of 31 DGRP lines and the Dahomey wild-type. To check for day-to-day variation, the Dahomey line was included on each day of the tests. Analysis of these Dahomey samples showed that there was no significant variation in expression of AMPs across days or across mating treatments (SP^0, SP^+ or virgins), consistent with previous reports for this strain (Gioti *et al.*, 2012). The LTR statistical model used to analyse the data accounted

for technical variation between qPCR plates and revealed that there was no qPCR plate-to-plate variation in this in this assay for all the DGRP and Dahomey samples tested.

For all three genes, expression was significantly different in each of the three types of mating treatment: *Mtk* (*P*=0.005355**, *F*=5.3622), *Dpt-B* (*P*=0.01195*, *F*=4.5214) and *IM1* (*P*< 0.005355**, *F*=5.3622) (Figure 2). Analysis showed that relative gene expression varied significantly across the DGRP lines, for *Mtk* (Glmer, Chisq= 100.2, *P*<2.2e-16***), *Dpt-B* (GLMER, Chisq= 86.58, *P*<2.2e-16***), and *IM1* (GLMER, Chisq= 100.2, *P*<2.2e-16***). The data showed that there was significant genetic variation in AMP expression in response to mating in all 3 pathways (Toll, Imd and both pathways combined). In addition, there was substantial variation in constitutive levels of expression of immune genes in unmated flies.

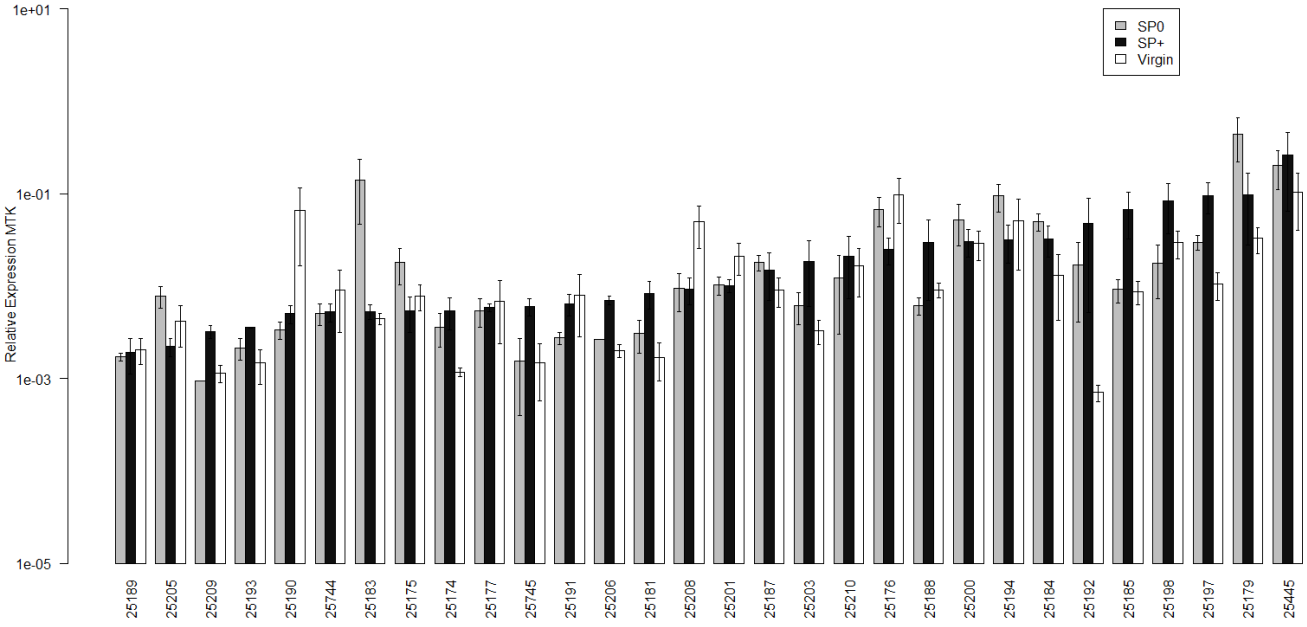
Table2: Analysis of variance table of assay 1 showing an analysis of the expression of different AMPs in *D. melanogaster* when unmated (virgins) or mated to SP^+, SP^0 males. P values <0.05 are considered significant (p < 0.0001 '***', P= 0.001 '**', P= 0.01 '*' 0.05).

Tests for gene expression differences				
Immune pathways	**Gene**	**Mating treatment SP^+/SP^0/Virgin**	**Line**	**Mating treatment SP^0/ SP^+ X Line**
Toll pathway	**IM1**	P =1.192e-11*** F=29.6503 Df=2, 157	P <2.2e-16*** F=7.4724 Df=29, 157	P =0.2074 F=1.1833 Df=58, 157
Immune Deficiency pathway (Imd)	**Dpt-B**	P =6.374e-06*** F=12.9166 Df=2, 158	P =1.124e-10*** F=4.6822 Df= 29, 158	P =0.01955* F=1.5351 Df=58, 158
	Drosocin	P =1.307e-07*** F=17.5519 Df=2, 158	P =3.333e-16*** F=6.8244 Df=29, 158	P =0.1013 F=1.3033 Df=58, 158
	PGRP-SB1	P =2.923e-06*** F=13.8282 Df=2, 158	P <2.2e-16*** F=24.3829 Df=29, 158	P =0.3302 F=1.0917 Df=58, 158
	Dpt-A	P =6.350e-05*** F=10.2805 Df=2, 158	P =6.351e-12*** F=5.1439 Df= 29, 158	P =0.104 F=1.2991 Df=58, 158
Toll and Imd pathway	**Mtk**	P= 3.613e-07*** F=16.3277 Df=2, 157	P= 6.927e-11*** F=4.7670 Df=29, 157	P= 0.008266** F= 1.6447 Df=58, 157

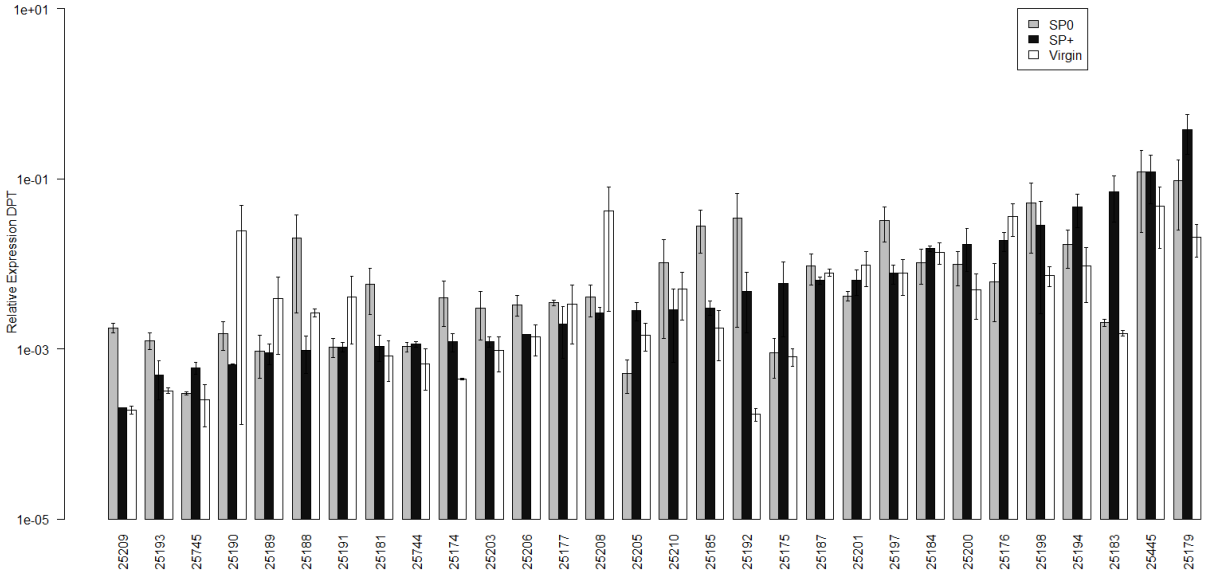
Table3: Statistical analysis table of assay 2 showing the expression of different AMPs in unmated females (virgins) or in females mated to SP^+, SP^0 males. P values <0.05 are considered significant. (p < 0.0001 '***', P= 0.001 '**', P= 0.01 '*' 0.05).

Tests for gene expression differences			
Immune pathways	**Gene**	**Mating treatment SP+/SP0/Virgin**	**Line**
Toll pathway	**IM1**	*P* = 0.005355 ** F= 5.3622 Df= 209, 99	P <2.2e-16*** *Chisq*= 100.2
Immune Deficiency pathway (Imd)	**Dpt-B**	*P* =0.01195 * *F*= 4.5214 *Df*= 209, 98	P <2.2e-16*** *Chisq*= 86.58
Toll and Imd pathway	**Mtk**	*P* = 0.005355 ** F= 5.3622 Df= 209, 99	P <2.2e-16*** *Chisq*= 100.2

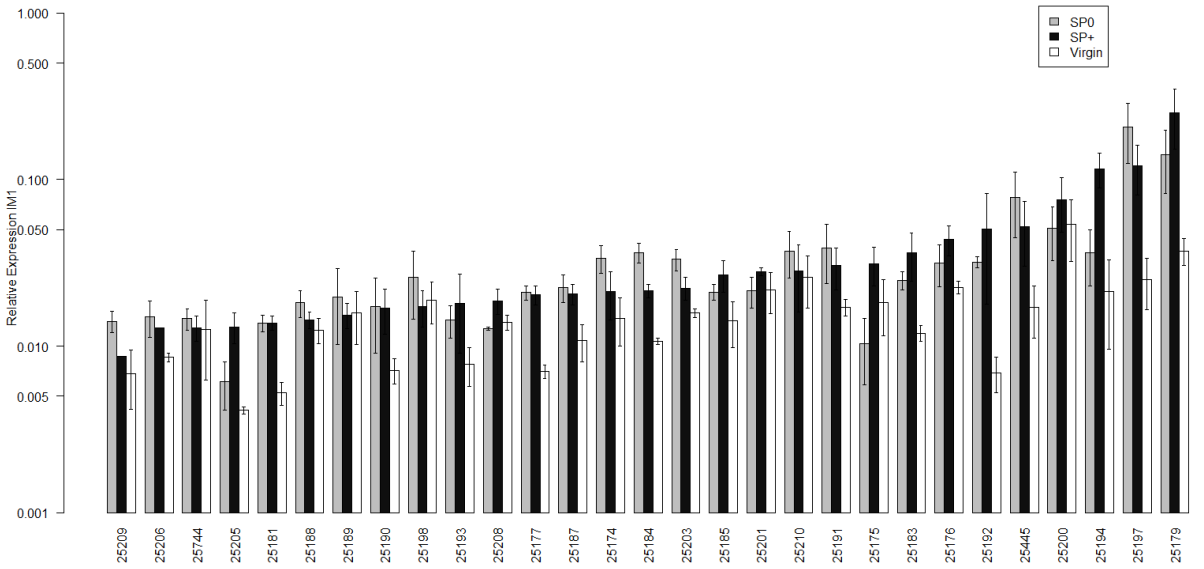
A



B

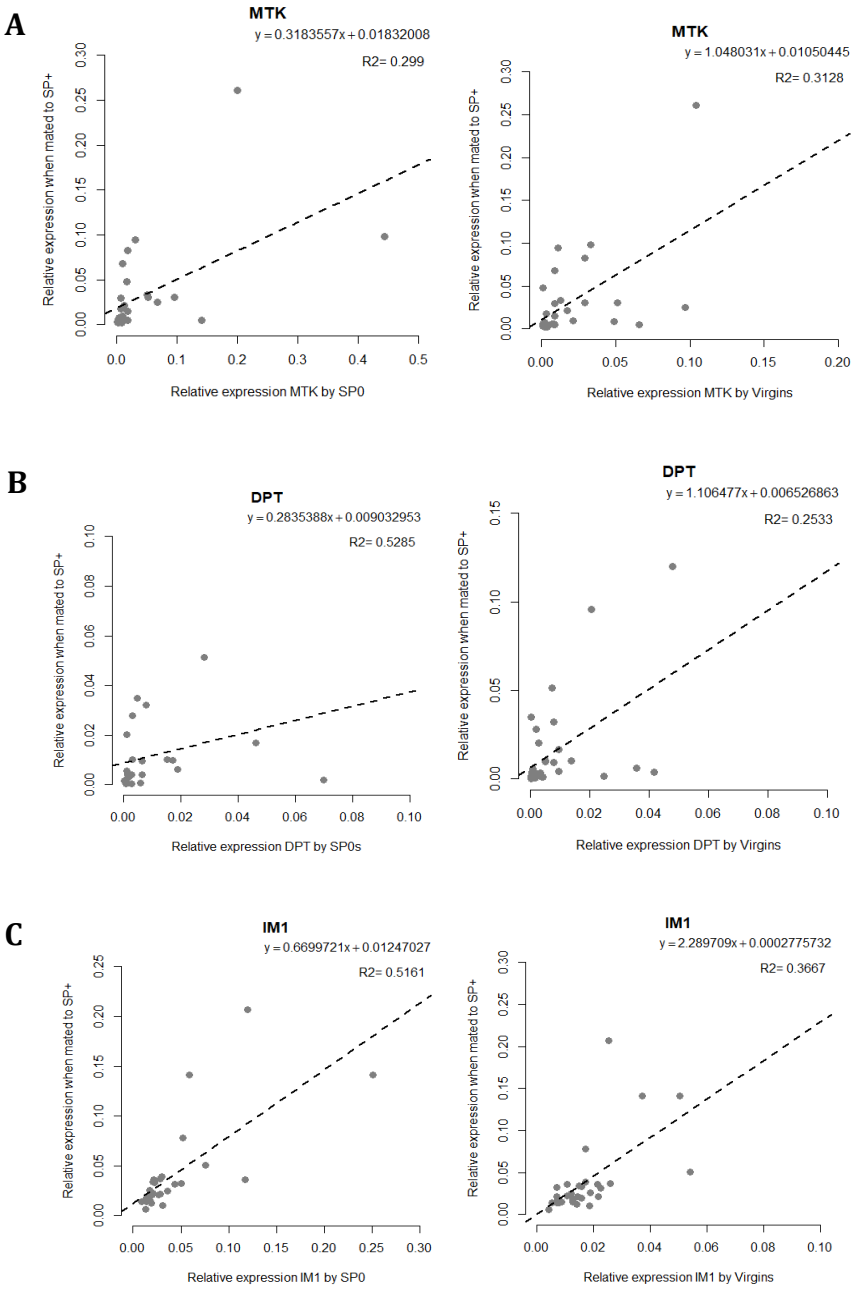


C



<u>Figure 2</u>: Relative expression of antimicrobial peptide genes. Bar plot presentation of the logarithmic relative expression (mean± SE of 3 replicate qPCR) of antimicrobial peptide genes (A) *Mtk*, (B) *Dpt-B* and (C) *IM1* in *D. melanogaster* virgin females and in females mated to either SP^0 or SP^+ males, 4 hours after the start of mating. Data points are plotted from lowest (left) to highest (right) level of expression in response to mating with SP^+ males.

Relative expression of AMPs and treatments



<u>Figure 3</u>: Correlation of AMP expression between different mating treatments. Correlation plots for average values per line of AMP expression between different treatments. (A) correlation of *Mtk* relative expression when females were mated to SP^0 and SP^+ males, and when females were mated to SP^+ males or remained virgins. (B) correlation of *Dpt-B* relative expression when females were mated to SP^0 and SP^+ males, and when females were mated to SP^+ males or remained virgins. (C) correlation of *IM1*

relative expression when females were mated to SP^0 and SP^+ males, and when females were mated to SP^+ males or remained virgins.

To determine whether AMP expression levels were correlated between the different mating treatments (SP^0 versus SP^+ males, or virgins versus SP^+ males), a correlation analysis was conducted for the assayed DGRP lines. The average gene expression for all three the antimicrobial peptides showed a positive correlation when females were mated to either SP^+ or SP^0 males *(Mtk: F*=11.95*, P*=0.001766*; Dpt-B: F*=31.39*, P*=5.36e-06*; IM1: F*=29.87*, P*=7.79e-06) (Figure 3). A significant correlation was also detected for the gene expression levels of all three AMPs between virgin and SP^+ male-mated females *(Mtk: F*=12.74*, P*=0.001315*; Dpt-B: F*=9.498*, P*=0.004582*; IM1: F*=16.21*, P*=0.0003912) (Figure 3). Since a correlation in AMP expression existed between the different treatments, we infer that the induced expression of the AMP genes in response to Sex Peptide can be partly a response to mating, as mating with and without Sex Peptide induced similar levels of AMP gene expression. Additionally, lines with a constitutively high level of AMP gene expression also tended to be the lines that responded most strongly to Sex Peptide receipt. Nonetheless, model simplifications that grouped the treatments for mating with SP^+ and SP^0 males together significantly reduced the fit of the model to the data (*Mtk*: F= 5.3622, *P*=0.005355; *Dpt-B:* F= 4.5214, *P* =0.01195; *IM1:* F= 5.3622, *P* = 0.005355). Thus, while mating and Sex Peptide receipt induced similar responses in the expression of AMP genes to some extent, at least for some lines upon the receipt of Sex Peptide.

GWAS analysis

A GWAS was performed to identify polymorphic (SNPs, insertion and deletion) markers that correlated with the variation in the expression differences of 3 AMPs (*IM1, Dpt-B* and *Mtk*) in females when mated to SP^+ or SP^0 males. The GWAS was performed separately for each gene, and for the SP^+ and SP^0 mating treatment, using the functionality of the DGRP website by providing the mean of the expression values of the AMPs for the 31 tested core set DGRP lines. Genes with SNPs, deletions and insertions that had statistical association with $P<10^{-5}$ were considered as candidate genes for

subsequent network mapping and gene ontology enrichment analysis. The GWAS results for the 3 AMP gene expression data in response to matings with SP^0 or SP^+ males is represented in a Manhattan plot (figure 4). This showed that significantly associated SNPs did not associate in one region of the genome.

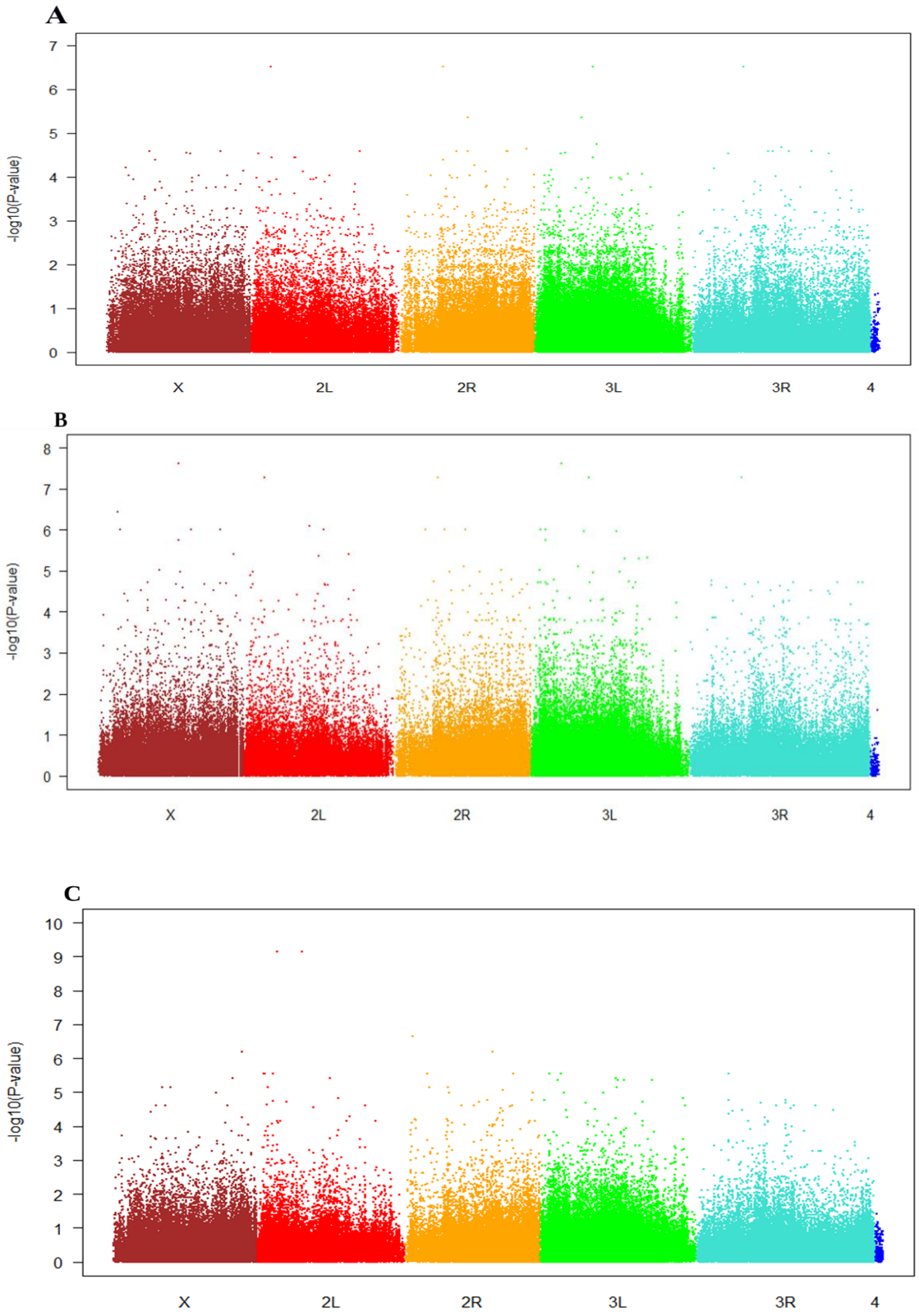
When females were mated with SP^+ males, the GWAS on the mean expression of each of the AMPs (*IM1, Dpt-B* and *Mtk*) yielded a list of significantly associated polymorphisms. For *Mtk* a total of 20 polymorphisms were significantly associated with expression variation, of which 6 SNPs were in introns and 14 SNPs were in or near 13 coding genes (Table 4). For *Dpt-B* a total of 110 significant polymorphisms were identified, of which 19 SNPs were in intergenic regions, 91 polymorphisms were located upstream or downstream or in 51 genes and included 15 synonymous and 3 non-synonymous SNPs (Table 4). For *IM1* expression, a total of 75 associated polymorphisms were identified, including 19 SNPs that were in intergenic regions, and 56 that were in or near 38 genes (Table 4).

When females were mated to SP^0 males, the GWAS on the mean expression of the 3 AMPs showed fewer polymorphisms significantly associated with expression level variation. For the variation in *Mtk* expression, the GWAS identified 18 polymorphisms, located in or near 14 genes. There was no gene region significantly associated with the expression level of *Dpt-B* (Table 4). For *IM1*, the GWAS identified 8 polymorphisms, located in or near 8 genes. A total of 9 candidate genes were significantly associated with variation in gene expression of more than 1 AMP and/or in more than 1 mating treatment (highlighted in grey, table 4). Overlapping genes generated by the GWAS when females where mated to SP^+ males for the three AMPs are summarized in the Venn diagram (Figure 5). These three AMPs (*Mtk*, *Dpt-B* and *IM1*) are representative of the different immune pathways (toll and Imd, Imd and Toll, respectively).

The GWAS for *Mtk* and *Dpt-B* expression shared a total of seven genes; one was shared between *Dpt-B* and *IM1.* There was no overlap in candidate genes between *Mtk* and *IM1* or between all three AMPs (Figure 5).

Table 4: GWAS candidate genes associated with variation in the expression of three AMPs after females were mated to SP^+ or SP^0 males. Highlighted are overlapping genes between the different mating treatments.

Immune pathways	Gene	SP+		SP0	
Toll and Imd pathway	*Mtk*	Mad	FBgn0011648	CG10440	FBgn0034636
		CG5068	FBgn0035951	sti	FBgn0002466
		CG10249	FBgn0027596	Neto	FBgn0265416
		mus312	FBgn0002909	qin	FBgn0263974
		CG10407	FBgn0038395	CG31221	FBgn0051221
		ird5	FBgn0024222	eIF5B	FBgn0026259
		Cyp6a20	FBgn0033980	RpS15Aa	FBgn0010198
		CG13323	FBgn0033788	HLH4C	FBgn0011277
		CG3168	FBgn0029896	CG33144	FBgn0053144
		LanB2	FBgn0267348	CG32982	FBgn0052982
		Gef64C	FBgn0035574	Dp1	FBgn0027835
		CG15731	FBgn0030390	CG12912	FBgn0033497
		CG14995	FBgn0035497	CG31475	FBgn0051475
				CG32082	FBgn0052082
Immune deficiency pathway (Imd)	*Dpt-B*	Gef64C	FBgn0035574		
		CG15731	FBgn0030390		
		Mad	FBgn0011648		
		CG5068	FBgn0035951		
		kirre	FBgn0028369		
		igl	FBgn0013467		
		CG8298	FBgn0033673		
		Dscam4	FBgn0263219		
		aret	FBgn0000114		
		CG8170	FBgn0033365		
		CG12090	FBgn0035227		
		ACXD	FBgn0040507		
		yellow-g2	FBgn0035328		
		dpr8	FBgn0052600		
		CG7358	FBgn0030974		
		CG12206	FBgn0029662		
		shakB	FBgn0085387		
		CG31705	FBgn0028490		
		Eip75B	FBgn0000568		
		bbg	FBgn0087007		
		CG43954	FBgn0264605		
		dpr	FBgn0040726		
		Ptp61F	FBgn0267487		
		mgl	FBgn0261260		
		CG4341	FBgn0028481		
		CG10249	FBgn0027596		
		mus312	FBgn0002909		
		EDTP	FBgn0027506		
		bru-3	FBgn0264001		
		CG13315	FBgn0040827		
		CG17199	FBgn0038775		
		LanB2	FBgn0267348		
		CG34127	FBgn0083963		
		CG3894	FBgn0035059		
		Ten-a	FBgn0267001		
		bun	FBgn0259176		
		ACXC	FBgn0040508		
		ACXB	FBgn0040509		
		Hira	FBgn0022786		
		dve	FBgn0020307		
		Eip63E	FBgn0005640		
		bru-2	FBgn0262475		
		sda	FBgn0015541		
		zfh1	FBgn0004606		
		cindr	FBgn0027598		
		lea	FBgn0002543		
		CG10051	FBgn0034437		
		Fas2	FBgn0000635		
		bab1	FBgn0004870		
		CG13917	FBgn0035237		
		CG44153	FBgn0265002		
Toll pathway	*IM1*	gkt	FBgn0260817	sm	FBgn0003435
		CG18304	FBgn0031869	CG9380	FBgn0035094
		grh	FBgn0259211	CG10249	FBgn0027596
		dpp	FBgn0000490	mus312	FBgn0002909
		CG30495	FBgn0050495	CHES-1-like	FBgn0029504
		VhaM8.9	FBgn0037671		
		Aats-trp	FBgn0010803		
		Vps16A	FBgn0261241		
		S	FBgn0003310		
		CG10527	FBgn0034583		
		stet	FBgn0020248		
		app	FBgn0260941		
		CG18265	FBgn0036725		
		CG43955	FBgn0264606		
		Ptp61F	FBgn0267487		
		salr	FBgn0000287		
		CCKLR-17D1	FBgn0259231		
		CG7744	FBgn0034447		
		AANAT1	FBgn0019643		
		luna	FBgn0040765		
		CG34356	FBgn0085385		
		disco-r	FBgn0042650		
		CG17652	FBgn0031361		
		CG17646	FBgn0264494		
		Optix	FBgn0025360		
		Grip163	FBgn0026432		
		CG16734	FBgn0037667		
		DopR	FBgn0011582		
		CHES-1-like	FBgn0029504		
		CG42256	FBgn0265296		
		CR43484	FBgn0263495		
		CR42646	FBgn0261429		
		CR43836	FBgn0264384		
		CG14459	FBgn0037171		
		sni	FBgn0030026		
		CG15611	FBgn0034194		
		sr	FBgn0003499		
		dpy	FBgn0053196		



<u>Figure 4:</u> Manhattan plot (−log10[P] genome-wide association plot) of a genome wide association study on antimicrobial peptide genes (A) *Mtk*, (B) *Dpt-B* and (C) *IM1* in *D. melanogaster* females mated to SP^+ males, 4 hours after the start of mating.

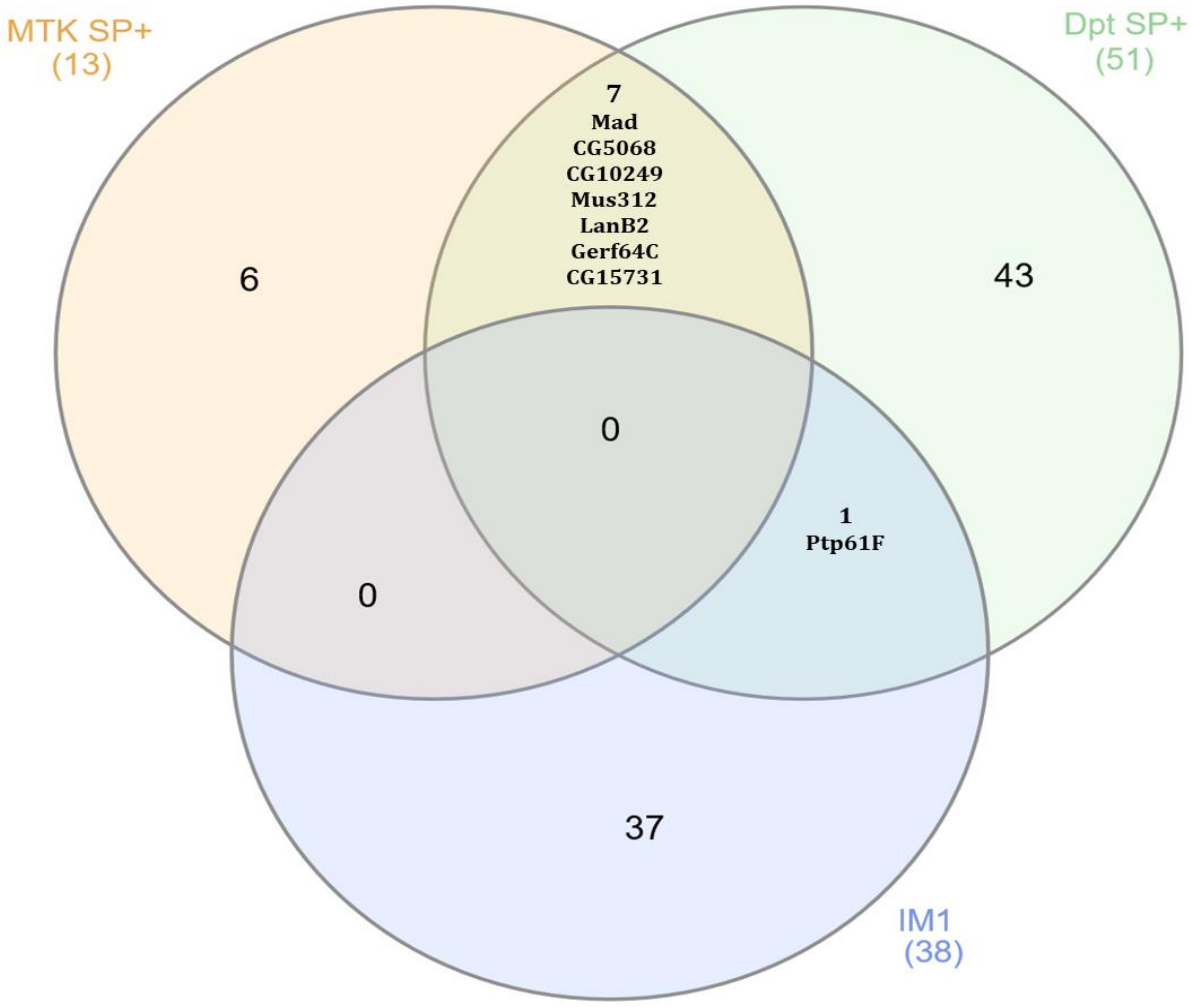


Figure 5: Venn diagram representing the overlap among the gene lists from GWAS analyses of the expression of three AMP genes in response to SP^+ males. Only one gene *Ptp61F* overlap between the GWASs list of *IM1* and *Dpt-B*. The diagram is drawn using the method of Heberle *et al.* (2015).

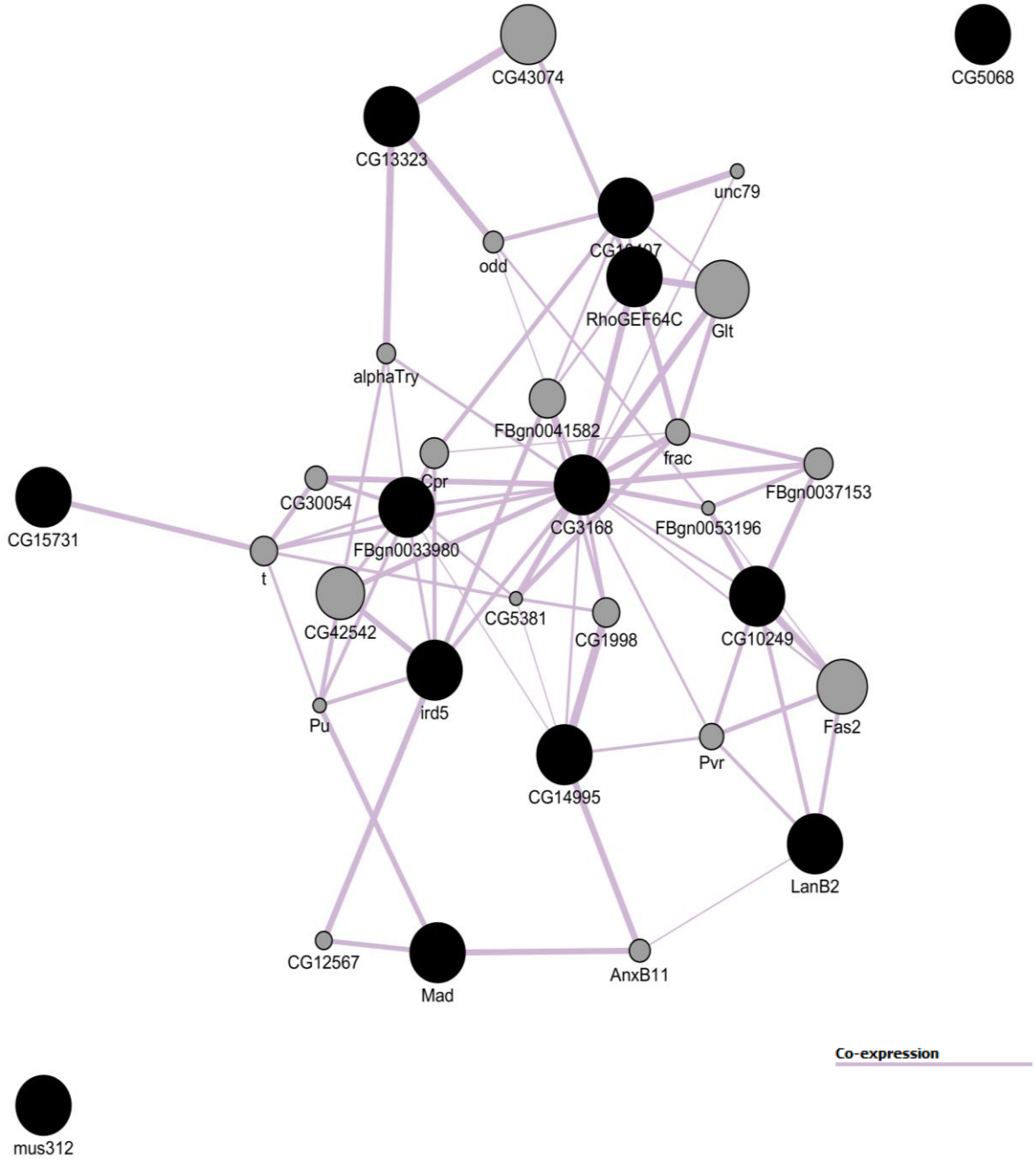
Functional gene networks

To further explore the functional significance of the candidate genes that were identified by the GWAS for the expression of three AMPs genes in females mated to SP^+ males, functional enrichment (DAVID; Huang *et al*., 2009) and gene network mapping analyses (using geneMANIA, Montojo *et al.*, 2010; Warde-Farley *et al.*, 2010) were performed.

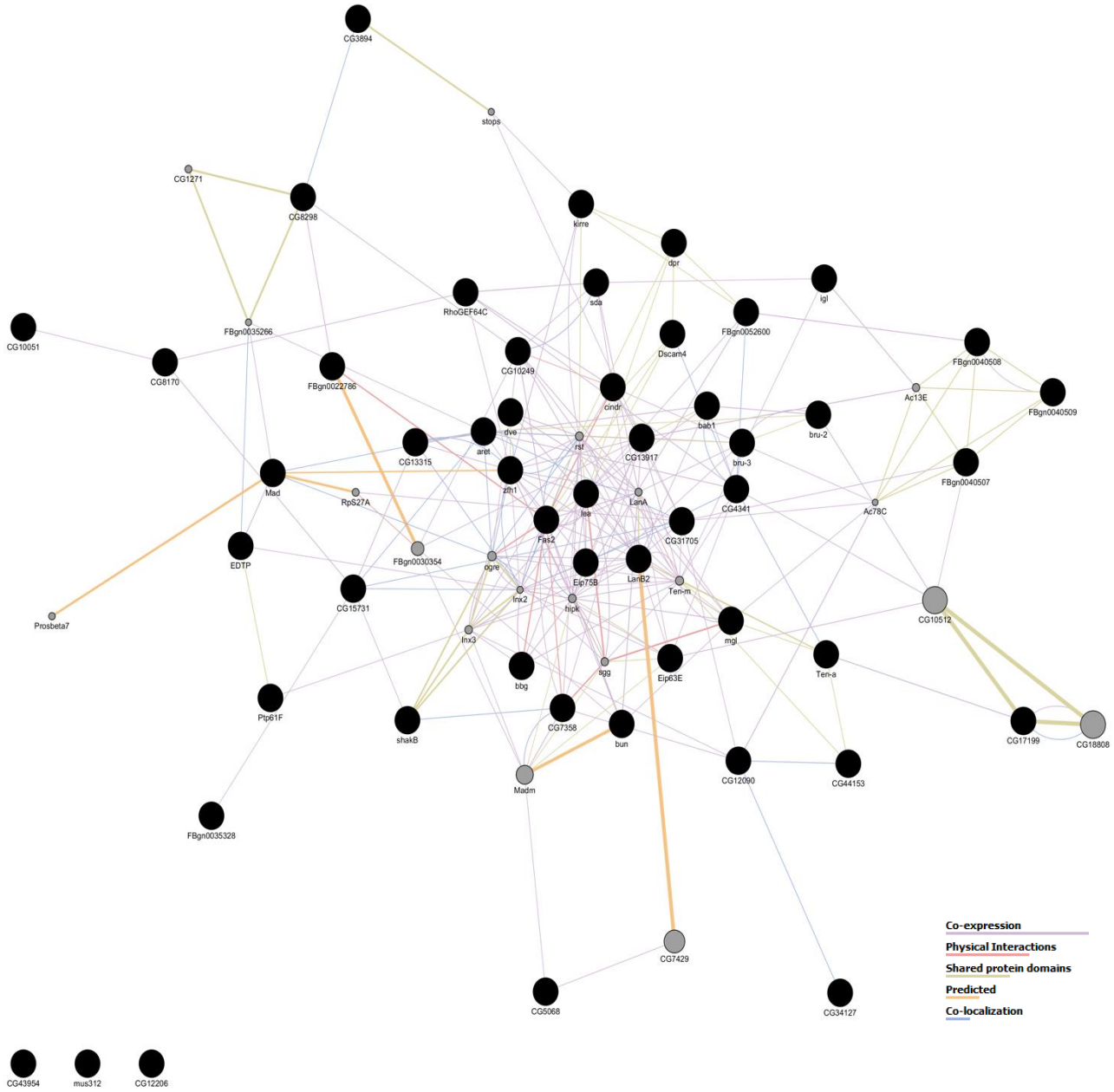
To obtain the functional annotations for each gene identified by the GWAS, and to perform a gene enrichment analysis on the candidate genes, we seeded the DAVID Bioinformatics Resource 6.8 program with the gene list for each of the 3 AMPs, separately. The gene annotations for each of the 3 AMP are displayed in Supplementary data tables 1, 2 and 3. The gene enrichment analysis for *Mtk* candidate genes showed an over-representation of the functional category defense response to Gram-negative bacteria (*P*=9.4E-02), morphogenesis (*P*=5.30E-02) and protein binding (*P*=8.10E-02) (supplementary data Table 4). For *Dpt-B,* the gene enrichment analysis revealed over-representation of genes coding for immunoglobulins (*P*=5.5E-06), phosphorus-oxygen lyase activity (*P*=3.4E-04), as well as genes in the antimicrobial humoral response (*P*=9.8E-02). Additionally, several genes were annotated with cAMP signaling, epidermal growth factors, and developmental proteins (supplementary table 4). As for *IM1,* enrichment was found for Zinc finger protein genes (*P*=6.0E-03), transcription factors (*P*=1.0E-02), pigmentation development (*P*=3.6E-02) and transmembrane protein genes (*P*=4.5E-02) (supplementary data Table 4).

For the candidate genes from the GWAS after mating to SP^0 males, the gene enrichment analysis with DAVID showed that for *Mtk,* genes coding for ZINC finger protein (*P*=4.8E-02) and transmembrane helix (*P*= 8.0E-1) were over-represented; none of these genes has previously been associated with immune function. As for IM1, genes for coiled coil proteins (*P*= 6.7E-2) were over-represented. One of these, *CG9380*, codes for a protein with a peptidoglycan recognition domain. However, the molecular and biological function of this particular gene is still unknown. The subsequent downstream analysis, described below, focused on the SP+ response only.

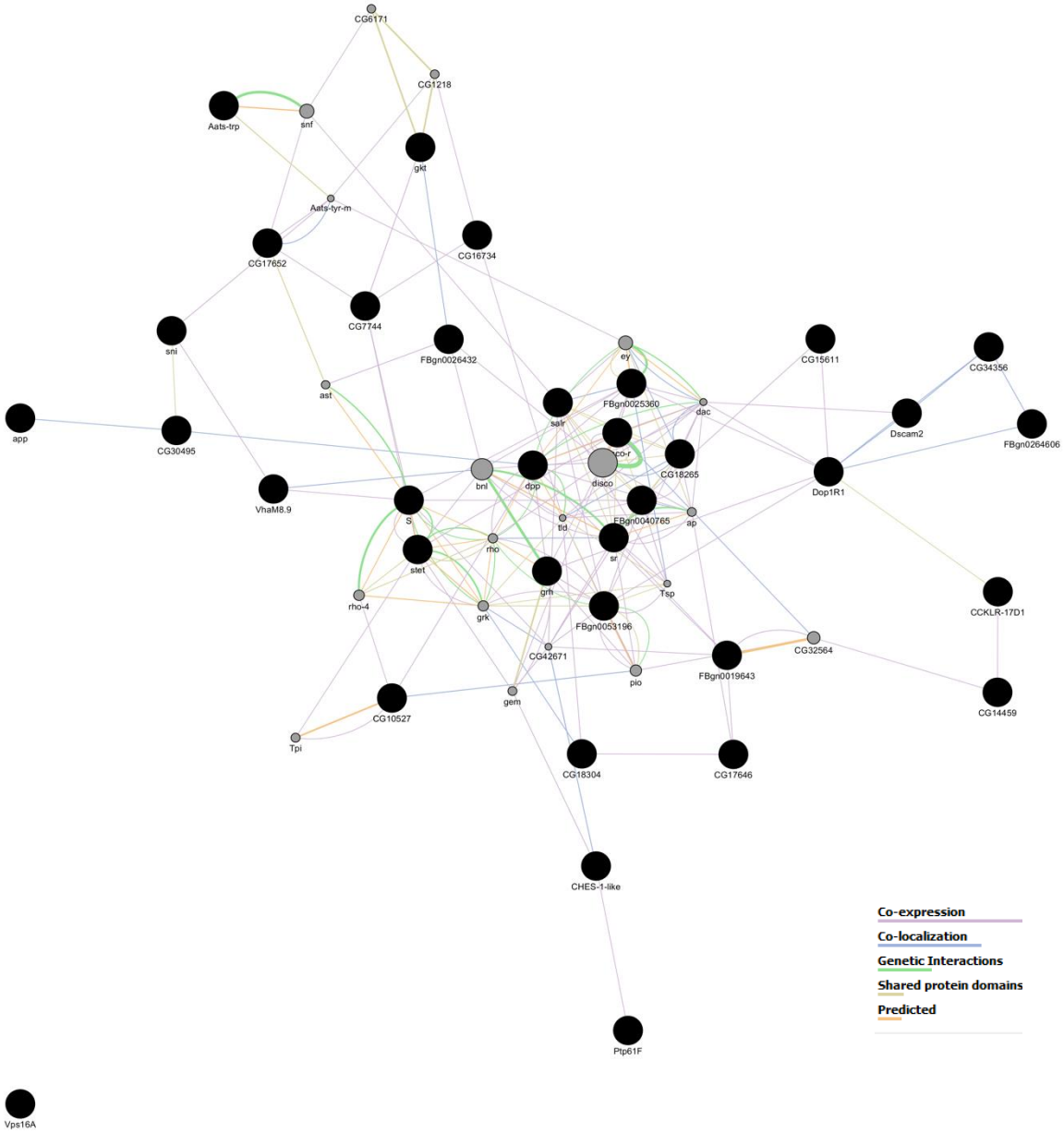
The functional gene network mapping was performed by using the GeneMANIA. The mapping of the 13 candidate genes resulted by the GWAS for *Mtk,* showed a network of 20 other related genes, the association represented by geneMANIA was 100% co-expression network where all the genes identified have similar expression levels (Figure 6). As for the mapping of the 51 candidate genes in the *Dpt-B* GWAS, geneMANIA generated a network of, in total 71 related genes. This association was based 61.07% on co-expression, 15.84% on physical interactions, 12.16% on connections based on shared protein domains, 6.34% on predicted networks and 4.60% on co-localisation (Figure 7). The network mapping of 38 candidate genes for the *IM1* GWAS, generated a network of 55 related genes, with 64.37% from co-expression network, 17.85% co-localisation network, 9.27% genetic interaction, 4.39% shared protein domains and 4.11% predicted links (Figure 8). The prediction of these gene networks generated by geneMANIA shows the different types of interactions that could occur between the GWAS-identified genes for all 3 AMPs and other related genes, using a very large set of functional associations (supplementary data Table 4).



<u>Figure 6:</u> Interaction network showing the relationships between *Mtk* GWAS candidate genes. Interaction networks of candidate genes identified by the GWAS for the expression of *Mtk,* when females were mated to SP^+ males. Black nodes depict candidate genes generated by the GWAS with significant SNPs from the DGRP analysis (Query genes). Grey nodes are other genes that are related to a set of input candidate genes (Non-query genes). The links representing the networks is based only on 100% on co-expression.



<u>Figure 7:</u> Interaction network showing the relationships between *Dpt-B* GWAS candidate genes. Interaction networks of candidate genes identified by the GWAS for the expression of *Dpt-B,* when females were mated to SP^+ males. Black nodes depict candidate genes generated by the GWAS with significant SNPs from the DGRP analysis (Query genes). Grey nodes are other genes that are related to a set of input candidate genes (Non-query genes). The links representing the networks in this case are based 61.07% on co-expression, 15.84% physical interactions, 12.16% shared protein domains, 6.34% predicted networks and 4.60% on co-localisation.



<u>Figure 8</u>: Interaction network showing the relationships between *IM1* GWAS candidate genes. Interaction networks of candidate genes identified by the GWAS for the expression of *IM1,* when females were mated to SP^+ males. Black nodes depict candidate genes generated by the GWAS with significant SNPs from the DGRP analysis (Query genes). Grey nodes are other genes that are related to a set of input candidate genes (Non-query genes). The links representing the networks in this case are based 64.37% on co-expression networks, 17.85% co-localisation networks, 9.27% genetic interactions, 4.39% shared protein domains and 4.11% on predicted links.

Table 5: Summary of the 16 candidates "immune genes", identified by the GWAS for the gene expression in females for three antimicrobial peptides in response to mating with SP^+ males. Each gene is considered to be a representative of a pathway that responds to immune challenge.

Pathway	AMP	Gene	Annotation	Immune function	Reference
Toll and Imd pathway	Mtk	Mad	FBgn0011648	Transcription factor involved in many biological processes, including the immune response. Represses Antimicrobial expression in case of wounding	Clark *et al.,* 2011
		ird5	FBgn0024222	Regulation of innate immune response of the (Imd) pathway by activation of Relish (regulator of antimicrobial peptide gene) and the induction of antimicrobial peptide genes after a gram-negative infection	Ertürk-Hasdemir *et al.,* 2009
		Cyp6a20	FBgn0033980	A cytochrome 450 monooxygenase, which may play a role in detoxification and defense response to Gram-negative bacteria	Daborn *et al.*, 2007; Berkey *et al* ., 2009
Immune Deficiency pathway (Imd)	Dpt-B	robo2	FBgn0002543	Immunoglobulin superfamily members, involved in cell communication, migration, and signalling events and in molecular recognition of self versus nonself, and involved in modulating the immune system	Vogel, 2003; Özkan *et al.*, 2013; Mandrioli *et al.*, 2015; Zinn and Özkan, 2017
		Fas2	FBgn0000635		
		CG44153	FBgn0265002		
		kirre	FBgn0028369		
		Dscam4	FBgn0263219		
		dpr8	FBgn0052600		
		dpr1	FBgn0040726		
		Mad	FBgn0011648	Transcription factor involved in many biological processes, including the immune response. Represses Antimicrobial expression in case wounding	Clark *et al.,* 2011
		bbg	FBgn0087007	Transcriptional repressor that contains two zinc finger clusters and a homeodomain. Modulate the Imd pathway of the innate immune response in mucosa	Bonnay *et al.*, 2013
		Eip75B	FBgn0000568	Steroid hormone involved in several biological processes, including modulating the innate immune signaling. More specifically it is a negative regulator of the Imd pathway.	Rus *et al.*, 2013; Xiong *et al.*, 2016
		Zfh1	FBgn0004606	Transcription factor, negative regulator of the Imd immune pathway	Myllymäki and Rämet, 2013
Toll pathway	IM1	dpp	FBgn0000490	Bone morphogenetic protein, modulates the innate immune mechanisms by regulating the transcription factor cascade. It is induced by either wounding or infection	Frandsen *et al.*, 2008; Clark *et al.*, 2011
		Dscam2	FBgn0265296	Immunoglobulin superfamily member, involved in many cell communication, migration, and signalling events and in molecular recognition of self versus nonself could be involved in modulating the immune system	Özkan *et al.*, 2013; Zinn and Özkan, 2017

The immunity genes

Based on the DAVID gene enrichment annotation and GeneMANIA network mapping, 16 candidate genes were selected for further exploration, based on their functional annotation in the immune response. A literature search of the genes involved in immunity is summarised in Table 5. Most of these immune genes belong to the immunoglobulin super family or are involved in modulating the *Imd* immune pathway. The exact role of each immunoglobulin and its contribution to the immune response of *D. melanogaster* is mostly unknown, but in general the immunoglobulins are capable of reacting to pathogens and may be involved in the defense against infection - hence they are candidates as immune effector molecules in insects (Mandrioli *et al*, 2015). Most of the polymorphisms involved in the variation in AMP expression seem to negatively modulate the Imd pathway.

Discussion:

Results summary

Our study showed that mating and the transfer of Sex Peptide can induce the expression of several AMP genes in females, but that there was significant phenotypic variation in these responses among lines. Immune gene induction and the variation was initially measured in isogenic lines of two different *D. melanogaster* populations (French and DGRP lines). Lines differed both in whether or not they induced the expression of AMPs after mating, and the extent to which they did so after receipt of Sex Peptide. Immune gene expression was not always upregulated in response to Sex Peptide. For some lines it was even down-regulated in females mated to SP^+ compared to virgin and/or female mated to SP^0 males. In other lines Sex Peptide had no effect at all, or none in addition to the response to mating. Furthermore, there were also differences among the three immune genes tested in detail, with those being regulated by the Imd pathway (*Dpt-B, Mtk*) being more responsive to Sex Peptide than the gene (*IM1*) under the regulatory control of the Toll pathway. The GWAS performed on the variation in expression of the antimicrobial AMPs in response to Sex Peptide in the DGRP population identified 13 candidate genes for *Mtk* (Toll and Imd pathway), 51 candidate genes for *Dpt-b* (Imd pathway) and 38 candidate genes for *IM1* (Toll pathway). The network analyses indicated that the majority of these genes are part of different networks, which suggests that most have several different functions in the organism, one role of which could be direct or indirect involvement in the immune response. For all these candidate genes, genetic variation was significantly associated with variation in the expression of AMPs after mating or Sex Peptide receipt. The functional annotation revealed that 8 of these candidate genes code for immunoglobulin superfamily proteins, and 8 modulate the Imd immune pathway, with 6 of these showing negative regulation.

Immune response

During mating, males transfer Sex Peptide to their mates and this is reported to boost the expression of AMPs in females during the first few hours after mating (Peng et al, 2005; Domanitskaya et al., 2007; Fedorka et al., 2007; Wigby et al., 2008). In most of these studies the genetic variation was been experimentally reduced to clearly outline Sex Peptide function.

However, to understand how Sex Peptide mediated interactions between males and females and how it affects the immune response after mating, we decided to follow the expression of several AMPs (covering different pathways in the innate immunity spectrum) in various isogenic lines of two different wild type populations.

In our first mating assay, we showed significant phenotypic variation in AMP expression across different lines in response to mating and to the receipt of Sex Peptide, and that different lines showed very different responses. This phenotypic variation was observed in both wild type populations and affected both the Toll and Imd pathways. For a more detailed test, and based on the results of the first assay, we selected a subset of three representative AMPs (*Mtk, Dpt-B* and *IM1)* from both immune pathways. In the second mating assay, we examined the expression for these 3 genes in 31 lines of the DGRP population. Overall, AMP gene expression varied significantly across the DGRP lines and expression was significantly different for the three types of mating treatment (virgin, SP^0 and SP^+). The data showed significant phenotypic and genetic variation in the expression of AMPs in response to mating in all 3 pathways (Toll, Imd and both pathways combined).

In both assays, mating with males that transferred Sex Peptide did not always result in altered transcription of AMP genes. Therefore, the canonical assumption that Sex Peptide always activates the innate immune response in *D. melanogaster* (Peng et al., 2005) is incorrect. In some lines, neither mating nor the receipt of Sex Peptide induced the expression of AMPs, while in other lines, mating without the receipt of Sex Peptide induced an equally strong activation of immune responses as did mating with the receipt of Sex Peptide. There seemed to be a correlation in the lines that responded most strongly to Sex Peptide, that expression of one AMP was associated with expression of another, and other lines that had weak AMP expression were consistently weak for all the AMPs tested. This indicated that some lines were more or less immune-responsive overall to Sex Peptide, contributing to the significant phenotypic variation observed.

The observed genetic variation in the PMR to Sex Peptide receipt could be the result of sexually antagonistic coevolution. The secreted proteins of the male accessory gland can impose mating cost, manipulating the females' physiology and inducing an "immune-like"

response to males' (Morrow and Innocenti, 2012). Females in turn may evolve mechanisms to mediate the manipulation of their physiology by males after mating. Many genes and proteins have been demonstrated to evolve rapidly, and prominent among these are many immunity genes. Hence it is possible that some of this evolutionary lability results from the actions of sexual conflict. To elucidate the association between intraspecific genetic variation in the immune response to Sex Peptide, the GWAS was carried out on the AMP expression of the DGRP lines, as discussed in the next section.

GWAS

To better understand the mechanisms underlying the phenotypic variation in immune response to Sex Peptide and the genetic variation, we used the GWAS analysis to generate a list of candidate genes that show polymorphisms correlated with the gene expression differences. Only a subset 31 out of 205 DGRP lines was used (MacKay *et al.*, 2012; Mackay and Huang, 2018). The GWAS was performed on the expression of three AMPs, one representative of the Toll pathway (*IM1*), one representative of the Imd (*Dpt-B*) and one regulated by both the Toll and Imd pathways combined (*Mtk*). Considerable genetic variation was found for the expression of each of the three AMPs, both in constitutive levels of expression and in response to mating and Sex Peptide receipt. However, there is a chance that some of these significantly associated SNPs identified by the GWAS could be false positives. Alternatively, there is a chance of missing relevant associations, due to the limited power of our assay, as we did not use the full DGRP set (Mackay and Huang, 2018), these candidates need to be followed up with other tests. Notwithstanding, a list of candidate genes was generated by the GWAS for each of the AMP, consisting of 13 candidate genes for *Mtk*, 51 candidate genes associated with *Dpt-B*, and 38 annotated genes for *IM1*. Most of the polymorphisms identified by the GWAS in this study were located in gene introns, some were synonymous and non-synonymous SNPs in coding regions. This suggests that much of the genetic variation identified would not cause changes in the protein composition, but rather result in variation in regulatory regions influencing the expression of potentially remote genes (Andolfatto, 2005; Cooper, 2010). This is not surprising given there is more SNP variation residing in intron sites for the DGRP lines (Mackay and Huang, 2018). Therefore, it is

unclear whether the lists of SNP variants sites identified by the GWAS are of direct functional significance in activating the immunity upon receipt of Sex Peptide or rather linked with other (as yet unidentified) functional SNPs. The list of these candidate genes was further examined, with a focus on genes with immune functions, as described below.

The candidate immune gene

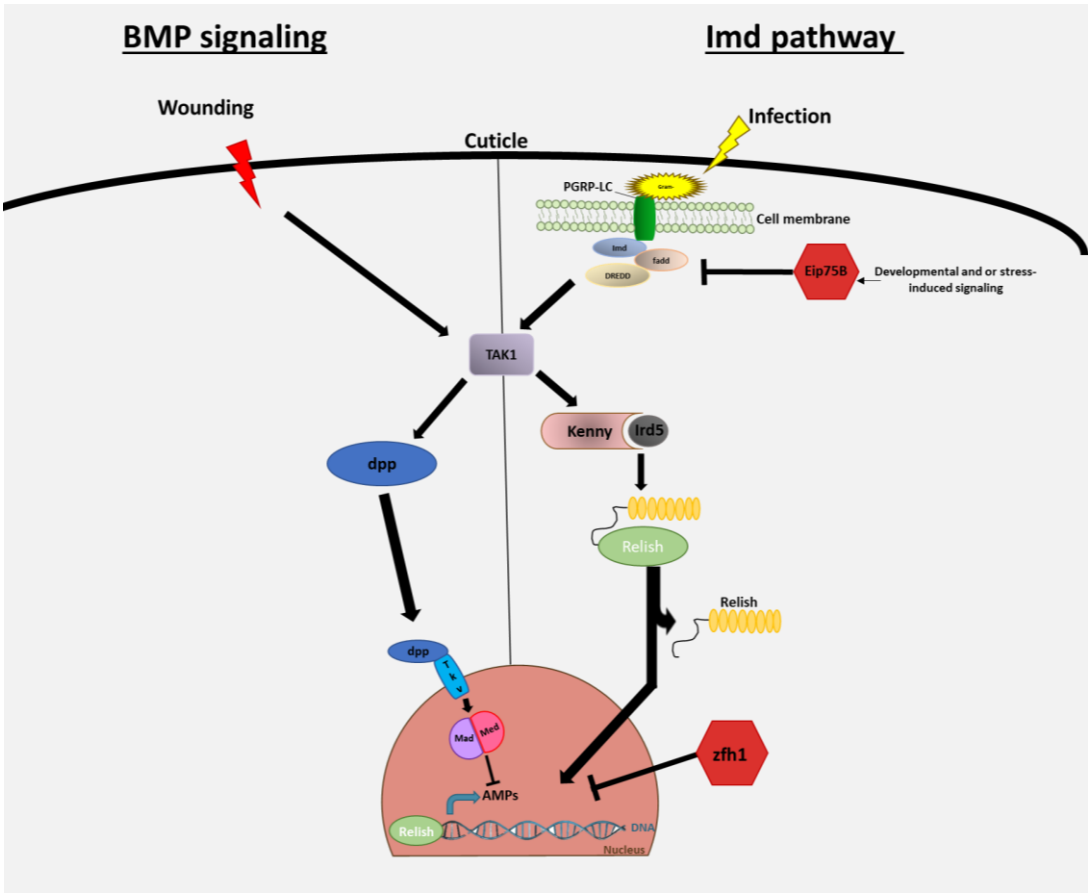


Figure 9: Simplified model of the Imd immune pathway and the BMP signaling; including the main genes of the Imd pathway and BMP signaling based on literature surveys**,** along with some of the immune genes identified by this study.

D. melanogaster has a highly sophisticated innate immune defense, which is based on the synthesis of potent antimicrobial peptides as well as immuno-competent cells that can provide protection against invading pathogens. The production of AMPs is mainly controlled by two pathways - Toll and Imd. The Toll pathway contributes to both immunity and developmental processes (Lemaitre and Hoffmann, 2007; Valanne *et al*, 2011), while Imd

signaling appears more restricted towards regulating the immune responses. Both of these pathways are highly conserved throughout evolution (Hoffmann, 2003) as well as being highly regulated by several mechanisms, to avoid chronic inflammation or unnecessary activation of the immune gene responses in the absence of infection. In this study most of the immune genes identified by the GWAS in response to Sex Peptide were regulators of the Imd pathway and for most genes this was negative regulation.

The Imd pathway is activated upon gram-negative bacterial infection. The bacteria are recognised by a membrane Peptidoglycan Receptor Protein, PGRP-LC. Following this, the PRGP-LC recruits the Death-domain protein IMD, which in turn interacts with another Death-domain protein FADD. FADD then binds to the caspase DREDD (Lemaitre and Hoffmann, 2007). This leads to the recruitment and activation of the TAK1 complex, which is responsible for the phosphorylation and activation of the Drosophila IKK complex. This complex contains 2 subunits: a catalytic kinase subunit encoded by *ird5* and a regulatory subunit encoded by *Kenny* (Myllymäki *et al*., 2014). The IKK complex then cleaves the transcription factor RELISH: an inhibitory domain remains stable in the cytoplasm and the other RELISH domain translocate to the nucleus where it binds to DNA leading to the transcription of antimicrobial peptides (Kleino *et al.*, 2005; Ertürk-Hasdemir *et al.*, 2009) (Figure 9). In our GWAS analysis, one of these core components of the signal transduction pathway *(ird5)* showed polymorphisms that were significantly associated with variation in candidate gene expression.

Some genes involved in the negative regulation of the Imd pathway showed polymorphisms that were significantly associated with the variation in gene expression of *Dpt-B* in response to Sex Peptide. The *Drosophila* Imd pathway is tightly regulated by several mechanisms to avoid unnecessary innate immune activation. This regulation occurs at different stages of the Imd pathway by numerous controlling factors. One candidate gene that was identified in the GWAS was *Eip75B*. The nuclear hormone receptor *Eip75B* is a regulator of the immune response, acting as a repressor of Imd signalling by interfering with PGRP-LC expression at the start of the immune response cascade (Rus *et al.*, 2013). Another immune regulatory mechanism involves the Zinc finger transcription factor *Zfh1*, is a transcriptional repressor that comprises of two zinc finger clusters and a homeodomain, which controls Imd signaling

pathway at the transcriptional level. The method by which *Zfh1* represses the Imd pathway is still unclear: it has been hypothesized that this occurs either by direct binding to an immune gene target gene promoter, perhaps in this way displacing or inhibiting Relish, or by inducing a repressor or repressing an activator of the Imd pathway (Myllymäki and Rämet, 2013). The gene *big bang* (*bbg*), encodes multiple membrane-associated PDZ (PSD-95, Discs-large, ZO-1) domain-containing protein isoforms (Bonnay *et al.*, 2013). It is required for maintaining a tight balance between immune response and immune tolerance toward the gut flora. Thus, *bbg* dampens the continuous activation of the Imd pathway by the endogenous flora in the anterior midgut (Bonnay *et al.*, 2013). Perhaps *bbg* is also involved in diminishing the activation of the immune response against the male's ejaculate transfer (semen and Sfps), as it is non-self, but still beneficial for offspring production. More research needs to be done to confirm this theory.

Among the genes with polymorphisms that were significantly associated with the variation in gene expression in response to Sex Peptide were several that are part of a signal transduction pathway that is induced by wounding – namely the bone morphogenic protein (BMP) signaling. BMP signaling modulates the innate immune mechanisms by regulating the transcription factor cascade. When wounded, the expression of the ligand *dpp* is upregulated, and the gene products then bind to the serine-threonine tyrosine kinase receptor Tkv receptor to activate the transcription factor *Mad* (Mothers against dpp). *Mad* then binds to *Medea (Med)* to down-regulate target gene expression. The complex *Mad/Med* silence elements near many antimicrobial peptides (Clark et al., 2011) (Figure 9). Two of the core proteins of this pathway were identified by the GWAS, namely *dpp* and *Mad*. The gene *dpp* inhibits immune genes expression directly through the help of *Mad-Med* silencer complex. *dpp* may thus be important following tissue damage in the absence of infection to avoid unnecessary immune responses (Clark *et al.*, 2011). Mating and transfer of the seminal fluid proteins activates the BMP signal, which may be a direct response to wounding and tissue damage during mating (Wigby *et al.*, 2008), or as an alternative immune response to the introduction of the foreign protein (Sex Peptide), that is not considered as an infection in the reproductive tract .

Several genes identified by the GWAS belong to the Immunoglobulin superfamily proteins. These proteins are known for their ability to specifically recognize and adhere to other molecules and for having a surveillance function with characteristics analogous to antibodies (Kurtz and Armitage, 2006). Several immunoglobulin proteins have been linked to immunity in invertebrates. For example, the gene Down syndrome cell adhesion molecule (*Dscam)* is implicated in defense against bacteria and *Plasmodium* parasites in the mosquito *Anopheles gambiae*, where *Dscam* can create a broad range of pattern-recognition receptors through alternative splicing (Dong *et al*, 2006). The gene *Hemolin* has a vital role in mediating the immune responses to bacteria in the Lepidoptera *Manduca sexta*, in particular in the ability of haemocytes to engulf bacteria through phagocytosis (Eleftherianos *et al.*, 2007). These studies show that invertebrates have specific recognition pattern and even 'memory' to recognise self and non-self via immunoglobulins (Wojtowicz *et al.*, 2007). Similarly, in *D. melanogaster* several *Dscam* genes have been identified. They were initially characterised for neural function, but recent studies suggest that *Dscam* acts as a signaling receptor or co-receptor during phagocytosis (Watson *et al.*, 2005). It has been shown that *Dscam* has the potential to express more than 18,000 isoforms by combining constant and variable exons through splicing, generating different *Dscam* receptors able to recognize diverse ligands and epitopes (Watson *et al.*, 2005; Wojtowicz *et al.*, 2007).

Immunoglobulin superfamily proteins are intriguing molecules and the extent and versatility of their roles in immunity *D. melanogaster* is still unknown. Additionally, whether and how they are physically interacting with Sex Peptide is not yet clear. We speculate that after mating, these immunoglobulins can recognise the transferred Sex Peptide as non-self and activate a set of PMRs in females, including the activation of the innate immune response through the Imd pathway. Alternatively, they may be acting as a first line of defense, where they may aid in discriminating between harmless molecules and infectious pathogens. Thus, it seems that these immunoglobulins are of importance in immunity and their abundance in this study suggests they play an important, but yet unidentified role, in the females' PMRs induced by Sex Peptide. Of the genes identified by the GWAS, 12 were of unknown function. Whether these candidate genes could form an important link between Sex Peptide and the immune response would be interesting to explore further.

From this study we can conclude that there is an immune response activated by Sex Peptide. The variation that we observed in the immune response to Sex Peptide is mostly associated with negative regulation of the Imd pathway. We speculate that the immunoglobulins play a role in female recognition of Sex Peptide, as these are significantly overrepresented among the genes that associated to the variation in immune gene expression in response to Sex Peptide. These immunoglobulins could have evolved as a defense mechanism to protect the female from non-self molecules that are of importance to female fitness - such as the activation of a response to Sex Peptide without triggering innate immunity unnecessarily. It is still unclear whether the activation of this immune response to Sex Peptide is in the best interest of the females, the males or both. Perhaps, this represents another manipulative strategy in the interest of males, that results in higher costs than benefits to females. This could be the foundation of a functionally evolved relationship between immunity and reproduction, specifically related to male-female interactions, in which females try to ameliorate the deleterious effects of Sex Peptide.

Conclusion

We have found significant variation in immune response to Sex Peptide in two sets of *D. melanogaster* lines, originating from two natural populations. A GWAS identified a set of candidate genes that were statistically related to variation in AMP expression in response to Sex Peptide and mating. Several of these candidate genes modulate the Imd signal transduction pathways. The molecular mechanism behind the regulation of these genes and their relationship with Sex Peptide remains to be studied.

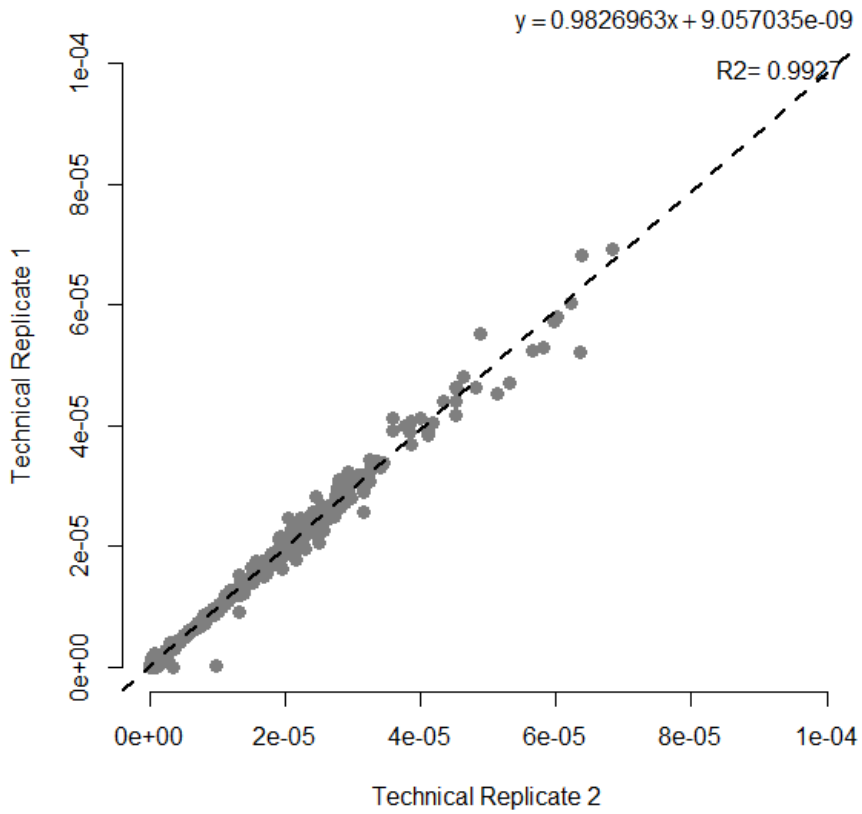
Acknowledgement

Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. We thank Jean Charles De Coriolis that assisted with the mating assays. This project was funded by Ubbo Emmius and UEA School of Biological Sciences.

Authors and Contributors

JR, TC and BW conceived the study, JR conducted the research, JR and BW analysed the data, JR wrote the chapter and JR, BW and TC revised the chapter.

Supplementary Data



<u>Figure 1:</u> Repeatbility of qPCR. Correlation of qPCR technical replicate sample measures. Scatter plot representing a significant correlation (*P* < 2.2e-16) of the same samples tested independently in two immuno-Q-PCR reactions. R^2 of 0.99.

Table 1: Functional annotation of genes involved in Mtk SP+ expression

Gene	Annotation	Gene ontology
Mad	FBgn0011648	Compound eye morphogenesis, transcription, DNA-templated, regulation of transcription from RNA polymerase II promoter, transforming growth factor beta receptor signaling pathway, dorsal closure, ventral cord development, open tracheal system development, imaginal disc-derived wing morphogenesis, imaginal disc-derived leg morphogenesis, histoblast morphogenesis, heart development, imaginal disc-derived wing vein morphogenesis, negative regulation of gene expression, ovarian follicle cell development, germ-line stem cell population maintenance, somatic stem cell population maintenance, trunk segmentation, germ-line stem cell division, regulation of cell differentiation, negative regulation of salivary gland boundary specification, positive regulation of synaptic growth at neuromuscular junction, positive regulation of transcription from RNA polymerase II promoter, wing disc anterior/posterior pattern formation, regulation of synapse structure or activity, transforming growth factor beta receptor signaling pathway involved in endodermal cell fate specification, BMP signaling pathway involved in Malpighian tubule cell chemotaxis, negative regulation of G1/S transition of mitotic cell cycle
CG5068	FBgn0035951	Protein demethylation
CG10249	FBgn0027596	Circadian rhythm, multicellular organism reproduction
mus312	FBgn0002909	Meiotic DNA double-strand break processing, resolution of meiotic recombination intermediates, nucleotide-excision repair, DNA damage recognition, DNA replication, DNA repair, mitotic G2 DNA damage checkpoint, reciprocal meiotic recombination, cell proliferation, reciprocal DNA recombination, interstrand cross-link repair, meiotic chromosome segregation
CG10407	FBgn0038395	Circadian rhythm, multicellular organism reproduction
ird5	FBgn0024222	Positive regulation of defense response to virus by host, protein phosphorylation, positive regulation of antibacterial peptide biosynthetic process, I-kappaB phosphorylation, Toll signaling pathway, peptidyl-serine phosphorylation, antibacterial humoral response, protein catabolic process, innate immune response, regulation of innate immune response, defense response to Gram-negative bacterium, defense response to virus, peptidoglycan recognition protein signaling pathway
Cyp6a20	FBgn0033980	Secondary metabolites biosynthesis, transport, and catabolism
CG13323	FBgn0033788	Unknown
CG3168	FBgn0029896	Transmembrane transport
LanB2	FBgn0267348	Salivary gland morphogenesis, midgut development, cell adhesion mediated by integrin, substrate adhesion-dependent cell spreading, system development, endodermal digestive tract morphogenesis, basement membrane assembly, extracellular matrix assembly
Gef64C	FBgn0035574	Inter-male aggressive behavior, axon guidance, imaginal disc-derived leg morphogenesis, positive regulation of Rho protein signal transduction, spiracle morphogenesis, open tracheal system
CG15731	FBgn0030390	Unknown
CG14995	FBgn0035497	Salivary gland cell autophagic cell death, autophagic cell death

Table 2: Functional annotation of genes involved in IM1 SP+ expression

Gene	Annotation	Gene ontology
gkt	FBgn0260817	DNA repair, central nervous system development, establishment or maintenance of epithelial cell apical/basal polarity
CG18304	FBgn0031869	Regulation of autophagy
grh	FBgn0259211	Epithelial cell morphogenesis, transcription, DNA-templated, plasma membrane organization, nervous system development, ganglion mother cell fate determination, ventral cord development, open tracheal system development, tracheal outgrowth, open tracheal system, primary branching, open tracheal system, R3/R4 cell fate commitment, regulation of cell shape, chitin-based embryonic cuticle biosynthetic process, regulation of tube architecture, open tracheal system, regulation of tube length, open tracheal system, chitin-based cuticle development, rhabdomere development, regulation of cell proliferation, positive regulation of transcription from RNA polymerase II promoter, membrane organization, regulation of wound healing
dpp	FBgn0000490	Cell fate specification, cell fate determination, ectodermal cell fate specification, compound eye morphogenesis, transforming growth factor beta receptor signaling pathway, germ cell development, chorion-containing eggshell formation, maternal specification of dorsal/ventral axis, oocyte, soma encoded, zygotic specification of dorsal/ventral axis, zygotic determination of anterior/posterior axis, embryo, amnioserosa formation, dorsal closure, dorsal closure, leading edge cell fate determination, ectoderm development, sensory organ development, open tracheal system development, epithelial cell fate determination, open tracheal system, epithelial cell migration, open tracheal system, foregut morphogenesis, hindgut morphogenesis, Malpighian tubule morphogenesis, imaginal disc development, imaginal disc growth, imaginal disc pattern formation, anterior/posterior pattern specification, imaginal disc, dorsal/ventral pattern formation, imaginal disc, eye-antennal disc morphogenesis, progression of morphogenetic furrow involved in compound eye morphogenesis, wing disc proximal/distal pattern formation, imaginal disc-derived wing vein specification, imaginal disc-derived wing morphogenesis, leg disc proximal/distal pattern formation, mesoderm development, heart development, hemocyte development, negative regulation of cell proliferation, germ cell migration, regulation of cell shape, imaginal disc-derived wing vein morphogenesis, anterior/posterior axis specification, dorsal/ventral axis specification, cardioblast differentiation, negative regulation of gene expression, positive regulation of pathway-restricted SMAD protein phosphorylation, stem cell division, stem cell population maintenance, BMP signaling pathway, ovarian follicle cell development, germ-line stem cell population maintenance, spectrosome organization, branch fusion, open tracheal system, fusion cell fate specification, regulation of tube diameter, open tracheal system, larval lymph gland hemocyte differentiation, eye-antennal disc development, genital disc development, labial disc development, wing disc pattern formation, genital disc anterior/posterior pattern formation, genital disc sexually dimorphic development, wing and notum subfield formation, female germ-line stem cell population maintenance, germ-line stem cell division, regulation of cell proliferation, regulation of apoptotic process, regulation of MAPK cascade, nurse cell apoptotic process, regulation of imaginal disc growth, regulation of cell differentiation, negative regulation of salivary gland boundary specification, regulation of organ growth, dorsal appendage formation, branched duct epithelial cell fate determination, open tracheal system, developmental pigmentation, wing disc anterior/posterior pattern formation, oogenesis, lymph gland development, embryonic hindgut morphogenesis, positive regulation of muscle organ development, head morphogenesis, SMAD protein signal transduction, pericardial nephrocyte differentiation, anterior Malpighian tubule development, BMP signaling pathway involved in Malpighian tubule cell chemotaxis
CG30495	FBgn0050495	Secondary metabolites biosynthesis, transport, and catabolism / General function prediction only
VhaM8.9	FBgn0037671	Establishment of imaginal disc-derived wing hair orientation, short-term memory, long-term memory, ATP hydrolysis coupled proton transport, Wnt signaling pathway, endosomal transport, imaginal disc-derived wing hair organization, phototaxis, establishment of body hair or bristle planar orientation, compound eye development, synapse organization, Wnt signaling pathway, planar cell polarity pathway, protein localization involved in establishment of planar polarity, axonal transport, presynaptic process involved in chemical synaptic transmission
Aats-trp	FBgn0010803	Tryptophanyl-tRNA aminoacylation, neurogenesis, dendrite morphogenesis
Vps16A	FBgn0261241	Intracellular protein transport, lysosomal transport, eye pigment granule organization, endosome to lysosome transport, cellular response to starvation, endosomal transport, regulation of vacuole fusion, non-autophagic, regulation of SNARE complex assembly, negative regulation of Notch signaling pathway, autophagosome maturation
S	FBgn0003310	Protein targeting to Golgi, compound eye photoreceptor cell differentiation, epidermal growth factor receptor signaling pathway, stomatogastric nervous system development, imaginal disc-derived wing morphogenesis, visual perception, ommatidial rotation, determination of genital disc primordium, epidermal growth factor receptor ligand maturation, regulation of epidermal growth factor receptor signaling pathway, R7 cell development, compound eye retinal cell programmed cell death, branched duct epithelial cell fate determination, open tracheal system, behavioral response to ethanol, oogenesis, stem cell fate commitment, epithelial cell proliferation involved in Malpighian tubule morphogenesis
CG10527	FBgn0034583	Cytoplasm
stet	FBgn0020248	Proteolysis, protein processing, sensory perception of pain
app	FBgn0260941	General function prediction only
CG18265	FBgn0036725	Unknown
CG43955	FBgn0264606	Positive regulation of neurotransmitter secretion, positive regulation of synaptic vesicle clustering
Ptp61F	FBgn0267487	Mitotic cell cycle, protein dephosphorylation, regulation of epidermal growth factor-activated receptor activity, germ-band extension, axon guidance, negative regulation of cell proliferation, negative regulation of phosphatidylinositol 3-kinase signaling, peptidyl-tyrosine phosphorylation, regulation of protein stability, regulation of protein localization, peptidyl-tyrosine dephosphorylation, negative regulation of MAP kinase activity, negative regulation of JAK-STAT cascade, negative regulation of Ras protein signal transduction, negative regulation of insulin receptor signaling pathway, oogenesis, negative regulation of peptidyl-tyrosine phosphorylation, cellular response to hypoxia
salr	FBgn0000287	Negative regulation of transcription from RNA polymerase II promoter, regulation of transcription, DNA-templated, transcription from RNA polymerase II promoter, signal transduction, sensory organ development, sensory perception of sound, male gonad development, imaginal disc-derived wing vein morphogenesis, neurogenesis, male genitalia development, antennal joint development
CCKLR-17D1	FBgn0259231	G-protein coupled receptor signaling pathway, positive regulation of cytosolic calcium ion concentration, neuropeptide signaling pathway, neuromuscular junction development, adult locomotory behavior, larval locomotory behavior, multicellular organismal response to stress
CG7744	FBgn0034447	Nuclear migration along microtubule
Dat	FBgn0019643	Catecholamine metabolic process, melatonin biosynthetic process, sleep, dopamine catabolic process, serotonin catabolic process, regulation of circadian sleep/wake cycle, sleep, octopamine catabolic process, developmental pigmentation
luna	FBgn0040765	Mitotic sister chromatid segregation, preblastoderm mitotic cell cycle, cell division
CG34356	FBgn0085385	Protein phosphorylation, detection of temperature stimulus involved in sensory perception of pain
disco-r	FBgn0042650	Metal ion binding
CG17652	FBgn0031361	Maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA), phagocytosis
CG17646	FBgn0264494	Defense mechanisms
Optix	FBgn0025360	Compound eye morphogenesis, compound eye photoreceptor cell differentiation, progression of morphogenetic furrow involved in compound eye morphogenesis, compound eye development, dendrite morphogenesis
Grip163	FBgn0026432	Microtubule nucleation, mitotic nuclear division, meiotic nuclear division, cytoplasmic microtubule organization, centrosome duplication, interphase microtubule nucleation by interphase microtubule organizing center, mitotic spindle assembly
CG16734	FBgn0037667	Unknown
DopR	FBgn0011582	Synaptic transmission, dopaminergic, adenylate cyclase-activating dopamine receptor signaling pathway, dopamine receptor signaling pathway, learning, memory, associative learning, olfactory learning, visual learning, response to sucrose, thermosensory behavior, response to starvation, thermotaxis, cellular response to sucrose stimulus, regulation of olfactory learning, regulation of presynaptic cytosolic calcium ion concentration, response to odorant
CHES-1-like	FBgn0029504	Regulation of transcription, DNA-templated, phagocytosis, mitotic G1 DNA damage checkpoint, embryonic heart tube development, regulation of cell division, cellular response to hypoxia
Dscam2	FBgn0265296	Homophilic cell adhesion via plasma membrane adhesion molecules, neuron projection morphogenesis
CR43484	FBgn0263495	Spermatogenesis
CR42646	FBgn0261429	Unknown
CR43836	FBgn0264384	Unknown
CG14459	FBgn0037171	Unknown
sni	FBgn0030026	Oxidation-reduction process
CG15611	FBgn0034194	Response to endoplasmic reticulum stress, positive regulation of Rho protein signal transduction
sr	FBgn0003499	Transcription / Cell division and chromosome partitioning
dpy	FBgn0053196	Epithelial cell development, regulation of transcription, DNA-templated, open tracheal system development, apposition of dorsal and ventral imaginal disc-derived wing surfaces, chitin-based embryonic cuticle biosynthetic process, chitin-based cuticle attachment to epithelium, regulation of imaginal disc-derived wing size, lateral inhibition

Table 3: Functional annotation of genes involved in *Dpt* SP+ expression

Gene	Annotation	Gene ontology
Gef64C	FBgn0035574	Inter-male aggressive behavior, axon guidance, imaginal disc-derived leg morphogenesis, positive regulation of Rho protein signal transduction, spiracle morphogenesis, open tracheal system
CG15731	FBgn0030390	Unknown
Mad	FBgn0011648	Compound eye morphogenesis, transcription, DNA-templated, regulation of transcription from RNA polymerase II promoter, transforming growth factor beta receptor signaling pathway, dorsal closure, ventral cord development, open tracheal system development, imaginal disc-derived wing morphogenesis, imaginal disc-derived leg morphogenesis, histoblast morphogenesis, heart development, imaginal disc-derived wing vein morphogenesis, negative regulation of gene expression, ovarian follicle cell development, germ-line stem cell population maintenance, somatic stem cell population maintenance, trunk segmentation, germ-line stem cell division, regulation of cell differentiation, negative regulation of salivary gland boundary specification, positive regulation of synaptic growth at neuromuscular junction, positive regulation of transcription from RNA polymerase II promoter, wing disc anterior/posterior pattern formation, regulation of synapse structure or activity, transforming growth factor beta receptor signaling pathway involved in endodermal cell fate specification, BMP signaling pathway involved in Malpighian tubule cell chemotaxis, negative regulation of G1/S transition of mitotic cell cycle
CG5068	FBgn0035951	Protein demethylation
kirre	FBgn0028369	Compound eye morphogenesis, homophilic cell adhesion via plasma membrane adhesion molecules, heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules, myoblast fusion, larval visceral muscle development, regulation of striated muscle tissue development, filtration diaphragm assembly, nephrocyte diaphragm assembly, garland nephrocyte differentiation, nephrocyte filtration, regulation of myoblast fusion
igl	FBgn0013467	Cell cortex
CG8298	FBgn0033673	Carbohydrate metabolic process, glycerol metabolic process, triglyceride metabolic process, glycerol-3-phosphate biosynthetic process
Dscam4	FBgn0263219	Cell adhesion
aret	FBgn0000114	Regulation of alternative mRNA splicing, via spliceosome, inter-male aggressive behavior, mRNA polyadenylation, germ cell development, spermatid development, negative regulation of oskar mRNA translation, imaginal disc-derived wing morphogenesis, regulation of myofibril size, negative regulation of translation, germarium-derived female germ-line cyst formation, positive regulation of exit from mitosis, female germ-line stem cell population maintenance, germ-line stem cell division, sarcomere organization, regulation of oskar mRNA translation, oogenesis, positive regulation of stem cell differentiation
CG8170	FBgn0033365	Proteolysis
CG12090	FBgn0035227	Regulation of autophagy, negative regulation of TOR signaling, cellular response to amino acid starvation, negative regulation of cell size, germarium-derived cystoblast division, regulation of TORC1 signaling
ACXD	FBgn0040507	cAMP biosynthetic process, adenylate cyclase-activating G-protein coupled receptor signaling pathway, adenylate cyclase-inhibiting G-protein coupled receptor signaling pathway, cyclic nucleotide biosynthetic process, cAMP-mediated signaling, intracellular signal transduction
yellow-g2	FBgn0035328	Melanin biosynthetic process, cuticle pigmentation
dpr8	FBgn0052600	Sensory perception of chemical stimulus, synapse organization
CG7358	FBgn0030974	Sensory perception of pain
CG12206	FBgn0029662	Cell redox homeostasis
shakB	FBgn0085387	Regulation of membrane depolarization, ion transport, phototransduction, jump response, response to light stimulus, intercellular transport, cell communication by electrical coupling, gap junction assembly, transmembrane transport
CG31705	FBgn0028490	Unknown
Eip75B	FBgn0000568	Negative regulation of transcription from RNA polymerase II promoter, transcription, DNA-templated, multicellular organism development, regulation of ecdysteroid metabolic process, molting cycle, chitin-based cuticle, regulation of gene expression, regulation of glucose metabolic process, ecdysis, chitin-based cuticle, antimicrobial humoral response, response to ecdysone, steroid hormone mediated signaling pathway, oogenesis
bbg	FBgn0087007	Innate immune response in mucosa, border follicle cell migration
CG43954	FBgn0264605	Integral component of membrane
dpr	FBgn0040726	Salt aversion, synapse organization, sensory perception of salty taste
Ptp61F	FBgn0267487	Mitotic cell cycle, protein dephosphorylation, regulation of epidermal growth factor-activated receptor activity, germ-band extension, axon guidance, negative regulation of cell proliferation, negative regulation of phosphatidylinositol 3-kinase signaling, peptidyl-tyrosine phosphorylation, regulation of protein stability, regulation of protein localization, peptidyl-tyrosine dephosphorylation, negative regulation of MAP kinase activity, negative regulation of JAK-STAT cascade, negative regulation of Ras protein signal transduction, negative regulation of insulin receptor signaling pathway, oogenesis, negative regulation of peptidyl-tyrosine phosphorylation, cellular response to hypoxia
mgl	FBgn0261260	Regulation of endocytosis, chitin-based cuticle development, regulation of adult chitin-containing cuticle pigmentation
CG4341	FBgn0028481	Unknown
CG10249	FBgn0027596	Neuron projection morphogenesis
mus312	FBgn0002909	Meiotic DNA double-strand break processing, resolution of meiotic recombination intermediates, nucleotide-excision repair, DNA damage recognition, DNA replication, DNA repair, mitotic G2 DNA damage checkpoint, reciprocal meiotic recombination, cell proliferation, reciprocal DNA recombination, interstrand cross-link repair, meiotic chromosome segregation
EDTP	FBgn0027506	Dephosphorylation, oogenesis
bru-3	FBgn0264001	Negative regulation of translation
CG13315	FBgn0040827	unknown
CG17199	FBgn0038775	Oxidation-reduction process
LanB2	FBgn0267348	Salivary gland morphogenesis, midgut development, cell adhesion mediated by integrin, substrate adhesion-dependent cell spreading, system development, endodermal digestive tract morphogenesis, basement membrane assembly, extracellular matrix assembly
CG34127	FBgn0083963	Lipid metabolism
CG3894	FBgn0035059	Intracellular signal transduction
Ten-a	FBgn0267001	Cell envelope biogenesis, outer membrane
bun	FBgn0259176	Compound eye photoreceptor cell differentiation, positive regulation of neuroblast proliferation, transcription, DNA-templated, regulation of transcription, DNA-templated, ovarian follicle cell migration, chorion-containing eggshell formation, peripheral nervous system development, imaginal disc-derived wing morphogenesis, decapentaplegic signaling pathway, positive regulation of cell proliferation, determination of adult lifespan, negative regulation of cell fate specification, mushroom body development, positive regulation of cell growth, sleep, ovarian follicle cell development, salivary gland cell autophagic cell death, segmentation, intestinal stem cell homeostasis, tissue regeneration, negative regulation of apoptotic process, negative regulation of Notch signaling pathway, dorsal appendage formation, autophagic cell death
ACXC	FBgn0040508	cAMP biosynthetic process, adenylate cyclase-activating G-protein coupled receptor signaling pathway, adenylate cyclase-inhibiting G-protein coupled receptor signaling pathway, spermatogenesis, cyclic nucleotide biosynthetic process, cAMP-mediated signaling, intracellular signal transduction
ACXB	FBgn0040509	cAMP biosynthetic process, adenylate cyclase-activating G-protein coupled receptor signaling pathway, adenylate cyclase-inhibiting G-protein coupled receptor signaling pathway, spermatogenesis, cyclic nucleotide biosynthetic process, cAMP-mediated signaling, intracellular signal transduction
Hira	FBgn0022786	DNA replication-independent nucleosome assembly, chromatin remodeling, transcription, DNA-templated, regulation of transcription, DNA-templated, covalent chromatin modification, sperm chromatin decondensation, fertilization, exchange of chromosomal proteins
dve	FBgn0020307	Negative regulation of transcription from RNA polymerase II promoter, regulation of transcription, DNA-templated, imaginal disc-derived wing morphogenesis, midgut development, copper ion import, imaginal disc-derived leg joint morphogenesis, photoreceptor cell maintenance, reproductive structure development
Eip63E	FBgn0005640	Embryonic development via the syncytial blastoderm, morphogenesis of an epithelium, instar larval development, protein phosphorylation, metamorphosis, developmental growth
bru-2	FBgn0262475	Negative regulation of translation
sda	FBgn0015541	Amino acid transport and metabolism
zfh1	FBgn0004606	Negative regulation of transcription from RNA polymerase II promoter, pole cell migration, nervous system development, mesoderm development, heart development, hemocyte development, motor neuron axon guidance, germ cell migration, gonad development, antimicrobial humoral response, somatic stem cell division, lymph gland development, garland nephrocyte differentiation
cindr	FBgn0027598	Compound eye morphogenesis, actin filament organization, border follicle cell migration, regulation of cytokinesis, intercellular bridge organization, lateral inhibition, positive regulation of receptor-mediated endocytosis, compound eye development
robo2	FBgn0002543	Neuron migration, outflow tract morphogenesis, axon guidance, ventral cord development, tracheal outgrowth, open tracheal system, salivary gland boundary specification, synaptic target recognition, gonad development, mushroom body development, positive regulation of cell-cell adhesion, embryonic heart tube development, cardioblast cell fate specification, central complex development, regulation of epithelial cell migration, open tracheal system
CG10051	FBgn0034437	Integral component of membrane
Fas2	FBgn0000635	Bolwig's organ morphogenesis, cell adhesion, homophilic cell adhesion via plasma membrane adhesion molecules, axonal fasciculation, neuromuscular junction development, learning or memory, learning, short-term memory, neuron recognition, motor neuron axon guidance, olfactory learning, regulation of cell shape, regulation of synaptic growth at neuromuscular junction, mushroom body development, regulation of tube architecture, open tracheal system, regulation of tube diameter, open tracheal system, regulation of tube length, open tracheal system, negative regulation of epidermal growth factor receptor signaling pathway, behavioral response to ethanol, regulation of synaptic plasticity, cell morphogenesis involved in neuron differentiation, imaginal disc-derived male genitalia morphogenesis, regulation of synapse structure or activity, synapse organization, photoreceptor cell axon guidance, terminal button organization, negative regulation of neuron remodeling
bab1	FBgn0004870	Transcription, DNA-templated, regulation of transcription, DNA-templated, regulation of transcription from RNA polymerase II promoter, eye-antennal disc morphogenesis, leg disc morphogenesis, imaginal disc-derived leg morphogenesis, sex differentiation, female gonad development, female sex differentiation, regulation of developmental pigmentation, sex-specific pigmentation, adult chitin-containing cuticle pigmentation, negative regulation of developmental pigmentation, negative regulation of male pigmentation

Table 4: GeneMANIA list of functional networks weighted by their ability to connect candidate genes to related genes, with their occurrence in the sample and in the genome.

Mtk SP+

Description	q-value	Occurrences in Sample	Occurrences in Genome
Monooxygenase activity	0.2254301	3	22
Imaginal disc-derived leg morphogenesis	0.9076552	3	81
Basement membrane	0.9076552	2	14
Genitalia morphogenesis	0.9076552	2	16
Genital disc morphogenesis	0.9076552	2	18
Oxidoreductase activity, acting on paired donors, with incorporation or Reduction of molecular oxygen	0.9076552	3	66
Male genitalia development	0.9076552	2	18
Imaginal disc-derived genitalia morphogenesis	0.9076552	2	14
Post-embryonic genitalia morphogenesis	0.9076552	2	14
Leg disc morphogenesis	0.9076552	3	82

Dpt-B

Description	q-value	Occurrences in Sample	Occurrences in Genome
Adenylate cyclase activity	7.56E-05	5	14
Phosphorus-oxygen lyase activity	0.0016847	5	28
Cyclase activity	0.0016847	5	30
Synapse assembly	0.0072316	7	122
Cell-cell junction	0.0072316	6	78
Synaptic growth at neuromuscular junction	0.0224314	6	98
Developmental growth	0.0335874	8	225
Cynapse organization	0.0482094	7	175
Cell adhesion	0.0490844	7	181
Neuromuscular junction development	0.0490844	6	127
Chemotaxis	0.0490844	8	252
Cell junction	0.0490844	6	126
Biological adhesion	0.0554151	7	193
Reproductive structure development	0.0698542	5	87
Reproductive system development	0.0698542	5	87
Axon development	0.0751684	8	288
Negative regulation of cellular protein metabolic process	0.0751684	5	90
Negative regulation of protein metabolic process	0.0751684	5	91
Heart development	0.0880686	5	96
Circulatory system development	0.0921487	5	99
Cardiovascular system development	0.0921487	5	99

IM1 SP+

Description	q-value	Occurrences in Sample	Occurrences in Genome
Leg disc pattern formation	3.53E-05	5	19
Leg disc proximal/distal pattern formation	3.53E-05	5	17
Transmembrane receptor protein tyrosine kinase signaling pathway	3.53E-05	9	147
Enzyme linked receptor protein signaling pathway	3.53E-05	10	212
Proximal/distal pattern formation, imaginal disc	5.94E-05	5	22
Proximal/distal pattern formation	1.22E-04	5	26
Imaginal disc-derived wing vein morphogenesis	0.0014534	5	43
Genital disc development	0.0016043	5	45
Eeg disc development	0.004067	6	99
Eye-antennal disc development	0.0050388	5	59
Sequence-specific DNA binding	0.0052721	7	166
Epidermal growth factor receptor signaling pathway	0.0084392	5	69
ERBB signaling pathway	0.0084392	5	69
Morphogenesis of an epithelium	0.0119857	8	273
Open tracheal system development	0.0135441	7	201
Respiratory system development	0.0158475	7	208
Epithelial tube morphogenesis	0.0230285	5	93
Compound eye morphogenesis	0.0230285	7	230
Cell fate specification	0.0230285	5	92
Tube development	0.0230285	6	155
Central nervous system development	0.0230285	7	225
Imaginal disc pattern formation	0.0289592	5	99
Eye morphogenesis	0.029058	7	242
Photoreceptor cell fate commitment	0.0334494	4	53
Photoreceptor cell differentiation	0.0366358	6	174
Morphogenesis of a branching structure	0.0411565	4	57
Antennal development	0.0515526	3	23
Tube morphogenesis	0.0525269	5	118
Neuron fate commitment	0.0694933	4	67
Response to organic substance	0.0721566	7	292
Regulation of tube architecture, open tracheal system	0.0814328	4	71
Cell fate determination	0.0835261	5	134
Cellular response to chemical stimulus	0.0841652	6	213
Cellular response to organic substance	0.0996427	5	141

Table 5: DAVID enrichment analysis. % indicates percentage of total genes assigned to term; Count indicates the number of genes in analysis assigned to each term.

***Mtk* SP$^+$**

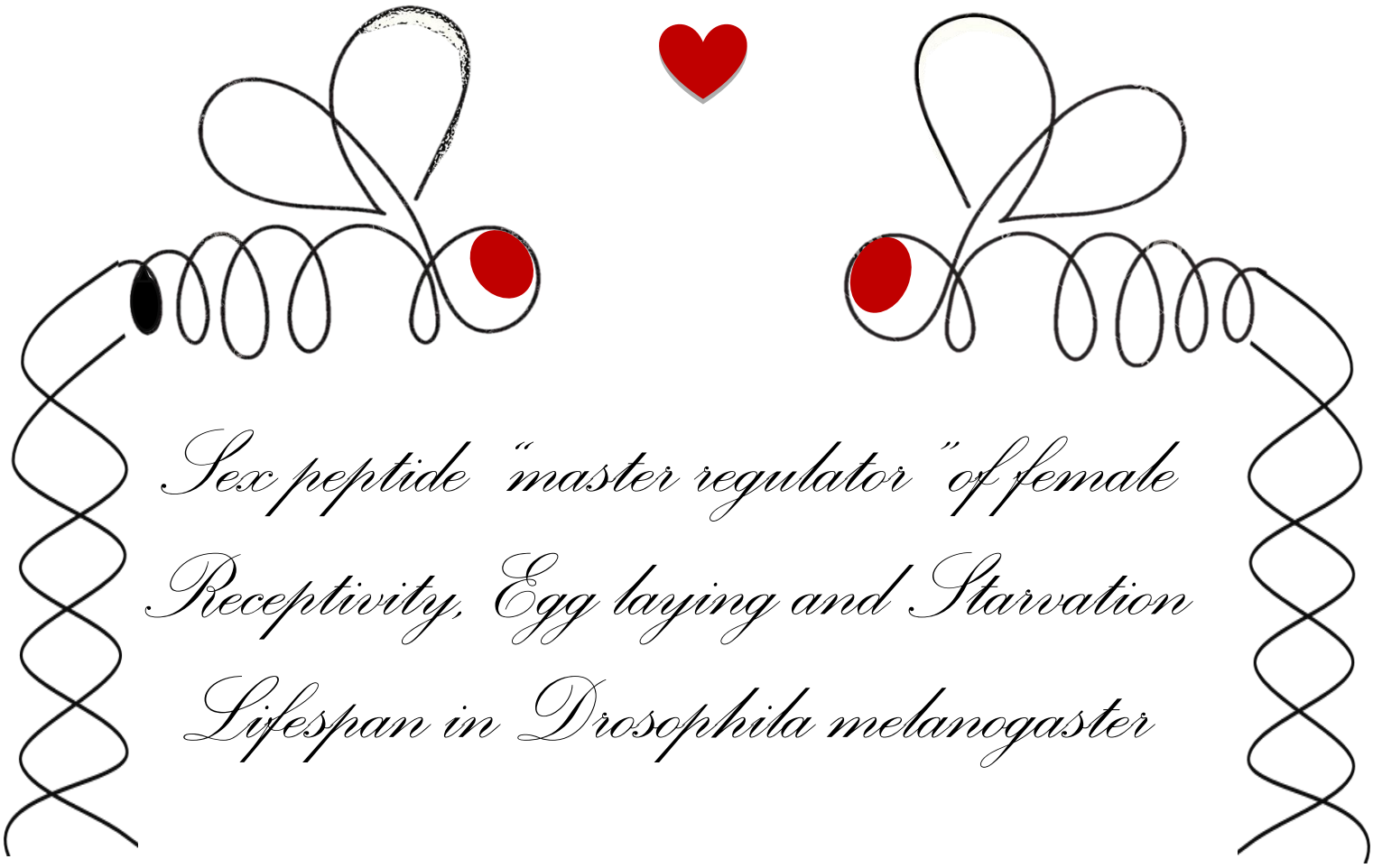
Category	Term	Count	%	P-Value	Benjamini
KEGG_PATHWAY	ECM-receptor interaction	2	15.4	1.20E-02	7.00E-02
GOTERM_BP_DIRECT	Imaginal disc-derived leg morphogenesis	2	15.4	5.30E-02	9.80E-01
GOTERM_MF_DIRECT	Protein binding	3	23.1	8.10E-02	9.00E-01
GOTERM_BP_DIRECT	Defence response to Gram-negative bacterium	2	15.4	9.40E-02	9.70E-01

***Dpt-B* SP$^+$**

Category	Term	Count	%	P-Value	Benjamini
INTERPRO	Immunoglobulin subtype	7	13.7	5.50E-06	6.00E-04
INTERPRO	Immunoglobulin-like domain	7	13.7	1.70E-05	9.30E-04
SMART	IG	7	13.7	4.70E-05	1.50E-03
INTERPRO	Immunoglobulin-like fold	7	13.7	7.80E-05	2.80E-03
INTERPRO	Immunoglobulin subtype 2	6	11.8	7.80E-05	2.10E-03
INTERPRO	Immunoglobulin I-set	5	9.8	1.50E-04	3.20E-03
GOTERM_MF_DIRECT	Phosphorus-oxygen lyase activity	3	5.9	3.40E-04	2.20E-02
SMART	IGc2	6	11.8	4.70E-04	7.70E-03
GOTERM_BP_DIRECT	Cyclic nucleotide biosynthetic process	3	5.9	9.20E-04	2.20E-01
GOTERM_BP_DIRECT	Synapse organization	4	7.8	1.40E-03	1.70E-01
GOTERM_MF_DIRECT	Adenylate cyclase activity	3	5.9	1.40E-03	4.60E-02
GOTERM_BP_DIRECT	cAMP biosynthetic process	3	5.9	1.40E-03	1.20E-01
GOTERM_BP_DIRECT	Adenylate cyclase-inhibiting G-protein coupled receptor signaling pathway	3	5.9	2.10E-03	1.30E-01
GOTERM_BP_DIRECT	Adenylate cyclase-activating G-protein coupled receptor signaling pathway	3	5.9	2.30E-03	1.20E-01
GOTERM_BP_DIRECT	cAMP-mediated signaling	3	5.9	2.90E-03	1.20E-01
GOTERM_CC_DIRECT	Plasma membrane	10	19.6	3.20E-03	1.70E-01
UP_SEQ_FEATURE	Splice variant	8	15.7	3.40E-03	2.90E-01
INTERPRO	Epidermal growth factor-like domain	4	7.8	3.40E-03	6.10E-02
INTERPRO	Adenylyl cyclase class-3/4/guanylyl cyclase	3	5.9	5.00E-03	7.60E-02
GOTERM_BP_DIRECT	Intracellular signal transduction	4	7.8	6.70E-03	2.20E-01
UP_KEYWORDS	Alternative splicing	8	15.7	7.00E-03	3.70E-01
SMART	EGF	4	7.8	7.40E-03	7.80E-02
UP_KEYWORDS	Coiled coil	13	25.5	9.40E-03	2.70E-01
GOTERM_BP_DIRECT	Negative regulation of translation	3	5.9	9.70E-03	2.80E-01
INTERPRO	Six-bladed beta-propeller, TolB-like	3	5.9	1.10E-02	1.40E-01
SMART	CYCc	3	5.9	1.10E-02	8.60E-02
UP_KEYWORDS	Cell adhesion	3	5.9	1.40E-02	2.70E-01
GOTERM_MF_DIRECT	Protein binding	8	15.7	1.50E-02	2.80E-01
GOTERM_BP_DIRECT	Garland nephrocyte differentiation	2	3.9	1.50E-02	3.60E-01
GOTERM_MF_DIRECT	Protein homodimerization activity	4	7.8	2.00E-02	2.80E-01
GOTERM_BP_DIRECT	Imaginal disc-derived leg morphogenesis	3	5.9	2.20E-02	4.50E-01
GOTERM_MF_DIRECT	Transcription factor activity, sequence-specific DNA binding	5	9.8	2.50E-02	2.90E-01
INTERPRO	EGF-like, conserved site	3	5.9	2.60E-02	2.70E-01
GOTERM_BP_DIRECT	Motor neuron axon guidance	3	5.9	2.60E-02	4.70E-01
UP_KEYWORDS	Transmembrane helix	18	35.3	2.70E-02	3.70E-01
UP_KEYWORDS	Transmembrane	18	35.3	2.80E-02	3.10E-01
INTERPRO	Fibronectin, type III	3	5.9	2.80E-02	2.70E-01
SMART	FN3	3	5.9	3.40E-02	2.10E-01
GOTERM_BP_DIRECT	Central complex development	2	3.9	3.80E-02	5.70E-01
GOTERM_BP_DIRECT	Mushroom body development	3	5.9	4.10E-02	5.70E-01
UP_KEYWORDS	Nucleus	9	17.6	4.80E-02	4.10E-01
GOTERM_BP_DIRECT	Imaginal disc-derived wing morphogenesis	4	7.8	5.00E-02	6.20E-01
GOTERM_BP_DIRECT	Cell adhesion	3	5.9	5.00E-02	6.00E-01
GOTERM_BP_DIRECT	Transcription, DNA-templated	5	9.8	5.40E-02	6.00E-01
GOTERM_MF_DIRECT	Nucleotide binding	4	7.8	5.50E-02	4.60E-01
GOTERM_BP_DIRECT	Regulation of synapse structure or activity	2	3.9	5.90E-02	6.20E-01
UP_KEYWORDS	Membrane	18	35.3	6.00E-02	4.40E-01
UP_KEYWORDS	Transcription regulation	5	9.8	6.20E-02	4.10E-01
GOTERM_CC_DIRECT	Presynaptic membrane	2	3.9	6.20E-02	8.50E-01
KEGG_PATHWAY	Purine metabolism	3	5.9	6.60E-02	5.30E-01
GOTERM_BP_DIRECT	Somatic stem cell population maintenance	2	3.9	7.00E-02	6.60E-01
UP_KEYWORDS	Transcription	5	9.8	7.40E-02	4.30E-01
GOTERM_CC_DIRECT	Presynaptic active zone	2	3.9	7.70E-02	7.90E-01
UP_SEQ_FEATURE	Compositionally biased region:Ser-rich	3	5.9	7.90E-02	9.80E-01
UP_KEYWORDS	Lyase	3	5.9	7.90E-02	4.20E-01
GOTERM_CC_DIRECT	Axon	3	5.9	8.90E-02	7.50E-01
UP_KEYWORDS	Disulfide bond	6	11.8	9.10E-02	4.40E-01
GOTERM_BP_DIRECT	Germ-line stem cell division	2	3.9	9.10E-02	7.40E-01
GOTERM_BP_DIRECT	Antimicrobial humoral response	2	3.9	9.80E-02	7.50E-01

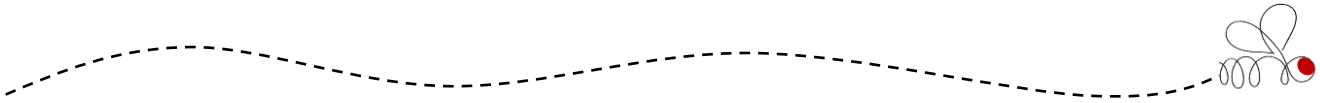
***IM1* SP$^+$**

Category	Term	Count	%	P-Value	Benjamini
INTERPRO	Zinc finger, C2H2-like	5	13.2	6.0E-3	3.9E-1
INTERPRO	Zinc finger, C2H2	5	13.2	7.9E-3	2.8E-1
SMART	ZnF_C2H2	5	13.2	9.9E-3	2.0E-1
GOTERM_MF_DIRECT	Transcription factor activity, sequence-specific DNA binding	5	13.2	1.0E-2	4.4E-1
GOTERM_BP_DIRECT	Establishment of body hair or bristle planar orientation	2	5.3	1.1E-2	9.2E-1
UP_KEYWORDS	Alternative splicing	6	15.8	1.8E-2	6.4E-1
GOTERM_BP_DIRECT	Progression of morphogenetic furrow involved in compound eye morphogenesis	2	5.3	2.3E-2	9.4E-1
GOTERM_BP_DIRECT	Branched duct epithelial cell fate determination, open tracheal system	2	5.3	2.3E-2	9.4E-1
GOTERM_BP_DIRECT	Open tracheal system development	3	7.9	2.9E-2	9.1E-1
UP_SEQ_FEATURE	Splice variant	5	13.2	3.1E-2	7.7E-1
GOTERM_BP_DIRECT	Developmental pigmentation	2	5.3	3.6E-2	8.9E-1
UP_KEYWORDS	Transmembrane helix	13	34.2	4.5E-2	7.3E-1
UP_KEYWORDS	Transmembrane	13	34.2	4.5E-2	5.9E-1
GOTERM_MF_DIRECT	Sequence-specific DNA binding	4	10.5	4.8E-2	7.6E-1
GOTERM_BP_DIRECT	Chitin-based embryonic cuticle biosynthetic process	2	5.3	5.6E-2	9.4E-1
GOTERM_BP_DIRECT	Ectoderm development	2	5.3	5.9E-2	9.1E-1
GOTERM_BP_DIRECT	Establishment of imaginal disc-derived wing hair orientation	2	5.3	6.4E-2	9.0E-1
GOTERM_CC_DIRECT	Integral component of membrane	13	34.2	6.6E-2	9.4E-1
GOTERM_BP_DIRECT	Cellular response to hypoxia	2	5.3	6.9E-2	8.9E-1
GOTERM_BP_DIRECT	Compound eye photoreceptor cell differentiation	2	5.3	7.1E-2	8.6E-1
GOTERM_BP_DIRECT	Endosomal transport	2	5.3	7.4E-2	8.4E-1
UP_KEYWORDS	Membrane	13	34.2	8.1E-2	7.1E-1
GOTERM_BP_DIRECT	Epithelial cell migration, open tracheal system	2	5.3	8.6E-2	8.6E-1
KEGG_PATHWAY	Neuroactive ligand-receptor interaction	2	5.3	9.2E-2	4.9E-1



Sex peptide "master regulator" of female Receptivity, Egg laying and Starvation Lifespan in Drosophila melanogaster

Jessy Rouhana, Wayne Rostant, Bregje Wertheim, Tracey Chapman



4

Abstract

A fundamental Darwinian insight is that natural selection is focussed on reproductive fitness rather than survival. This puts a premium on reproductive success for both sexes. However, this does not necessarily equate to sexual co-operation and there are many instances of conflict, in which each sex may gain by optimising their mating strategies at the cost of the other sex. The fruit-fly *Drosophila melanogaster* provides an excellent exemplar of sexual conflict. Each sex may display conflicting mating strategies and males may impose costs on females as a side-effect of the adaptive manipulation of seminal fluid proteins transferred during mating. These proteins induce several marked changes in female reproductive functions and post-mating behavior. One seminal fluid in particular, the "Sex Peptide", plays a central role in these post mating responses in females: it increases egg laying and feeding and reduces receptivity, longevity and sleeping behavior. In this study we characterised the genomic variation associated with some of these key female phenotypic responses to Sex Peptide. Using a core panel of genome-sequenced lines from the Drosophila Genome Reference Panel (DGRP) we first showed that the phenotypic variation associated with receptivity, egg laying, starvation lifespan, mating latency and mating duration in response to receipt of Sex Peptide was associated with significant underlying genetic variation. We then performed a genome wide association study (GWAS). This highlighted several candidate gene regions of interest and showed that the phenotypic variation in different female post mating responses to Sex Peptide was controlled by different genes and mechanisms.

Keywords

Sex Peptide, Receptivity, Egg laying, starvation lifespan, *Drosophila melanogaster*, DGRP, GWAS, Sexual selection, Genetic variation

Introduction

Success in sexual reproduction can be typified by both conflict and cooperation between males and females. Both sexes cooperate to produce fit and viable offspring. However, due to differences in the evolutionary interests of each sex (Parker, 1979) males and females may also be in conflict over the optimal values of reproductive traits such as gamete size, parental investment, available resource levels and particularly over the mode and frequency of mating. Such conflict potentially leads to an evolutionary arms race between males and females (Boorman and Parker, 1976; Arnqvist and Rowe, 2002; Gage, 2004). Females may benefit from multiple mating by increasing fecundity and the genetic diversity of their offspring (Arnqvist and Nilsson, 2000). However, this can be costly for males if it leads to a reduction in the number of offspring sired (Chapman *et al.*, 2003). Set against this, the physiological costs of extra matings are also divergent, being much higher in females than males. Consequently, across a broad range of taxa, males have evolved different strategies to manipulate female pre- and post-mating behavior, and thereby secure higher lifetime reproductive success for themselves. These tactics include guarding females and physically preventing them from mating with other males (Elias *et al.*, 2014; Jarrige *et al.*, 2016) and perfuming females with pheromones that render them unattractive to other males (Scott and Richmond, 1987; Andersson *et al,* 2000). Males may even take remote control of females through the transfer of seminal fluid proteins that render females less receptive to further courting, trigger egg production and have the potential to reduce female fitness (Chapman, 2001; Wigby *et al.*, 2009; Avila *et al.*, 2011; Xu and Wang, 2011). Variation in the responses of females may result from differential sensitivity and/or ability to resist the potential manipulative effects of males (Wigby and Chapman, 2004a).

In *D. melanogaster,* both males and females are promiscuous. This has resulted in an evolutionary arms race between the two sexes, whereby males gain by securing and maximizing their lifetime reproductive success even if it is costly to females, and where females gain by resisting the mating costs inflicted by males while maintaining the optimal quality and quantity of offspring (Arnqvist and Nilsson, 2000; Chapman *et al.*, 2003). *Drosophila melanogaster* has been an pivotal model to uncover the mechanisms influencing reproduction and female post-mating behavior and to provide a window into the marked

changes in mated females caused by the activation of diverse sets of genes (Gioti *et al.*, 2012; Laturney and Billeter, 2014). These post mating changes in females are largely induced by the seminal fluid proteins transferred by males during mating. These male proteins physically support sperm transfer during mating, but also elicit post-mating responses that increase male reproductive success whilst sometimes simultaneously generating costs in females. This can lead to a tug-of-war, in which males employ seminal fluid proteins to facilitate successful sperm storage and to guarantee that females make a significant investment in the current brood and withhold from re-mating with other males. Collectively these effects can be costly and can even shorten female lifespan (Chapman *et al.*, 1995; Chapman, 2001; Ram and Wolfner, 2007; Avila *et al.*, 2011; Sirot *et al.*, 2015).

The effects of one enigmatic seminal fluid protein, known as the 'Sex Peptide', have been studied in some detail. Sex Peptide is transferred to females and bound to sperm within females (Peng *et al.*, 2005). It causes a substantial reprogramming of female behaviour and physiology including increased egg laying, increased food intake, slowed intestinal transit and water balance, altered immunity, reduced sleep patterns, reduced sexual receptivity to re-mating and increased aggression (Manning, 1967; Chen *et al.*, 1988; Liu and Kubli, 2003; Carvalho *et al.*, 2006; Barnes *et al.*, 2008; Isaac *et al.*, 2010; Ribeiro and Dickson, 2010; Isaac, *et al.*, 2014; Bath *et al.*, 2017). Sex Peptide also directly influences sperm usage and sperm release in the female reproductive tract (Avila *et al.*, 2010). Reflecting these many effects, Sex Peptide is also reported to alter the expression of a diverse array of genes in females both across time and in different parts of the body (Gioti et al., 2012).

In *D. melanogaster* the arena of the sexual conflict described above often occurs within the mated female's body, with males trying to increase the magnitude of female responses while females try to dampen them down. However, to understand the pace, dynamics and trajectory of the co-evolution arising from this potential manipulation of gene expression in one sex by the other, it is necessary to understand the molecular interactions between males and females. As a first step towards this, we need to identify which genes and proteins may be involved in the regulation of female responses to Sex Peptide.

In this study we performed an in-depth investigation to identify the genetic variation and genome regions associated with female phenotypic variation in response to receipt of Sex

Peptide. Specifically, we measured the response to Sex Peptide receipt on female re-mating behavior, egg laying and longevity under starvation conditions. We did this in a core set of genome-sequenced lines, the *Drosophila* Genome Reference Panel (DGRP; Mackay *et al.* 2012). A Genome Wide Association (GWAS) approach was then used to identify the genome regions associated with these responses (re-mating, egg laying and starvation lifespan) to Sex Peptide. We mapped the variation in each of these phenotypes to genomic variation using the DGRP website and examined the functional descriptions of the genomic variation and underlying genes identified.

Material and methods

Fly stocks

i. Iso-female lines

The core set captures most of the genetic variation existing in the *D. melanogaster* isofemale lines from the *Drosophila* Genomic Reference Panel (DGRP) (Mackay *et al*, 2012). These lines were used to measure variation in the different Sex Peptide response phenotypes. These are inbred lines generated from isofemales that were originally derived from individuals collected from North Carolina, USA and were obtained from the DGRP collection via the Bloomington stock centre. The genomes of all the DGRP lines have been fully sequenced and are publicly available for testing for genetic variation in focal traits as well as genome wide association studies (dgrp2.gnets.ncsu.edu).

ii. Sex Peptide mutant lines

Sex Peptide lacking (knockout, SP^0) and genetically matched control males (SP^+) were used in this study and were derived as described in Liu & Kubli (2003). The experimental SP^0 ($SP^0/\Delta 130$) males bear a non-functional Sex Peptide gene, produced by crossing (*SP^0/TM3 Sb ry*) males, whereby SP^0 is the Sex Peptide gene knockout, to SPΔ2-7 females (*Δ130/TM3 Sb ry*) in which Δ130 is a deletion of amino acid 2 to 7 in the N-terminal region of the Sex Peptide gene. The SP^+ control line males contained the knockout SP^0 and the wild type Sex Peptide genes in tandem (SP0, SP+/Δ130). These males produce normal levels of sex peptide (Liu & Kubli 2003). They were generated by crossing *SP0, SP+/TM3 Sb ry* males to *Δ130/TM3 Sb ry* females. All stocks had been back-crossed into the Dahomey wild type genetic background prior to these tests, to increase the vigour of the males and to introduce a wild type genetic background for both SP^+ and SP^0 males (Fricke, Bretman and Chapman, 2010).

iii. Wild type lines

The *D. melanogaster* Dahomey wild type flies were originally collected in the 1970s in Dahomey (now Benin) and have been kept since then in a large population cages with overlapping generation on sugar yeast agar (SYA) medium (100g brewer's yeast, 50g sucrose, 15g agar, 30ml of 10% w/v Nipagin solution and 3ml propionic acid, per litre of medium). They were reared at 25°C, 50% humidity on a 12L:12D cycle. Each stock cage

was supplied every week with three new bottles of 70ml SYA and bottles were removed after 28 days.

Fly rearing and collection

All flies were grown on standard SYA medium in a 25 °C room, with a 12:12 h light: dark cycle and 50% humidity. Larval density in the cultures from which the DGRP females were derived was controlled by rearing 100 larvae per vial on SYA medium. At eclosion, virgin females were collected on ice anaesthesia and were kept in individual vials 4-5 days until mating. Larval density was also controlled in the cultures from which males were collected; SP^0 and SP^+ males were reared in bottles in which 50 males and 50 female parents had been placed for a period of 24 h. Parents were then transferred daily to new bottles to synchronise the cultures and standardise density. After eclosion, SP^0 and SP^+ males were stored in single sex groups of 10 males per vial on SYA medium until the day of mating, at 4-5 days post eclosion. The wild type Dahomey were reared in the same way as the DGRP females; upon eclosion, wild type females were kept individually in vials and wild type males were pooled in groups of 10 until mating took place at 4-5 days of age.

Receptivity assay

To determine the effect of Sex Peptide on female receptivity, re-mating assays were conducted over 2 days. On the first day of the tests, SP^+, SP^0 and Dahomey males were mated once with females from each of 30 DGRP lines, as well as females from the Dahomey wild type. The final number of lines tested in each type of assay differed as some lines sometimes grew poorly and did not yield a sufficient number of flies to perform a mating assay. For each line and mating treatment, 40 females were initially set up. Immediately after the end of mating males were discarded and females were maintained individually in vials. After 24 hours, 1 fresh Dahomey male was introduced to each of the mated females and the number of females that re-mated was recorded. On both days females were given a 3-hour time window of exposure to males in which to mate. Mating latency and duration was recorded for first and second mating (see below). Dahomey females were used as a control for random environmental variation and were tested in each of the 7 experimental blocks of mating tests.

Egg laying and starvation lifespan assays

To detect the effect of Sex Peptide on female egg laying and once-mated female lifespan, virgin females from each of 32 DGRP lines were exposed to either SP^0 or SP^+ males and were allowed to mate once. For each line and mating treatment 40 females were initially set up. Immediately afterwards females were individually transferred into fresh SYA vials and allowed to lay eggs for 24 hours and again transferred to new vials for another 24 hours. After 48 hours from the initial mating, females were pooled in groups of 10 and maintained on agar only (15g of agar, 1L of water) medium until death. This assay allowed us to test the female starvation survival of each of the DGRP lines subjected to single matings with either SP^0 or SP^+ males. This starvation measure shows a strong correlation with survival on standard food media (Zwaan *et al.,* 1991). The number of deaths in each line and treatment was recorded 2 times a day until all the flies were dead. Dahomey females were used to control for random environmental variation and were tested in each of the 4 experimental blocks of mating tests.

Latency and duration of mating

The latency of the start of mating for each virgin and mated female in the receptivity assays was recorded. This was derived from the time when males were introduced to the females until the mating started. The duration of each mating was also recorded and was the time from the observed start of mating until the pair separated. These data were collected for both the receptivity and the egg laying and starvation lifespan assays.

Body weight

To document the difference in mass between the different lines, the body weight of each of the lines was measured using a laboratory scale (Sartorius MC1). Flies were reared at a standard density on SYA and derived from vials in which 5 males and 5 female parents were maintained for 1 day, and then transferred daily to new vials for 5 days, to synchronize cultures and standardize density. At eclosion females and males were pooled separately, per DGRP line, 3 pools of ten were instantly frozen in liquid nitrogen, and then weighed to the nearest 1.0 mg with the molecular scale.

Statistical analysis

All statistical analyses were conducted using RStudio (Version 0.99.903) (RStudio, 2016). Different statistical approaches were required to analyse the different trait data measured in this manuscript due to the different data distributions, as described below:

i. Mating latency and duration

To compare the mating latency and the mating duration among DGRP lines, we analysed the data from both the receptivity assay and the egg laying assay, using linear mixed models, implemented as "lmer" by REML in the "lme4" package. The significance of factors was determined by step-wise model reduction from the maximal model via likelihood ratio tests (LRT), whereby the deviance (D) is the difference between the log likelihood of the reduced model and the log likelihood of the full model, using the Kenward Roger method for *F*-tests for assigning significance of Sex Peptide fixed effect. The maximal model included the cross (mating to a SP^+ and SP^0 male) as a fixed effect, and the DGRP lines and dates as random factors. In the simplified model, the random effect of DGRP lines was omitted in order to test for significant variation among DGRP lines, and the fixed effect of cross was omitted to test for differences in latency and duration for mating with SP^+ and SP^0 males. DGRP line was included as a random effect because we were not interested in the specifics of each line but instead in the overall effect of variation among the lines in the expression of Sex Peptide phenotypes. The mating latency was log-transformed for analysis to improve normality.

ii. Receptivity

Day-to-day variation was assessed in a separate analysis, comparing the responses of Dahomey females on each of the experimental mating days. For each mated female, it was recorded whether or not they remated (0/1) when a Dahomey male was introduced for 3 hours, 24h after the first mating. The variation in receptivity was analysed, using a generalized linear model "GLM" (McCullagh and Nelder, 1989) with binomial errors. The model included experiment date as a fixed effect and the interaction with cross (mating to a SP^+, SP^0 or WT Dahomey male), and the Chi-square test was used for assigning significance.

Variation among the 30 DGRP lines for the proportion of females that re-mated was analysed using the "Glmer" function on the "lme4" package (Bates *et al.*, 2014), specifying binomial errors. The significance of factors was determined by step-wise model reduction from the maximal model via likelihood ratio tests (LRT), whereby the deviance (D) is the difference between the log likelihood of the reduced model and the log likelihood of the full model, using the Chi-square test for assigning significance. The maximal model included the cross as a fixed effect, and the DGRP lines and dates as random factors. In the simplified model, the random effect of DGRP lines was omitted. Model parameters are summarised in supplementary data Table 1.

iii. Weight

To determine whether DGRP line variation in female weight was associated with variation in the number of eggs laid, the correlation between female weight and numbers of egg laid was analysed using a linear model "Lm" (Lindley and Smith, 1972). The analysis was done separately for the data from females crossed to either SP^0 males or SP^+. In addition, we determined whether there was an interaction between female weight and the magnitude of the Sex Peptide effect on the numbers of eggs laid, also using linear models.

iv. Egg laying

To check for day-to-day variation in egg laying, the Dahomey samples were analysed in a generalized linear model "GLM" (McCullagh and Nelder, 1989), implemented in package "lme4" (Bates et al. 2015), specifying a zero-inflated negative binomial distribution. The maximal models included cross (either to SP^0 or SP^+) as a fixed effect and date and the interaction between date and cross as a random effect. In the simplified models the interaction term between cross and date was dropped as it was non-significant, while the random effect of date was significant when models were compared using LRT.

To test for variation among DGRP lines in egg laying after mating to SP^+ or SP^0 males, the statistical analyses were performed using the Generalized linear mixed-effects models using Template Model Builder "GlmmTMB" function on the "CRAN" package (Brooks et al., 2017). A maximum likelihood approach was used to compare and find the best distribution to fit the data, which was a zero inflated negative binomial model (Zuur et al., 2009). The full model included cross (either to SP^0 or to SP^+) as a fixed effect, and lines,

date and weight as a random effect. A stepwise model simplification of the maximal model with analysis of deviance was used to determine significant terms. In this analysis, weight and dates were dropped from the model, as they did not significantly contribute to explaining the variation in egg laying. Model parameters are summarised in supplementary data Table 1.

v. Survival resistance

Prior to analyses, the 'bbmle' (Bolker, 2016) package, was used to compare 10 mixed effects models and find the best model fit to study multiple random effect. The Sex Peptide effect on survival was performed using linear mixed effects "lme", implemented in the "nlme" package (Pinheiro *et al.*, 2018), with the maximum likelihood approach. The maximum model included a main effect of male (SP^0 or SP^+), modelled variance as function of date to solve heteroscedasticity, included random interaction effect of Line on male, but no random effects of date. The first simplified model excluded the random interaction of males on lines to test for significant genetic variation for Sex Peptide mediated effect on the different tested lines. In the second simplified model, the fixed effect of male was dropped to confirm the main effect of males. Since the survival resistance assay data satisfied the proportional hazards assumption, the Cox Proportional Hazards method was implemented, using the "coxme" package (Therneau, 2015) to generate hazard ratios subsequently used as inputs for the GWAS. The models were specified to test for the effects of Sex Peptide and the relevant hazard ratios were calculated for each of the DGRP lines and male type. The hazard ratio indicates the ratio of the instantaneous hazard (mortality) rates of SP^0 and SP^+ for each of the tested lines.

Genome-wide association study

Phenotype trait values per DGRP line for re-mating, egg laying, survival resistance assay, mating duration and mating latency in response to receipt of Sex Peptide were uploaded in the DGRP2 analysis webserver (dgrp2.gnets.ncsu.edu) (MacKay *et al.*, 2012; Huang *et al.,* 2014). A genome-wide association study (GWAS) was performed for each trait by using the DGRP pipeline to identify candidate genes, polymorphisms and pathways associated with the query phenotypes. From this output the top polymorphism (SNPs and indels) with allele

frequencies ≥ 0.05 and significant associations ($P < 10^{-5}$) with the trait values were then considered for functional enrichment analysis (see below). These GWAS analyses accounted for effects of *Wolbachia* infection, cryptic relatedness due to major inversions, and residual polygenic relatedness (Mackay et al., 2012). The analyses were performed separately on the different phenotypes that were tested.

Functional enrichment and gene mapping

All the candidate genes generated by the GWAS for the phenotype measures associated with re-mating, egg laying, survival resistance assay, mating duration and mating latency were subject to functional enrichment analysis using DAVID bioinformatic resources 6.8, NIAID/NIH (Huang *et al.,* 2009) to identify which functions were overrepresented among these genes associated with the variation in responses to Sex Peptide. The candidate genes were also used for network mapping, using the geneMANIA Cytoscape 3.4.0 plugin (Data Version: 13/07/2017) (Shannon *et al.*, 2003; Montojo *et al.*, 2010). The geneMANIA server predicts a functional network by associating genes based on several databases (Mostafavi *et al.*, 2008), including 1) gene co-expression, where genes are linked when their expression level is similar across the same conditions; 2) genetic interactions, with two genes being functionally associated if the effects of perturbing one are associated with perturbations to a second, 3) physical interactions, where the proteins are linked if they were found to interact in a protein-protein interaction study, 4) co-localisation, where two genes are linked if they are both expressed in the same tissue or if their gene products are both identified in the same cellular location, 5) shared protein domains, where genes are linked if they have the same protein domain, and 6) the predicted network specifies a functional relationship between genes, often protein interactions, that have orthologs in different organisms. As noted previsouly in Chapters 2 and 3, this analysis account for random associations set of 0.01% of the association weights and setting the rest of the associations to zero (Mostafavi *et al.*, 2008).

Results

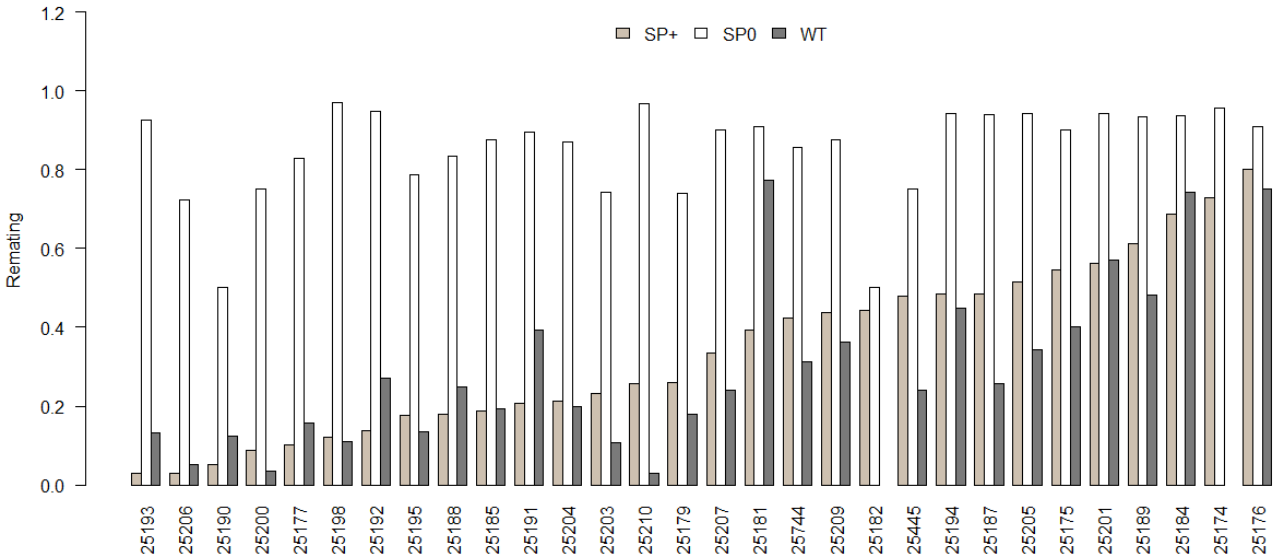
Receptivity

Firstly, we tested for day-to-day variation in Dahomey females that were included during each of the experimental assay blocks. The statistical analysis showed no significant effect of dates on the response to Sex Peptide receipt in terms of receptivity in Dahomey females across the tested days *(Df=7, P=0.1485).* Therefore, date was not included in the subsequent statistical analyses of DGRP line receptivity.

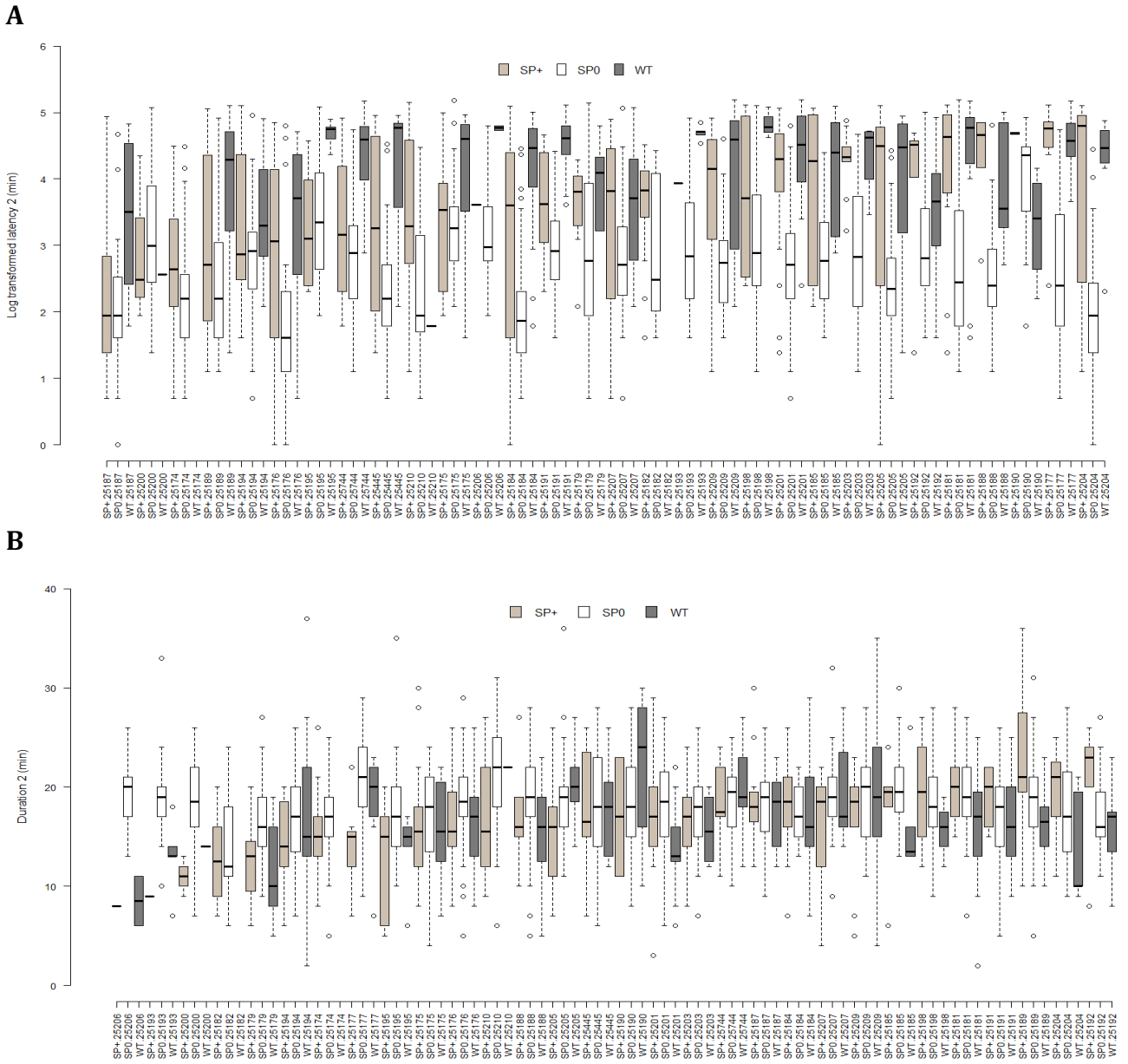
Highly significant variation was detected among the DGRP lines in re-mating percentages, and as expected, males genotype (SP^+, SP^0 or WT) had a major effect on female receptivity (Figure 1). In all the DGRP lines, females that did not receive Sex Peptide had significantly higher re-mating rates (varying from 50% to 97.1%), than did control females mated to SP^+ (ranging from 3% to 80%) or to fully wild type males (2.6% to 77.4%). Interestingly, the DGRP lines showed significant variation in the extent to which receipt of Sex Peptide diminished re-mating rates in females (*Chisq*=20.45, *P*=0.002).

For the second mating, the latency results revealed significant variation for lines (*Chisq=4.9305, P=0.02639)* and Sex Peptide had an effect on the latency of the second mating (*F=1, P=0.01513*). Females that were mated to SP^0 males on the first day generally re-mated more rapidly with those mated to wild type males on the second day, exhibiting reduced latency compared to females who had mated to SP^+ or to wild type males (Supplementary data). As for the duration of the second mating, lines differed significantly from each other (*Chisq*=13.95, *P*=0.0001878), while the different mating treatments also had a significant effect on the second-mating duration (*Chisq*=6.119, *P*=0.01337) (Figure 2).

These results indicated significant variation among the DGRP lines in the effects of Sex Peptide on female receptivity: the percentage of females that re-mated after receiving Sex Peptide differed markedly among the DGRP lines, as did latency time until re-mating.



<u>Figure 1:</u> Variation in re-mating percentages of females from 30 DGRP lines, 24 hours after mating to SP^+, SP^0 or WT males. Bar plot representing the percentage of DGRP females that re-mated with WT males 24 hours after a first mating to SP^0, SP^+ or WT males. For each line and treatment, n=40.



<u>Figure 2:</u> Re-mating latency and re-mating duration of females from 30 DGRP lines, 24 h after mating to SP⁺, SP⁰ and WT males. (A) Boxplots of the latency for the second mating (plotted on logarithmic scale) of the DGRP females to WT males, 24 hours after the same females were mated to either SP⁰, SP⁺ or WT males. (B) Boxplots of the second mating duration of DGRP females when mated to WT, 24 hours after being mated to either SP⁰, SP⁺ or WT males. Median represented by horizontal line within box, with box representing the interquartile range (IQR) and whiskers the highest/lowest value within. Outliers are represented by points. The labels on the x-axes is a concatenation of the DGRP line identifier and the mating treatment for the first mating (to WT, SP⁺ or SP⁰ mating).

GWAS

To identify polymorphism regions that correlate with female reduced receptivity to re-mating 24 hours after the receipt of Sex peptide, a GWAS was performed on the female re-mating percentage using the functionality of the DGRP website. The Manhattan plot showed significantly associated SNPs on chromosome X, 2R and 3R of the genome (Figure 3). Genes with SNPs, deletions and insertions that had statistical association with $P<10^{-5}$ were considered as candidate genes for subsequent network mapping and gene ontology enrichment analysis. In total 8 significantly associated polymorphisms were identified, of which 1 SNPs was in intergenic region and 6 SNPs and 1 INS in the upstream and intron regions of 2 genes *Socs16D* (FBgn0030869) and *CG9747* (FBgn0039754).

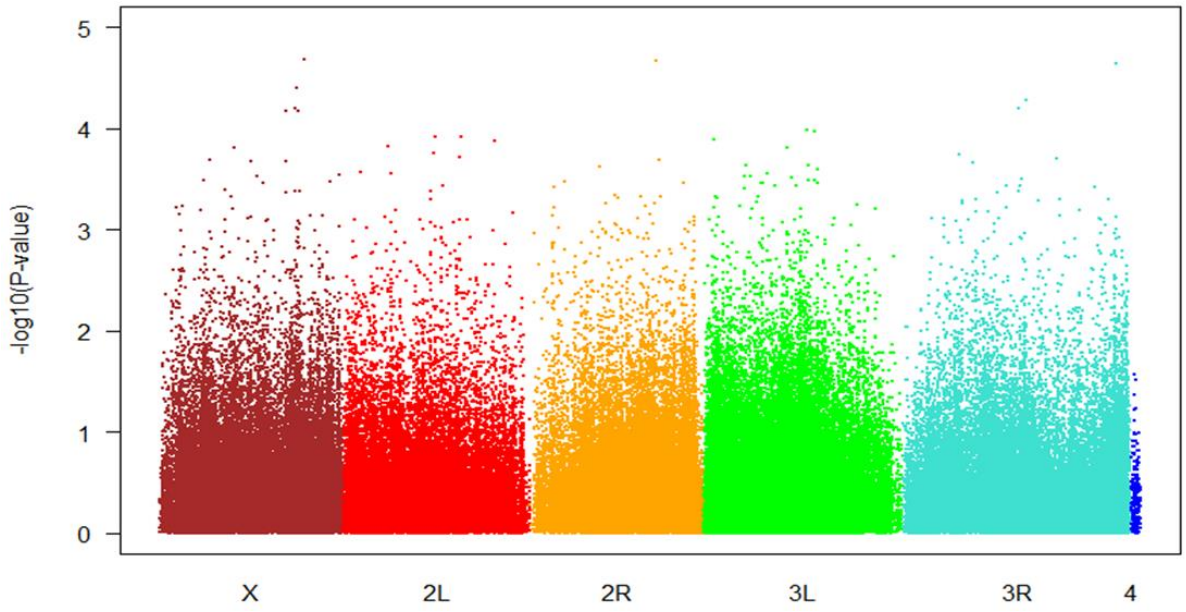
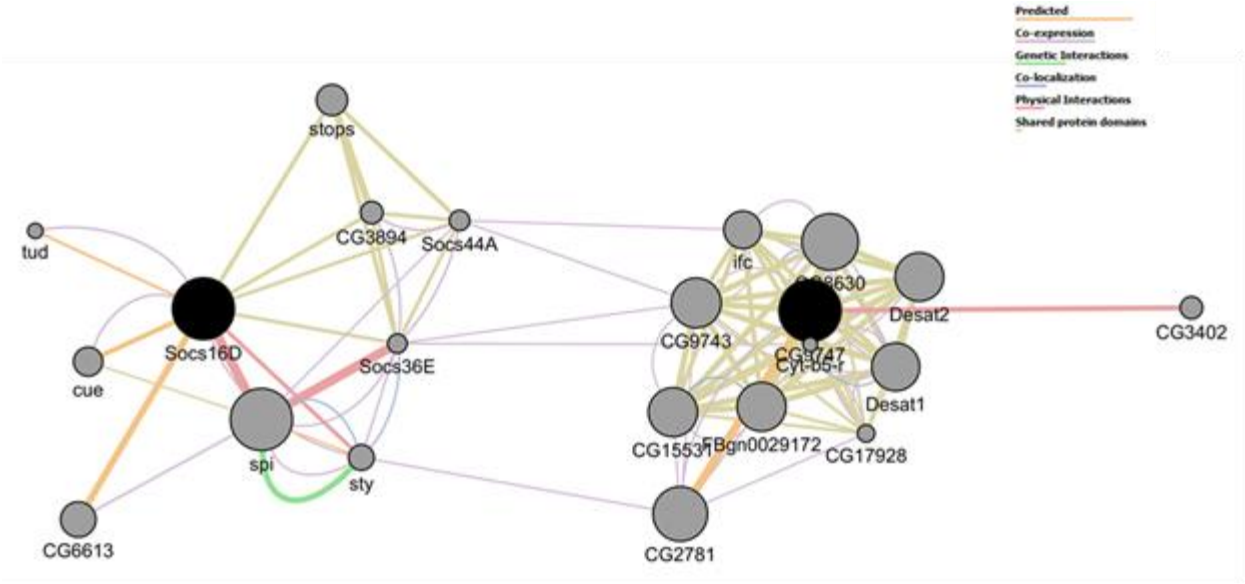


Figure 3: Manhattan plot (−log10[P] genome-wide association plot) of a genome wide association study on receptivity to remating in females from the DGRP lines 24 hours after initial matings with SP^+ males.

Functional gene networks

The functional annotations for the 2 genes identified by the GWAS, was performed by DAVID Bioinformatics Resource 6.8 program. According to DAVID, gene *CG9747* is involved in lipid metabolic process, unsaturated fatty acid biosynthetic process, long-chain fatty acid biosynthetic process, oxidation-reduction process. As for gene *Socs16D*, it negatively regulates the protein kinase activity and the JAK-STAT cascade and mediates the TORC1 signaling and the cytokine signaling pathway. Additionally, the functional gene network mapping was also performed on the 2 candidate genes by using the GeneMANIA app in Cytoscape (Montojo *et al.*, 2010; Warde-Farley *et al.*, 2010).

The 2 candidate genes that were identified by the GWAS on the re-mating percentages as effect of Sex Peptide revealed a network of 20 other related genes. The network represented by geneMANIA was based 37.47% on network prediction, 25.50% on co-expression network, 16% genetic interactions, 9.20% physical interactions, 9.73% colocalization and 2.10% on shared protein domains (Figure 4). The network generated by geneMANIA for these 2 genes estimates the different types of interaction that could occur between the GWAS-identified genes and other related genes, using a very large set of functional associations. The two candidate genes identified are part of a network involved in oxidoreductase activity and regulation of ERBB signaling pathway (Supplementary data Table 2).



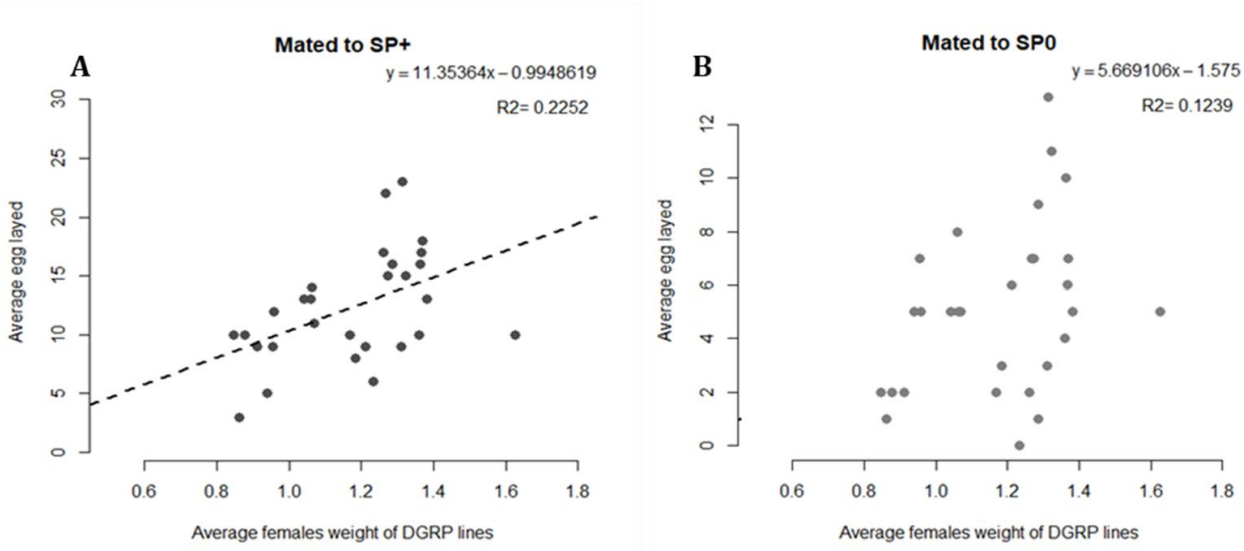
<u>Figure 4:</u> Interaction network of the 2 candidate genes associated with the variation in re-mating responses to Sex Peptide. Interaction networks of the 2 candidate genes identified by the GWAS on the proportion of females that re-mated when females were first mated to SP^+ males. Black nodes depict candidate genes generated by the GWAS with significant SNPs from the DGRP analysis (Query genes). Grey nodes are other genes that are related to a set of input candidate genes (Non-query genes). The links representing the networks in this case are based 37.47% on prediction, 25.50% on co-expression networks, 16% genetic interactions, 9.20% physical interactions, 9.73% colocalization and 2.10% on shared protein domains.

Body weight

A significant positive correlation was detected between mean female size and the mean number of eggs laid by females of each DGRP line after mating to SP^+ males *(F*=7.849, *P*=0.009291*)* (Figure 5A). A similar positive trend existed for the relationship between mean female size and the number of eggs laid when females were mated to SP^0 males (*F*=3.818, *P*=0.06115) (Figure 5B).

To test whether weight effects on egg numbers interacted with the egg-laying responses to Sex Peptide across the different DGRP lines, we plotted the difference between egg numbers laid by females mated to SP^+ males and females mated to SP^0 males (here considered to be the "Sex Peptide effect" on egg laying) against weight. The analysis showed no significant

correlation between weight and the Sex Peptide effect on fecundity (F=2.632, P=0.1163) (Figure 6). Combined, these tests indicate that the number of eggs laid by females, but not the effect of Sex Peptide on fecundity per se, was positively correlated to body size variation among the DGRP lines. In addition, we compared the GlmmTMB zero inflated negative binomial models including and excluding the interaction of weight and Sex Peptide as a random effect: the comparison of the two models was non-significant. Based on these results, and since we did not obtain the individual weight of the DGRP females in the egg laying assay, weights were not incorporated in the subsequent analysis of the egg laying data.



<u>Figure 5</u>: Correlation between DGRP line mean female weight and the mean numbers of eggs laid after mating to SP^0 or SP^+ males. (A) Scatter plot representing the significant correlation (P=0.0092) between DGRP line female body size and the number of eggs laid after mating with SP^+ males. (B) Scatter plot representing a non-significant correlation (P=0.06115) between DGRP line female body size and the number of eggs laid following mating to SP^0 males.

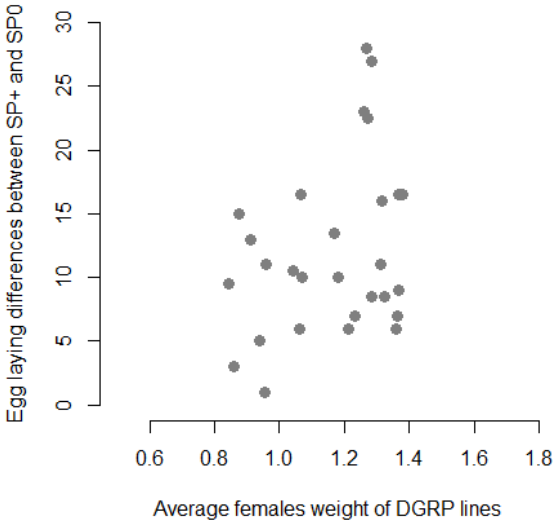


Figure 6: Correlation of DGRP female weight and the 'Sex Peptide effect' on fecundity (the difference of number of eggs laid between females of the same DGRP lines when mated to SP^+ males to SP^0 males). Scatter plot representing the non-significant correlation (*P=0.1163)* between female body size (weight) and the Sex Peptide effect on fecundity (difference in the number of eggs laid following matings with SP^+ or SP^0 males).

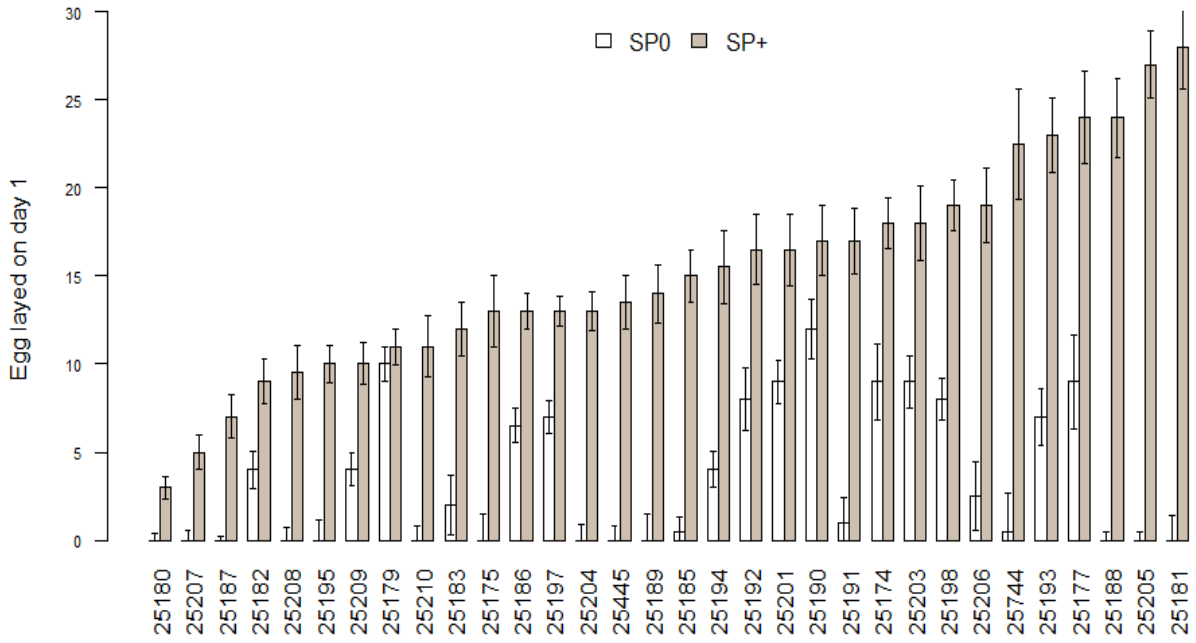
Egg laying

The number of eggs that resulted from these matings were scored 24 hours after mating (and 48 hours after mating, see supplementary data Figure 3). To account for day-to-day variation, the Dahomey line was also tested on each assay day. For the day-to-day effects, we compared zero inflated negative binomial models, including and excluding date as a random effect. In this comparison, date was non-significant *("GlmmTMB", Chisq=3.1411, P=0.07634*). Therefore, date was removed from subsequent analysis of the egg-laying data.

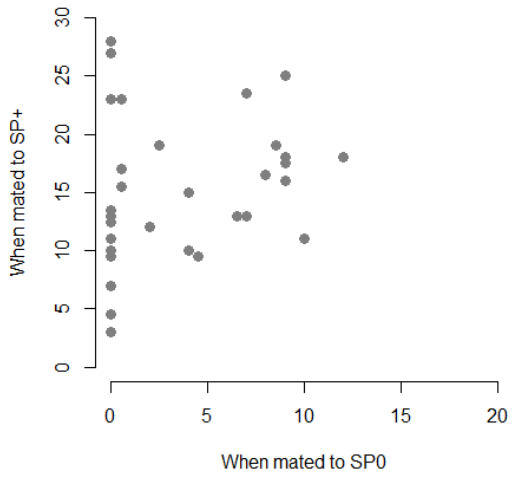
There was a significant effect of the interaction term between Sex Peptide × lines for egg laying on day 1 ("GlmmTMB", *Chisq=4.9037, P=0.0268*) (Figure 7). All the DGRP lines tested showed higher numbers of eggs laid when mated to SP^+ males than following matings with SP^0 males. Also, on the second day after mating, the numbers of eggs laid by females mated to SP^+ males were higher in most DGRP lines compared to matings with SP^0 males. The numbers of eggs laid on day 1 was approximately twice the number of eggs laid on day 2 (Supplementary data Figure 3). To determine whether the numbers of egg laid by females mated to SP^0 males was correlated with that following matings to SP^+ males, a linear regression analysis was

conducted on the 32 DGRP lines for egg laying on day 1 (Figure 8). The results showed a non-significant correlation (*F=1.438, P=0.2398*). Thus, DGRP lines do not systematically differ in how many eggs were laid on day 1 after mating, but instead varied in the egg laying responses to receipt of Sex Peptide. For day 2 there was a significant correlation in the number of eggs laid by the females of same lines when mated to either SP^0 or SP^+ males (*F=9.16, P=0.005041*) (Supplementary data Figure 4). These patterns could imply that the egg laying data on day 2 better reflected the variation among lines in egg laying rate *per se* than it did the variation among lines in their egg laying responses to Sex Peptide. Based on this information, the downstream analysis focused on the number of eggs laid 24 hours after mating.

Two phenotypic measures were calculated from the data on day 1 to describe the egg laying response to Sex Peptide: i) the median difference in the numbers of eggs laid by the females when mated to SP^+ and SP^0 for each of the 32 DGRP lines, to obtain an estimate of the absolute increase in the number of egg laid due to the receipt of Sex Peptide; and ii) the ratio of the numbers of eggs laid by females when mated to SP+ and to SP^0 males for the 32 DGRP lines, to obtain an estimate of the relative change in the number of eggs due to receipt of Sex Peptide. These two measures were used in a subsequent GWAS analysis to identify which genes were associated with the variation in egg laying responses to receipt of Sex Peptide.



<u>Figure 7</u>: Mean number of eggs laid by the DGRP females within 24 hours after mating to SP⁺ or SP⁰ males. Bar plot representing the median numbers of eggs laid by DGRP females within the first 24 hours after mating to SP⁰ or SP⁺ males. The lines are ordered in ascending order of SP+ fecundity. Error bars indicate s.e.m. For each line and mating treatment, n=40.



<u>Figure 8</u>: Correlation of the number of eggs laid by females of the DGRP lines within 24 hours after mating to SP⁰ or SP⁺ males. Scatter plot representing a non-significant correlation (*P=0.2398*) between the fecundity of DGRP females in the 24h following matings to either SP⁺ males (Y axis) or SP⁰ males (X axis).

Egg laying GWAS

i. GWAS on the increase in the numbers of egg laid

The GWAS performed on the median difference in the number of eggs laid by females from each of the DGRP lines when mated to SP^+ or when mated to SP^0 males. The Manhattan plot showed that significantly associated SNPs were in more than one region of the genome (Figure 9). A total of 30 polymorphisms were significantly associated with egg laying variation, of which 7 SNPs were in intergenic regions and 22 SNPs and 1 insertion in or near 15 genes (Supplementary data Table 3).

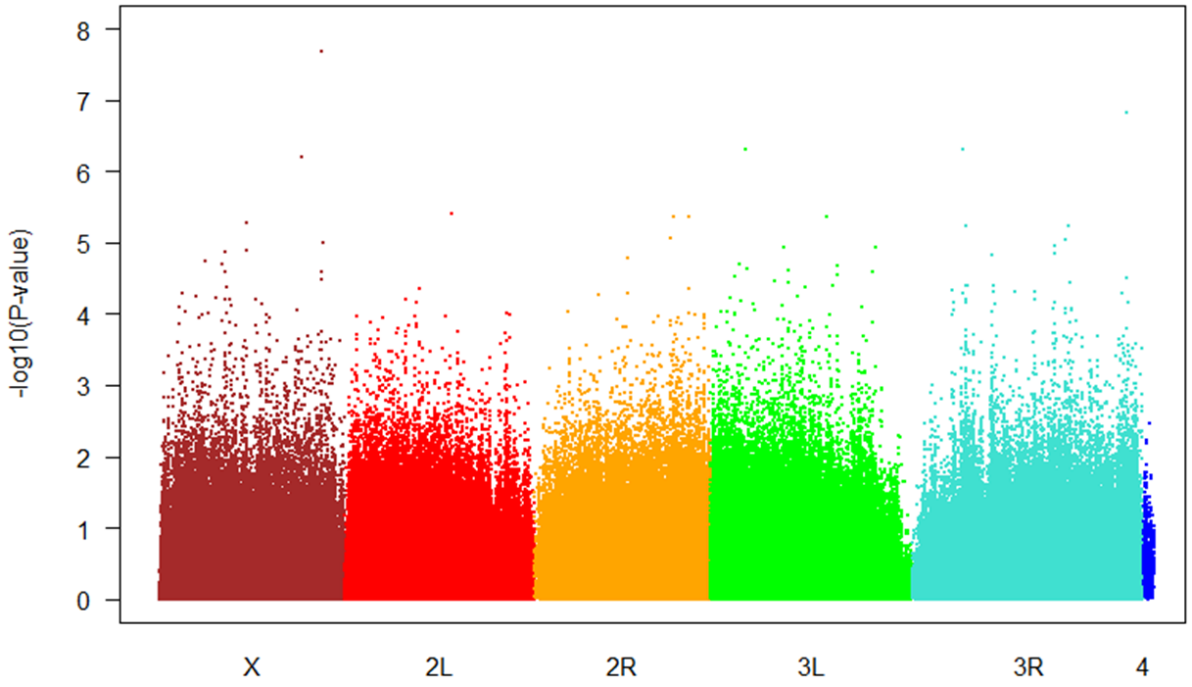


Figure 9: Manhattan plot (−log10[P] genome-wide association plot) of a genome wide association study on the increase of number of eggs laid by females from the DGRP lines following matings to SP^+ or SP^0 males.

ii. GWAS on the relative change in the numbers of eggs laid

The GWAS was performed on the ratio of the mean numbers of eggs laid by females from each of the DGRP lines when mated to SP^+ or to SP^0 males. The Manhattan plot showed that significantly associated SNPs did not associate in one region of the

genome (Figure 10). The GWAS provided a list of 200 polymorphisms that were significantly associated with the relative change in egg laying upon receipt of Sex Peptide, of which 45 SNPs were in intergenic regions and 144 SNPs, 5 INS and 6 DEL in or near 90 genes (Supplementary data Table 4).

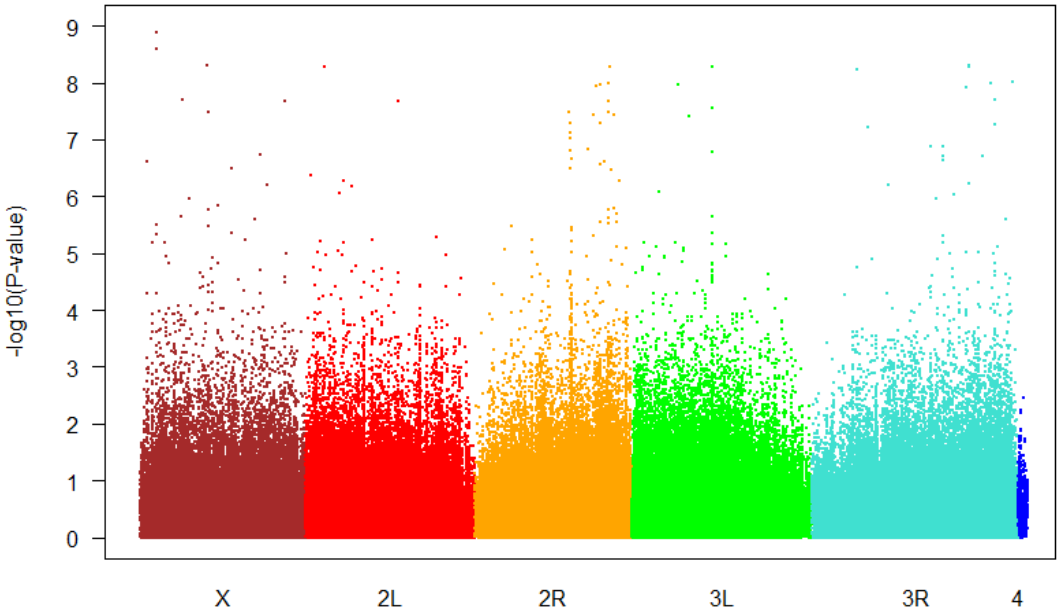


Figure 10: Manhattan plot (−log10[P] genome-wide association plot) of a genome wide association study on the ratio of the mean numbers of eggs laid by females from each of the DGRP lines when mated to SP^+ or to SP^0 males.

iii. Overlapping genes in both of the above GWAS analyses

The two GWAS analyses performed on the DGRP variation in egg laying in response to Sex Peptide identified one overlapping gene: FBgn0262617 (*CG43143*).

Functional gene networks

To obtain the functional annotations for each gene identified by the GWAS, and to perform a gene enrichment analysis on the candidate genes, we seeded the DAVID Bioinformatics Resource 6.8 program with the two gene lists from the GWAS. The gene annotations for the

increase in the numbers of egg laying and the relative change of numbers of eggs laid are displayed in Supplementary data table 5. The DAVID gene enrichment analysis for candidate genes from the GWAS on the increase in egg numbers showed an significant over-representation of coiled coil proteins (*P=1.10E-02*) that serve a mechanical role in forming stiff bundles of fibres, proteins with a Pleckstrin homology-like domain (*P=1.30E-02*) that are involved in intracellular signaling or constituents of the cytoskeleton, and phagocytosis proteins (*P=1.80E-02*) that are initially contained within phagocytic vacuoles and then fuse with primary lysosomes to effect digestion of foreign particles.

For the candidate genes from the GWAS performed on the relative change in egg laying, the gene enrichment analysis showed a significant over-representation of a set of genes involved in development, coiled coil proteins (*P=9.2E-8*), splicing proteins (*P=4.8E-5*), plasma membrane proteins (*P=8.1E-4*) and Insulin-like growth factor binding proteins (*P=1.0E-4*). These proteins are key regulators of cell proliferation, differentiation and transformation (supplementary data Table 6).

The functional gene network mapping was performed by using the GeneMANIA app in Cytoscape (Montojo *et al.*, 2010; Warde-Farley *et al.*, 2010). The 15 candidate genes that were identified by the GWAS for the increase in numbers of eggs revealed a network of 20 other related genes. The network represented by geneMANIA was based 89.19% on a co-expression network where the genes have similar expression levels and 10.81% on proteins with shared protein domains (Figure 11). For the 90 candidate genes identified in the GWAS on the relative change in egg laying, geneMANIA generated a network of a total of 110 related genes. These network associations were based 63.74% on co-expression, 28.79% on predicted interactions, 4.39% on co-localisation and 3.08% on connections based on shared protein domains (Figure 12). The predictions of these gene networks generated by geneMANIA suggested the different types of interaction that could occur between the GWAS-identified genes and other related genes, using a very large set of functional associations (Supplementary data Table 7).

The functions of these genes were further explored by searching the Flybase database for general annotations and relevant literature (Gramates *et al.*, 2017), and the FlyAtlas database for a description on the tissue- and developmental stage-specific expression (Chintapalli et al., 2007). More than half of the candidate genes identified by the GWAS have peaks of expression in embryonic stages (Supplementary data Table 8), of which 9 are of unknown biological function (Supplementary data Table 9). In addition, 41 of these genes have a developmental function, for example neural system development (*CG15765*), compound eye development (*Sobp, a*), imaginal disk (*sp1)* and structural constituent of cytoskeleton (*CG34347*). Other candidate genes are involved in signaling pathways, for example *Pde6* and *Pde8* are components of the cAMP signalling cascade (Ganguly and Lee, 2013) and others have molecular functions in proteolysis (*CG11836, CG14227, CG31427, Fur2*), or in Calcium ion binding (*CG11041*).

The candidate "egg laying" genes

Based on the DAVID gene enrichment annotation, GeneMANIA network mapping and Flybase search, 13 candidate genes were selected for further exploration, based on their functional annotations that indicated their involvement in the oocyte maturing and egg development. A literature search of the genes involved in oocyte and egg formation and development is summarised in Table 1. Most of the 13 genes of interest are directly linked to the process of formation and maturation of an ovum, from a primordial female germ cell to an egg, such as *Axn* and *Doa* that are involved in oogenesis; *bun* and *Kst* that play a role in ovarian follicle cell development; *mfr, prage* and *par-1* that are involved in egg patterning, development and activation.

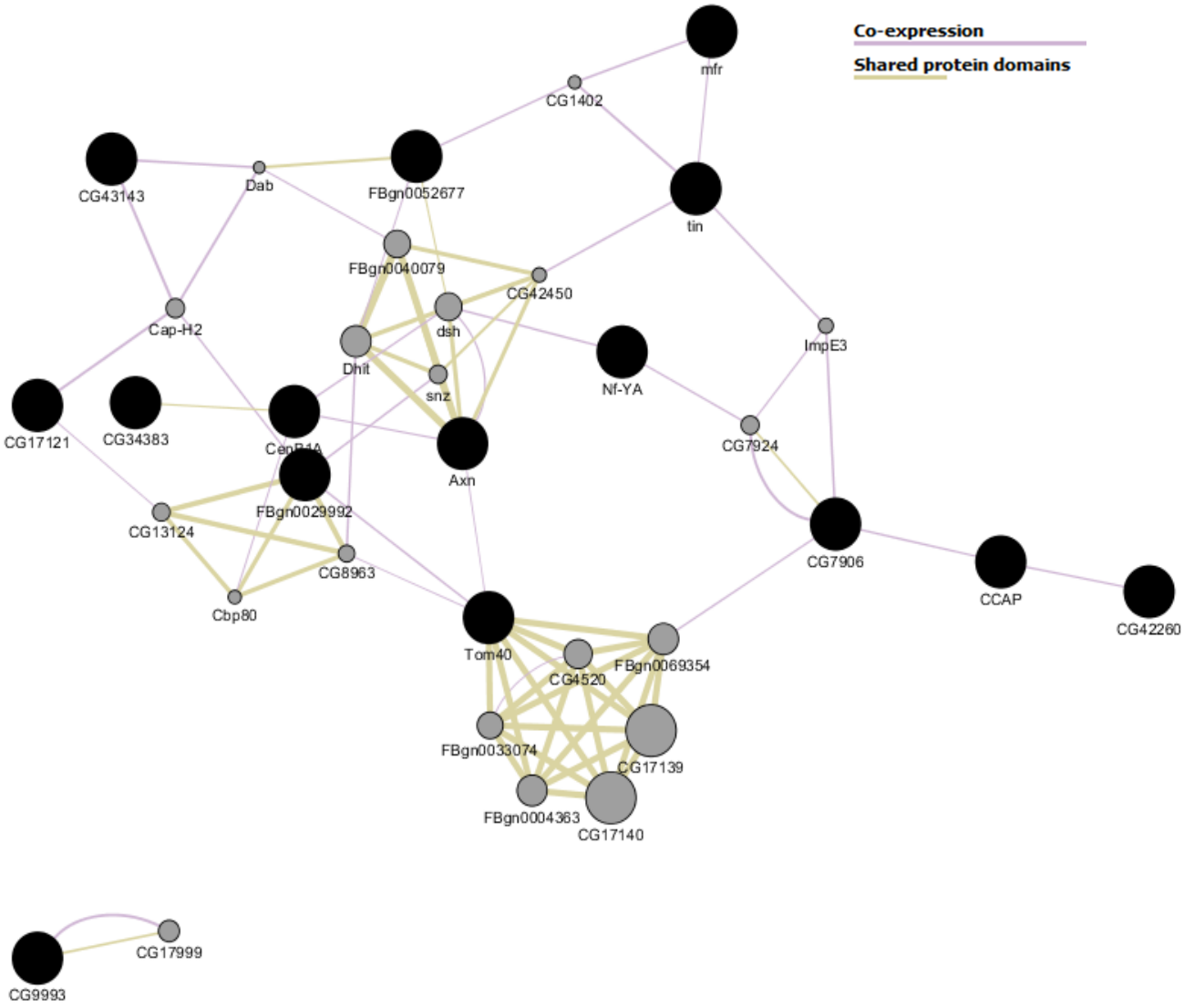


Figure 11: Interaction network of candidate genes involved in the increase in the numbers of eggs laid in response to Sex Peptide. Interaction networks of candidate genes identified by the GWAS for the increase of the numbers of eggs laid when females were mated to SP^+ males, compared to when they were mated to SP^0 males. Black nodes depict candidate genes generated by the GWAS with significant SNPs from the DGRP analysis (Query genes). Grey nodes are other genes that are related to a set of input candidate genes (Non-query genes). The links representing the networks in this case are based 89.19% on co-expression networks and 10.81% on shared protein domains.

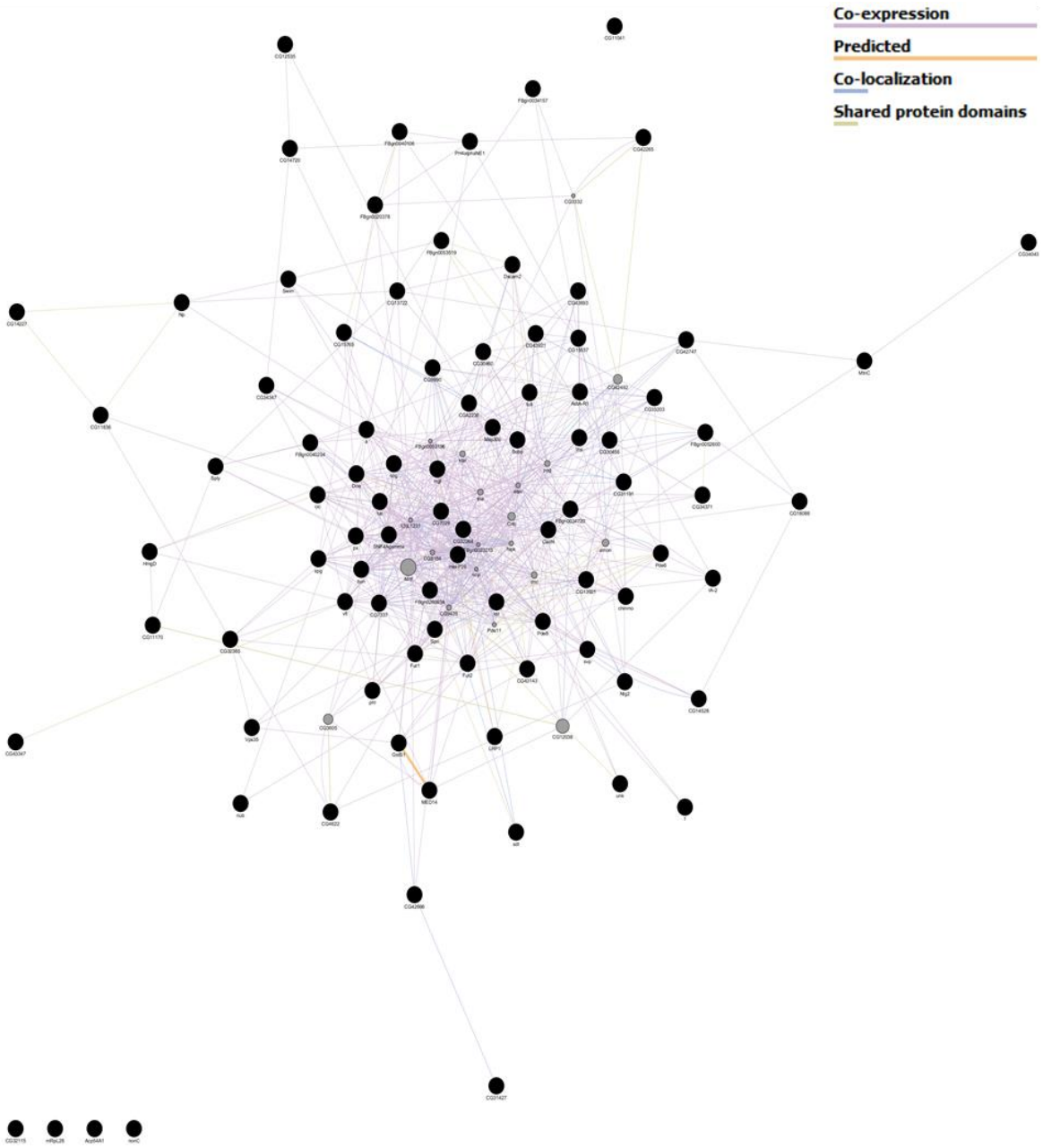


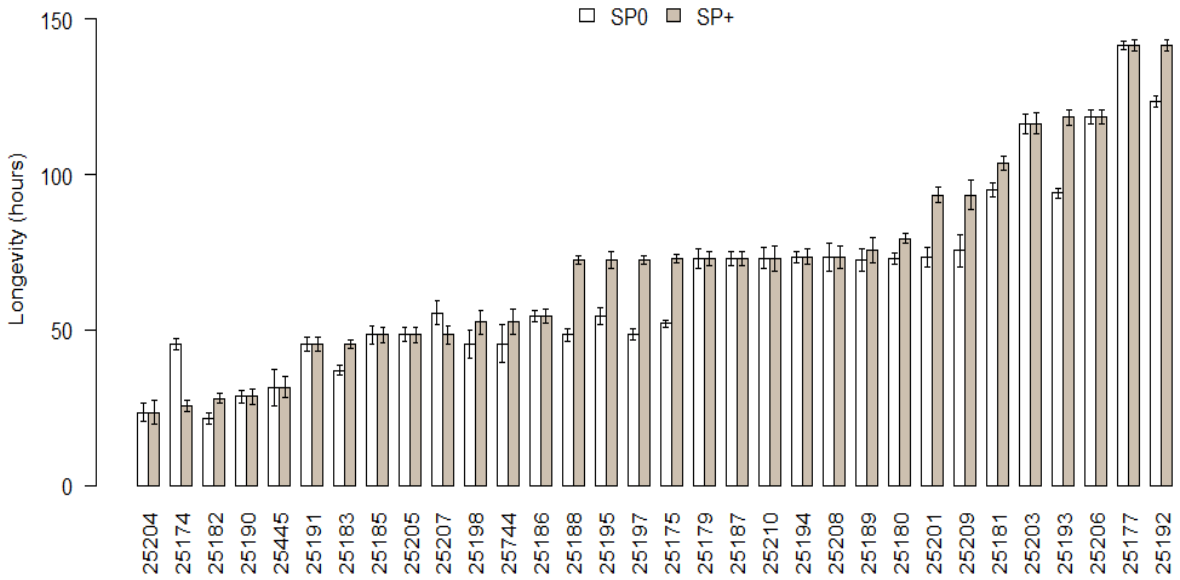
Figure 12: Interaction network of candidate genes involved in the relative change in the numbers of eggs laid in response to Sex Peptide. Interaction networks of candidate genes identified by the GWAS for the ratio in the numbers of egg laid when females were mated to SP^{+} males compared to when they were mated to SP^{0} males. Black nodes depict candidate genes generated by the GWAS with significant SNPs from the DGRP analysis (Query genes). Grey nodes are other genes that are related to a set of input candidate genes (Non-query genes). The links representing the networks in this case are based 64.74% on co-expression networks, 28.79% on predicted interactions based on orthologs, 4.39% on co-localisation and 3.08 % on shared protein domains.

Table 1: Summary of the 13 candidates "egg laying genes", identified by the two GWAS for the variation in egg laying in females.

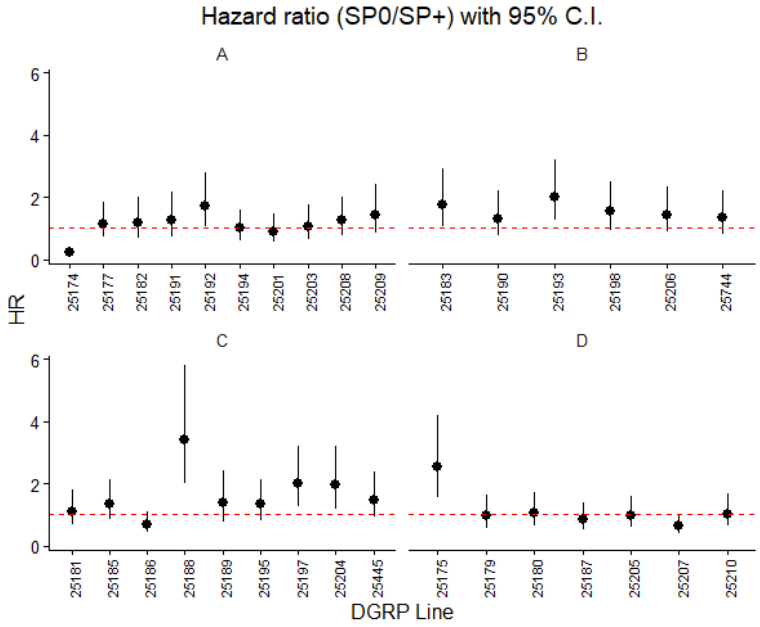
Phenotype	Gene	Annotation	Function	References
Increase in numbers of eggs laid	***mfr***	FBgn0266757	A membrane protein involved in egg patterning, and early embryogenesis	Smith and Wakimoto, 2007
	Axn	FBgn0026597	Maintaining the proliferation of follicle cells	Song, 2003
	tin	FBgn0004110	Involved in germ cell migration, and is required for proper development of gonadal mesoderm	Moore *et al.*, 1998
Relative change in numbers of eggs laid	***Msp300***	FBgn0261836	Maintenance of the structural integrity of the ring canals connecting the female germline cyst	Yu *et al.*, 2006
	Raf	FBgn0003079	Polarization of the ovarian follicle cells along the dorsal/ventral axis	Brand and Perrimon, 1994
	bun	FBgn0259176	Ovarian follicle cell structuring and development	Dobens and Raftery, 2000; Dobens *et al.*, 2005
	Kst	FBgn0004167	Involved in constricting of the follicle cells during mid-oogenesis and ovarian follicle cell migration	Zarnescu and Thomas, 1999
	mei-P26	FBgn0026206	Germ cell development	Page *et al.*, 2000
	sog	FBgn0003463	Polarization of the oocyte along the dorsal-ventral axis	Carneiro *et al.*, 2006
	tai	FBgn0041092	Involved in follicle cells migration and the maintenance of an internal steady state within the germ-line stem-cell niche.	Mathieu et al., 2007; König et al., 2011
	prage	FBgn0283741	Egg activation	Tadros *et al.*, 2003
	par-1	FBgn0260934	Involved in oocyte polarization, development and differentiation	Cox *et al.*, 2001; Doerflinge *et al.*, 2006
	Doa	FBgn0265998	Involved in oogenesis and oocyte karyosome formation	Morris *et al.,* 2003

Survival resistance

Day-to-day variation was accounted for by including Dahomey females during each of the experimental assay blocks. The statistical analysis showed no significant effect of dates on the starvation survival in response to mating (either SP0 or SP+) in Dahomey females across the tested days (*Chisq=1.6964, P=0.6377*). Therefore, date was not included in the subsequent statistical starvation analysis of DGRP line Interestingly, the results showed a significant fixed effect of Sex Peptide (*LR= 7.867, P=0.005*), with females that received Sex Peptide during their single mating being more resistant to subseqwuent starvation (i.e. surviving longer) than those that did not. The variation for the starvation resistance effect of Sex Peptide was highly significant among the DGRP lines tested (*LR=49.99, P<0.0001*) (Figure 13 and 14).



<u>Figure 13:</u> Starvation survival analysis of DGRP females following single matings to SP⁰ or SP⁺ males. Bar plot of the subsequent median starvation survival (hours of lifespan on agar only medium) of females from 32 DGRP lines, following single matings to SP⁰ or SP⁺ males. Lines are presented in ascending order of survival in response to receipt of SP+.



<u>Figure 14:</u> A forest plot showing the hazard ratio and 95% confidence intervals for starvation survival of females from 32 DGRP lines, following single matings to SP⁰ or SP⁺ males. Circles represent the hazard ratio of females when mated to SP⁺ and the vertical bars extend from the lower limit to the upper limit of the 95% confidence interval of the estimate of the hazard ratio. Dotted lines indicate the overall average effect of matings with SP0 males on female survival. (A, B, C and D indicate the experimental blocks)

GWAS

A GWAS was performed using as input data the survival hazard ratios of females from the 32 DGRP lines, using the functionality of the DGRP website, to identify polymorphism regions that correlate with starvation survival after the receipt of Sex Peptide. The Manhattan plot significantly associated SNPs were on chromosome 2L, 2R, 3L and 3R (Figure 15). Genes with SNPs, deletions and insertions that had statistical association with $P<10^{-5}$ were considered as candidate genes for subsequent network mapping and gene ontology enrichment analysis. In total 4 significantly associated polymorphisms were identified, of which 1 SNPs and 1 DEL was in intergenic regions and 1 SNPs and 1 INS in the upstream and intron regions of 2 genes, *daw* (FBgn0031461) and *CG34027* (FBgn0054027).

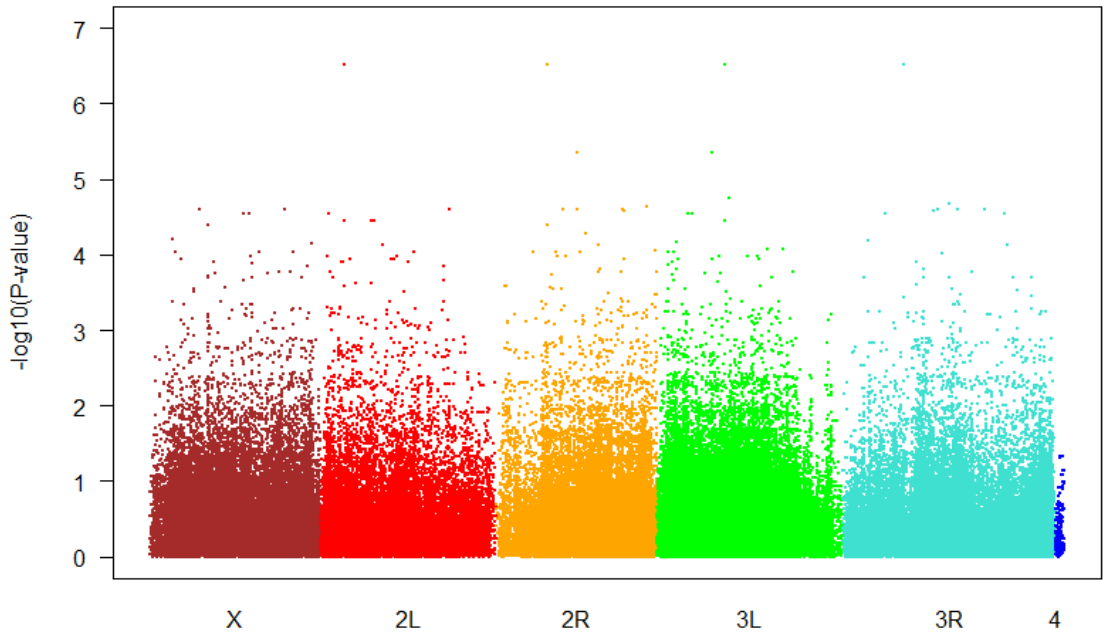
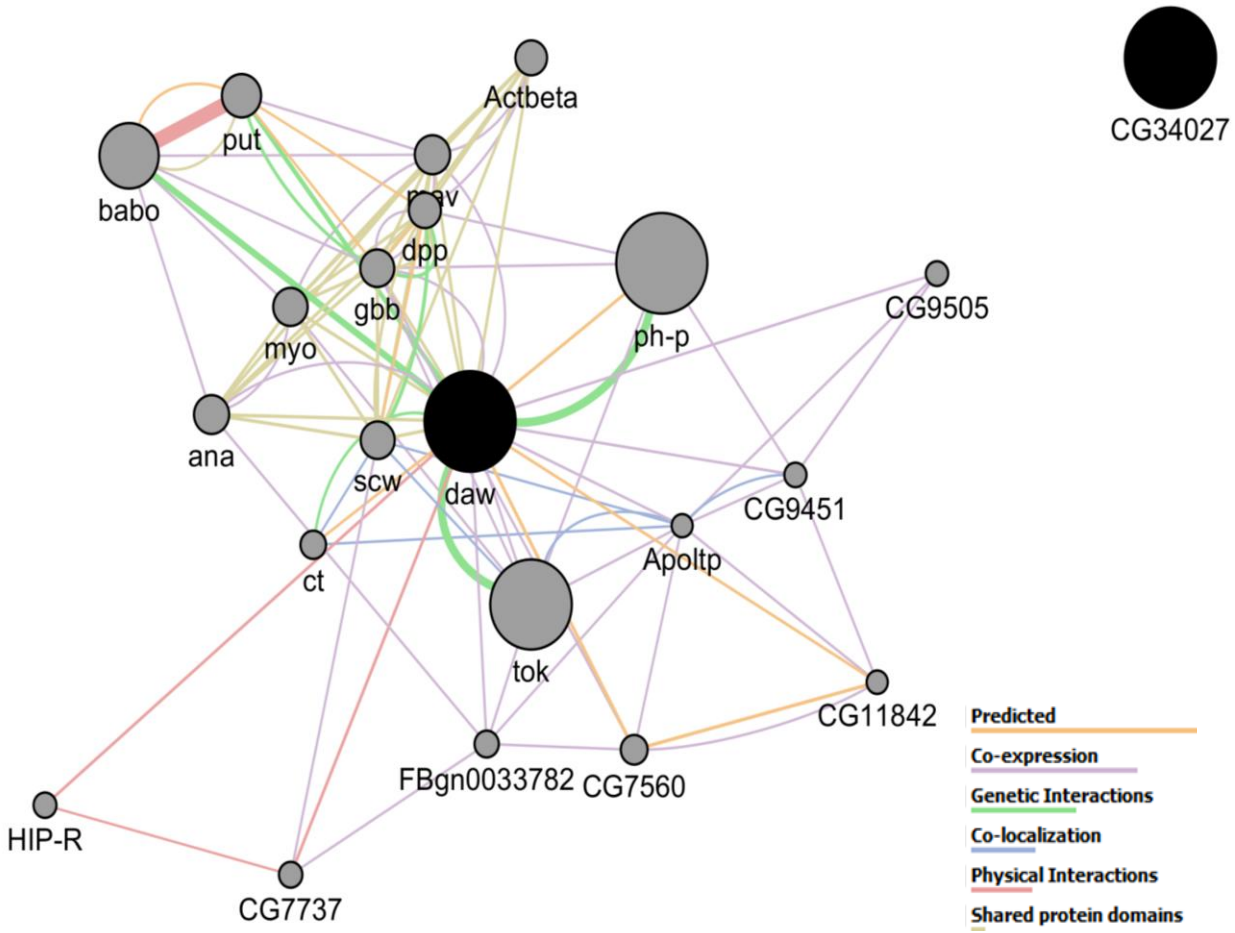


Figure 15: Manhattan plot (−log10[P] genome-wide association plot) of a genome wide association study on the survival hazard ratios of females from the 32 DGRP lines, under starvation survival after the receipt of Sex Peptide.

Functional gene networks

The functional annotations for the 2 candidate genes identified by the GWAS, was performed by DAVID Bioinformatics Resource 6.8 program. Based on the DAVID analysis, the *daw* gene identified is implicated in several signaling pathways (growth factor beta receptor pathway, SMAD protein phosphorylation pathway, activin receptor signaling pathway, MAPK cascade and insulin secretion). One of the major functions of *daw* is the determination of adult lifespan as well as regulation autophagy and apoptotic process. As for *CG34027,* it is an integral component of the cell membrane, but its molecular and biological processes are unknown.

The functional gene network mapping was also performed on the 2 candidate genes by using the GeneMANIA app in Cytoscape (Montojo *et al.*, 2010; Warde-Farley *et al.*, 2010). The 2 candidate genes involved in starvation survival following Sex Peptide receipt revealed a network of 20 other related genes. The network represented by geneMANIA comprised 37.47% on prediction, 25.50% on co-expression networks, 16% genetic interactions, 9.20% physical interactions, 9.73% colocalization and 2.10% on shared protein domains (Figure 16). Since the biological and the molecular function of gene CG34027 is unknown, geneMANIA could not associated it with a network. However, the *daw* candidate gene is part of a network involved in several processes summarized in Supplementary Data Table 10.



<u>Figure 16:</u> Interaction network of the 2 candidate genes involved in the starvation survival GWAS analysis. Interaction networks of the 2 candidate genes identified by the GWAS of the starvation survival of females mated once to SP^+ males. Black nodes depict candidate genes generated by the GWAS with significant SNPs from the DGRP analysis (Query genes). Grey nodes are other genes that are related to a set of input candidate genes (Non-query genes). The links representing the networks in this case are based on 37.47% predictions, 25.50% co-expression networks, 16% genetic interactions, 9.20% physical interactions, 9.73% colocalization and 2.10% on shared protein domains.

Discussion

Results summary

Our results suggested that across the tested DGRP lines, the transfer of Sex Peptide had a clear overall effect to significantly reduce female re-mating, increase female egg laying and increase female starvation lifespan following a single mating. However, the extent of these effects varied significantly across lines. This phenotypic variation in response to Sex Peptide was tracked through GWAS, revealing a set of genes putatively involved in determining each of these phenotypes. For receptivity, two candidate genes were identified by the GWAS, but it is as yet unclear how they act to reduce receptivity. For egg laying a total of 104 candidates were identified by the GWAS, of which 13 genes are already known to show direct involvement in egg development and in the regulation of egg laying. Half of the rest of the candidate genes are highly expressed in early embryonic stages. Finally, the GWAS performed on the starvation survival hazard ratio revealed two candidate genes, of which *daw* is already known to determine adult lifespan. These results confirm the pleiotropic effects of Sex Peptide in controlling female post-mating behaviour and physiology, while also showing the extent of genetic variation for each of these effects.

Mating latency and duration

In both egg laying and receptivity analysis, we measured the latency and duration of virgin matings in females from 32 DGRP lines. We found consistent results: significant phenotypic variation in mating latency, and no significant variation in mating duration. The latency to virgin mating occurred irrespective of the genotype of the mating males (SP^0, SP^+ or WT). In contrast, the genotype of male had a significant effect on virgin mating duration. These results are consistent with the finding of a similar study on the DGRP females (Gorter, 2018) which showed significant variation in mating latency of virgin females. This variation in latency is not surprising as females play an important role in exerting mate choice (Bastock and Manning, 1955). If females are more or less receptive to male courtship (Spieth, 1974), it could determine indirectly the latency of mating. As for duration, the lack of variation among the females of the DGRP lines is in agreement with another study, which indicates that males, not females primarily determine copulation duration (MacBean and Parsons, 1967).

Especially in matings with the wild type males, the first mating duration tended to be slightly longer than the second.

In the case of a second mating (i.e in the receptivity assay), females from the DGRP lines showed significant variation for both latency and duration following their initial matings to males of the different genotypes. In addition, females that were mated with SP^0 males on day one had a shorter latency than those mated to SP^+ or WT males and tended to have a slightly longer mating duration. Thus, the transfer of Sex Peptide in the first mating had a significant effect on both latency and duration of the second mating. For latency, this was not surprising, since Sex Peptide is known to reduce female receptivity to other males, by increased female rejection of courting males (Manning, 1967). The effect on second mating duration is perhaps less expected but could suggest that males can perceive whether or not the females received Sex Peptide in their first mating and adjust their investment accordingly in the second.

GWAS

We performed GWAS mapping analyses to identify candidate genes, polymorphisms, and pathways affecting the variation in female post mating response to Sex Peptide. We used an unbiased GWAS on 30-32 DGRP lines (MacKay *et al.*, 2012; Mackay and Huang, 2018). We detected significant genetic variation among the lines for the receptivity, egg laying and survival resistance assay. However, it is important to be aware of the potential for false positives arising from the use of a relatively modest number of lines in comparison to the full set (Mackay and Huang, 2018).

Receptivity

One of the most obvious forms of potentially selfish manipulation by males via the actions of Sex Peptide is achieved by the reduction of female sexual receptivity to further matings. The mechanistic basis of how Sex Peptide reduces female receptivity is not yet known. In this study we tracked the re-mating variation in response to Sex Peptide in 30 DGRP isofemale lines and associated it through GWAS to genes that could be involved in controlling female receptivity.

i. Re-mating variation

Overall, females of the 30 DGRP lines showed significant variation for re-mating rate. In addition, Sex Peptide had a significant main effect on re-mating rates. Females that received Sex Peptide had very low re-mating compared to females that did not, with the re-mating rates in the latter being close to 100% in many lines. This variation in re-mating due to Sex Peptide suggests that females express different resistance levels to the effect of Sex Peptide on receptivity. To better understand the genes involved in this sexual antagonistic evolution, a GWAS was performed on the re-mating rates following receipt of Sex Peptide.

ii. GWAS

As a result of the GWAS, we found 8 polymorphisms, located on the 2R, 3R and X chromosomes, that were significantly associated with Sex Peptide effect on re-mating. Of these polymorphisms one did not occur in any known protein-coding gene or within 1kb up- or downstream of their location. The 7 remaining polymorphisms (6 SNPs and 1 insertion) were linked with 2 protein-coding genes, *Socs16D* and *CG9747*.

iii. Functional enrichment

The functional enrichment analysis conducted using geneMANIA and DAVID, on the 2 candidate genes from the GWAS, revealed that gene *Socs16D* regulates negatively the protein kinase activity and the JAK-STAT cascade and mediate the TORC1 signaling and the cytokine signaling pathway. *CG9747* also known as stearoyl-coA 11-desatura, is a cuticle hydrocarbon involved in lipid metabolism, unsaturated fatty acid biosynthesis, long-chain fatty acid biosynthesis, oxidation-reduction and pheromone synthesis. Very little is known about *CG9747* or how it could be involved in reducing female re-mating. We suggest once Sex peptide is release in mated females. We hypothesise it may act on as a pheromone to render females unattractive to males, thereby reducing the re-mating rate. The gene *Socs16D* codes for a SOCS (Suppressor of Cytokine Signaling) protein. These are regulators of the JAK-STAT pathway that participate in a negative feedback loop that is transcriptionally activated by JAK-STAT signaling (Rawlings *et al.*, 2004). However, *Socs16D* is as yet uncharacterised. Another member of the SOCS protein *Socs36E,* has been

well studied. *Socs36E* attenuates *STAT* activation through a negative feedback loop (Monahan and Starz-Gaiano, 2013). Successively, STAT is involved in a regulatory circuit that regulate expression of *mir-279* (Yoon *et al*., 2011). Subsequently, *mir-279* regulates the activity of the follicle cells in the egg chamber (Yoon *et al*., 2011; Monahan and Starz-Gaiano, 2013). Intriguingly, a study by Fricke *et al* (2014) showed that females lacking *mir-279* are less efficient in suppressing re-mating 24 hours after the receipt of Sex Peptide.

Based on all this information, we suggest that Sex Peptide activates *Socs16D*, which in turn regulates the JAK-STAT pathway in a negative feedback loop. JAK-STAT consecutively regulates *mir279* through another feedback loop, that is involved in reducing female re-mating and receptivity. More research now needs to be done on *Socs16D* to characterise its function and involvement in post-mating responses in females (Figure 17).

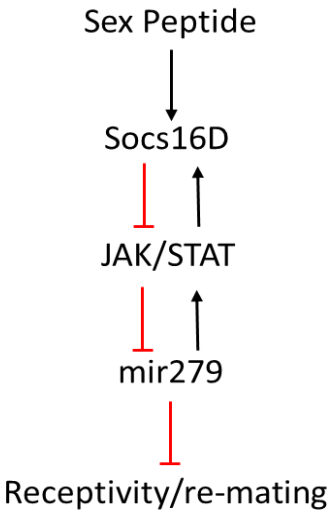


Figure 17: Proposed model in which *Socs16D* is integral to a genetic circuit that attenuates STAT activity to reduce receptivity and re-mating.

Egg laying

One of the main post-mating responses affected by Sex Peptide is the egg laying rate (Chapman *et al.*, 2003). Sex Peptide is known to stimulate egg laying in females after mating, by the release of juvenile hormone III-biosepoxide (JHB3) (Moshitzky *et al.*, 1996; Soller *et al*, 1997, 1999; Kubli, 2003). In these studies, genetic variation was excluded to clearly outline Sex Peptide function. To better understand the evolutionary dynamics in the response to Sex Peptide on egg laying, we analysed the egg-laying rate in 32 isofemale lines of the DGRP, when mated to males that transfer sperm and semen with or without Sex Peptide. This identified a set of genes through GWAS that were potentially associated to the response in egg laying.

i. Egg laying variation

Our results showed a significant phenotypic variation in egg-laying across 32 DGRP lines in response to mating with males without Sex Peptide, and in response to the receipt of Sex Peptide. Females that mated to males that transferred Sex Peptide always produced more eggs than when mated to males without Sex Peptide. In addition, the numbers of eggs laid on day 1 was approximately twice the number of eggs laid on day 2 after mating. This coincides with the finding (Chapman *et al.*, 2003) that Sex Peptide effect on egg laying peaks 24 to 28 hours after mating. Therefore, we conclude that Sex Peptide induces a universal egg laying response in *D. melanogaster.* However, the extent of the egg laying response varied significantly in the tested lines. This indicates that some lines have evolved different sensitivity or resistance to the effect of Sex Peptide on egg laying. This genetic variation in egg laying response to Sex Peptide receipt could be the result of sexually antagonistic coevolution. To clearly understand the association between intraspecific genetic variation in the egg laying response to Sex Peptide, we performed a GWAS on the increase of the numbers of eggs laid, and on the relative change in the numbers of eggs laid of the DGRP lines when mated to males with and without Sex Peptide. The results of this analysis are described in the next section.

ii. GWAS

The GWAS on the increase of the numbers of eggs laid in DGRP females identified a total of 30 polymorphisms, spread across all chromosomes, that were significantly associated with the increase of egg laying as result of the receipt of Sex Peptide. Of these polymorphisms 7 SNPs with significant associations did not code for any known protein coding gene within

1kb up or downstream of their location. The 23 remaining polymorphisms showing significant associations were in or near 15 protein-coding genes.

The GWAS performed on the relative change in numbers of eggs laid in the 32 DGRP females, revealed a total of 200 significantly associated polymorphisms, also spread across the genome. Among these polymorphisms were 45 SNPs with significant associations located in the intergenic regions that did not fall within any known protein coding gene or within 1kb up- or downstream of their location. The 155 remaining polymorphisms showing significant associations were linked with 90 protein-coding genes, and within these coding genes, 17 had an unidentified function.

iii. Functional enrichment

The functional enrichment analysis using GeneMANIA and DAVID on the candidate genes from the two GWASs showed that 41 coded for developmental proteins, including imaginal disc morphogenesis, neural development and membrane organization. For example, *Axin, Doa, svp, unk, arc* and *sobp* genes are involved in the morphogenesis of eye imaginal discs and eye development and *chinmo, Raf, bun, cic, px, sog, forked, vfl and unk* are involved in wing imaginal disc development. Several genes, such as *tin, spn, unk, sdt, svp* and *Dscam2* are also involved in neuron development and differentiation. A number of genes code for proteins involved in regulation of transcription, such as *tin, Chimno, MED14, cic, tai and nub*. According to FlyAtlas and Flybase, 29 of these developmental genes have peaks of expression in embryonic stages. Our results are in accordance with findings of Gioti *et al* (2012), which showed that the receipt of Sex Peptide induces significant alterations in genes involved in egg and early embryo development. These developmental maternal genes are differentially expressed in females, as they will be deposited in the fertilized eggs and will direct early embryonic development (Hooper *et al.*, 2007; Liu and Lasko, 2015). We might speculate that Sex Peptide could induce females to increase their investment in maternally expressed genes during oogenesis, to increase the short-term production of offspring, as it has been previous suggested that male *D. melanogaster* influence maternal investment in offspring by affecting the offspring size (Pischedda *et al.*, 2011). Alternatively, we could speculate that the alteration of the maternal gene investment is a side or indirect effect of the increase in egg laying due to Sex Peptide.

The GWAS also revealed 15 genes that are involved in the neural signaling pathways. Among these signaling genes there were also two (*Pde6* and *Pde8*) of the six genes that code for cyclic nucleotide phosphodiesterases (PDEs), which collectively have important roles in the cAMP and cGMP signalling (Day *et al.*, 2005). A recent study on post-mating receptivity in females showed that silencing *Pde8* reduces post-mating receptivity (Gorter, 2018). Furthermore, sequence variation of *Pde6* seems to be involved in mated female egg retention (Horváth and Kalinka, 2018). Both these genes thus appear to be involved in post-mating behavior in females.

The functional annotation of the genes identified by the GWAS also revealed that 29 of the genes are involved in protein and RNA processing, including protein phosphorylation, such as *CG43143, CG33519, CG7029, par-1 and tom40*. For 17 genes, there were no identified functions or annotations, 9 of which had peaks of expression in the embryonic stages. Possibly, some of these genes might have a role in the post mating response with respect to Sex Peptide. Further studies are required to understand their function.

While the JAK/STAT pathway is known to be required for oogenesis (Beccari *et al.*, 2002), none of the candidate genes in the GWAS for egg laying were part of this signal transduction pathway. However, several of the genes had Epidermal Growth Factor (EGF) domains or are known to be part of the Ras/Raf/MAPK pathway. This pathway is known to interact with the JAK/STAT pathway to ensure development of mature eggs (Xi *et al*., 2003). Intriguingly, we did find that several genes identified by the GWAS interact with JAK/STAT pathway. Of these: *Raf* interacts with the JAK/STAT pathway and results in increased numbers of lamellocytes in the blood (Hombría and Brown, 2002). The JAK/STAT pathway also interacts with components of the DPP/BMP pathway, one of these components was identified by the GWAS as *bun* (Arbouzova, 2006). Finally, the Drosophila protein *Tin* has been shown to modulate the JAK/STAT pathway in the developing mesoderm and also promotes proper heart precursor diversification (Liu *et al.*, 2009; Johnson *et al.*, 2011). How these proteins and the JAK/STAT pathways regulate oogenesis and egg development collectively is not yet clear.

iv. The candidate "egg laying" genes

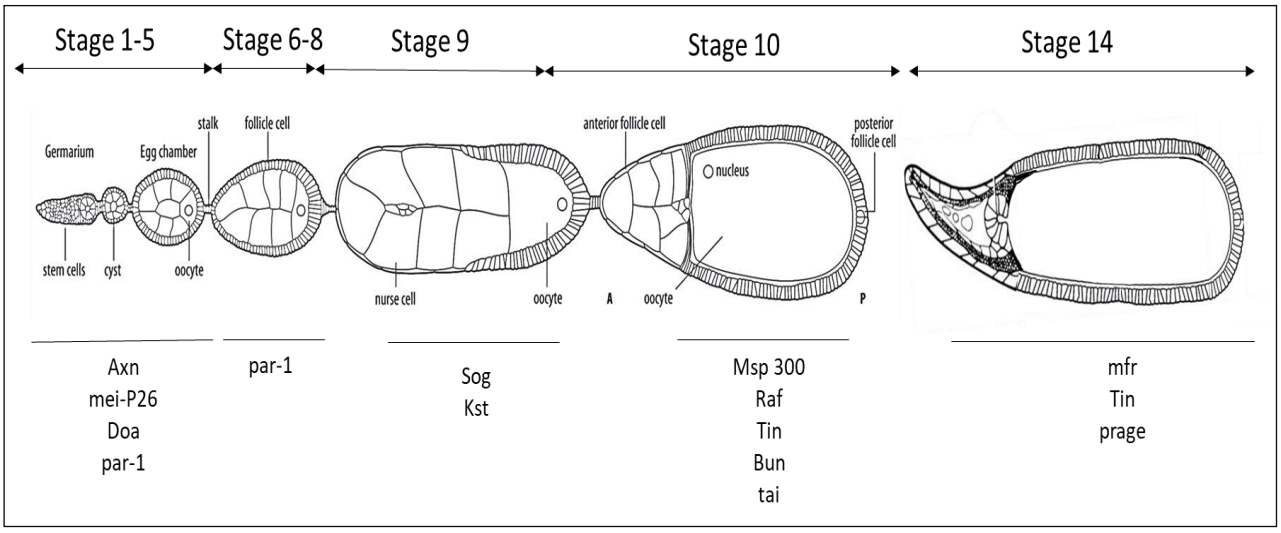


Figure 18: ***Drosophila melanogaster*** **oogenesis**. Different stages of developing eggs within the oocytes are shown, together with the known temporal expression patterns of the "egg laying" candidate genes identified by the GWAS.

The development of germ cells into mature eggs requires 14 morphologically distinct stages, which occur in different egg chambers of the ovarioles. At the start, a single cystocyte undergoes four synchronous divisions where one of these cells differentiates into the oocyte and enters meiosis, while the rest become nurse cells that are critical to the development of the oocyte and the future embryo. Each egg chamber contains follicle cells enveloping one oocyte and 15 nurse cells interconnected by actin-rich cytoplasmic bridges referred to as ring canals (Becalska and Gavis, 2009). These ring canals allow the transport of the cytoplasm from the nurse cell to the oocyte ensuring a proper development of a mature oocyte (Figure 18).

The GWAS performed on the variation in egg laying revealed 13 genes expressed throughout oogenesis that are directly linked to the oocyte and egg development (Figure 18). Among these candidates, *mei-P26* and *Doa* are involved in oogenesis and oocyte differentiation: *mei-P26* is required for meiotic recombination and germline differentiation (Page *et al.*, 2000); *Doa* regulates the cell cycle in nurse cells and oocytes (Morris *et al.*, 2003). The genes *Axn, Kst, bun* and *tai* are important for the follicle cell development and migration: *Axn* is required in the regulation cascade responsible for maintaining normal proliferation and differentiation of follicle cells, and mutations in this

gene cause an over-proliferation of the follicle cells and failed encapsulation of the germline (Song, 2003); *Kst* is required for a normal apical contraction of the follicle cells during mid-oogenesis and ensures structured egg chambers (Zarnescu and Thomas, 1999); *bun* is a transcription factor responsible for the signaling in anterior follicle cells and is required to pattern the anterior eggshell structures during oogenesis (Dobens *et al.*, 2005); *tai* is required for border cell migration and is involved in ovarian stem cell niche formation and early germline differentiation by controlling cellular proliferation and death (Mathieu *et al.*, 2007; König *et al.*, 2011). By controlling cellular proliferation and death, *tai* controls the metabolic function that allows the egg stem cell niche to continue to function.

Another set of candidates identified by the GWAS is responsible for polarising the oocyte: *Raf*, *par-1* and *sog*. *Raf* is important in specifying the dorsoventral polarity of the egg, and it acts in the somatic follicle cells at stage 10 (Brand and Perrimon, 1994). The *par-1* gene has two roles in oogenesis, it is required early oogenesis in the organization of the microtubule cytoskeleton to ensure proper germline cyst polarity and subsequently oocyte differentiation (Cox *et al.*, 2001), and late in oogenesis it is required for oocyte anterior/posterior axis specification (Doerflinger *et al.*, 2006). *Sog* is expressed by follicle cells during mid-oogenesis (stage 8 to 10), where it is secreted into the perivitelline space and is stockpiled there for subsequent patterning during early embryogenesis; in addition *Sog* is differentially cleaved generating the dorsal–ventral asymmetry in embryos (Carneiro *et al.*, 2006).

Another key element to oogenesis that was identified by the GWAS was the gene *Msp300*, which is essential for transport of the cytoplasm from nurse cells to the oocyte during the late stages of oogenesis (Yu *et al.*, 2006). The gene *tin* is required for germ cell migration to find their target and ensure development of gonadal mesoderm (Moore *et al.*, 1998). Lastly, *mfr* and *prage* are involved in egg activation, process in which the egg become metabolically active. The *mfr* gene is expressed in ovaries, beside regulating the egg patterning and development, it is also involved in the development of maternally controlled stages in early embryogenesis (Smith and Wakimoto, 2007). The gene *prage* is involved in meiotic progression in early embryos (Tadros *et al.*, 2003).

Survival resistance

Sex Peptide is also known to influence female fitness and survival. Previous studies have shown that elevated and continual exposure to Sex Peptide in multiple matings decreases female fitness and lifespan (Wigby and Chapman, 2005; Mueller *et al.*, 2007). However, the mechanisms by which Sex Peptide reduces female survival are still unknown, as is the number of matings required to mediate costly effects. To understand how Sex Peptide affects starvation lifespan in single matings, we monitored the starvation survival of 32 DGRP isofemale lines after single matings to SP^0 or SP^+ males. We then performed a GWAS to associate starvation survival variation with genes that might modulate female starvation lifespan.

i. Starvation survival variation

Our data showed significant variation in starvation lifespan in the tested DGRP lines. Overall, Sex Peptide did not reduce female lifespan as is does following multiple matings in unlimited food conditions (Wigby and Chapman, 2005). In 20 DGRP lines, females that received Sex Peptide resisted starvation and outlived females that did not, while in 3 DGRP lines females showed higher survival during starvation when they had not received Sex Peptide. These results are consistent with previous studies, in some cases continual receipt of Sex Peptide reduces lifespan (Fowler and Partridge, 1989; Wigby and Chapman, 2005), and in others, the Sex Peptide effect on lifespan is diet dependent (Fricke *et al.*, 2010). Receipt of Sex Peptide has been shown to decrease survival upon repeated matings on standard diets. When diets are varied, there is a spectrum of effects from positive to negative on survival (Fricke *et al.*, 2010). In this study there was significant variation in the effect of Sex Peptide on survival. To understand further how female lifespan responds to receipt of Sex Peptide, it is important to understand the genes involved.

ii. GWAS

The GWAS associated 4 polymorphisms, located on the 2L, 3L and 3R, chromosomes, associated with the effect of Sex Peptide on female starvation survival. Of these polymorphisms two did not code for any known protein coding gene within 1kb up or downstream of their location. The 2 remaining polymorphisms (1 SNPs and 1 insertion) were linked with 2 protein coding genes *CG34027* and *daw*.

iii. Functional enrichment

The functional enrichment analysis with GeneMANIA and DAVID on the 2 candidate genes from the starvation survival resistance GWAS, revealed that gene *CG34027,* which codes for an integral component of membrane with unknown molecular and biological function. The gene *daw* is involved in many signaling pathways (growth factor beta receptor pathway, SMAD protein phosphorylation pathway, activin receptor signaling pathway, MAPK cascade and insulin secretion). One of the major biological processes that *daw* is known to be implicated in, is the determination of adult lifespan.

daw is an Activin-like ligand of the transforming growth factor beta pathway (TGF-β) superfamily. When the insulin/IGF signalling is reduced, dFOXO binds to the promotor region and transcriptionally represses *daw*. The inactivation of *daw* and of its downstream signaling partners *babo* and *Smox* extend lifespan in *D. melanogaster* (Bai *et al.*, 2013).

Drosophila female lifespan can be extended either by reducing diet, reducing insulin signalling (Broughton *et al.*, 2005), or by reducing mating frequency (Priest *et al.*, 2008). In this study, insulin signaling was reduced due to the starvation treatment, and only one mating occurred, which might be beneficial to trigger stress protective responses, but might be costly in the longer term. How Sex Peptide increase fitness in case of starvation is still unknown, we suggest that Sex Peptide could intervenes either in inhibiting the Activin pathway or reducing the insulin signaling pathway, whichever leading to increase in lifespan.

Conclusion

The results confirm that Sex Peptide increases female egg laying, reduces female receptivity and affects female survival following starvation. Overall, it is becoming clear that there is more than one female response to Sex Peptide effect, and instead a wide range of phenotypic variation in response to Sex Peptide, and a great number of genes involved. This phenotypic variation and the interpretation of the genetic variation and underpinning gene interactions represents a key step in furthering our understanding of Sex Peptide effects on females.

Acknowledgement

Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. We thank Emily Fowler, Elizabeth Duxbury, Janet Mason, Will Nash, Michael Bolton, Michael Darrington, Kris Sales, Stewart Leigh, Philip Leftwich, Naomi Clarke, Becky Lewis and Ben McDonald for practical help with mating experiments. This project was funded by the Ubbo Emmius Scholarship and the UEA School of Biological Sciences.

Authors and Contributors

JR, TC and BW conceived the study, JR conducted the research, JR, BW and WR analysed the data, JR wrote the chapter and JR, BW and TC revised the chapter.

Supplementary data

Latency and mating duration

For both the receptivity and the egg laying assays, we scored for each female the latency from introducing a male into the vial until the first mating, and the duration of the mating. The latency until the first mating was highly variable within and among lines, ranging from 0 min to 4 hours. The "lmer" analysis on the latency to first mating in the receptivity assay, showed that there is a significant variation of latency between lines (*Chisq*= 8.3262, *P*=0.003908) but overall, no Sex Peptide effect on the first latency (*Chisq*=0.3058, *P*=0.5803). As for the first mating duration in the receptivity assay showed no significant variation between the lines (*Chisq*=0, *P*=1) although a significant effect of Sex Peptide on duration (*Chisq*=8.0164, *P*=0.004636) (Supplementary Figure 1). In the egg laying assay, there was no significant effect of male genotype on mating latency or duration (latency: *Chisq*=0.1046, *P*=0.7464; duration: *Chisq*=5.5909, *P*=0.01805), whereas the lines themselves did differ significantly in overall latency to mating (*Chisq*=4.7876, *P*=0.02866) and in mating duration (*Chisq*=0, *P*=1) (Supplementary Figure 2). This indicates that the DGRP lines varied largely both within and among lines in latency to first mating, but that there was no significant difference in latency towards males transferring Sex Peptide. However, there was a significant difference in mating duration among DGRP lines when mated to either SP^{+}, SP^{0} or WT males.

Receptivity: first mating latency and duration

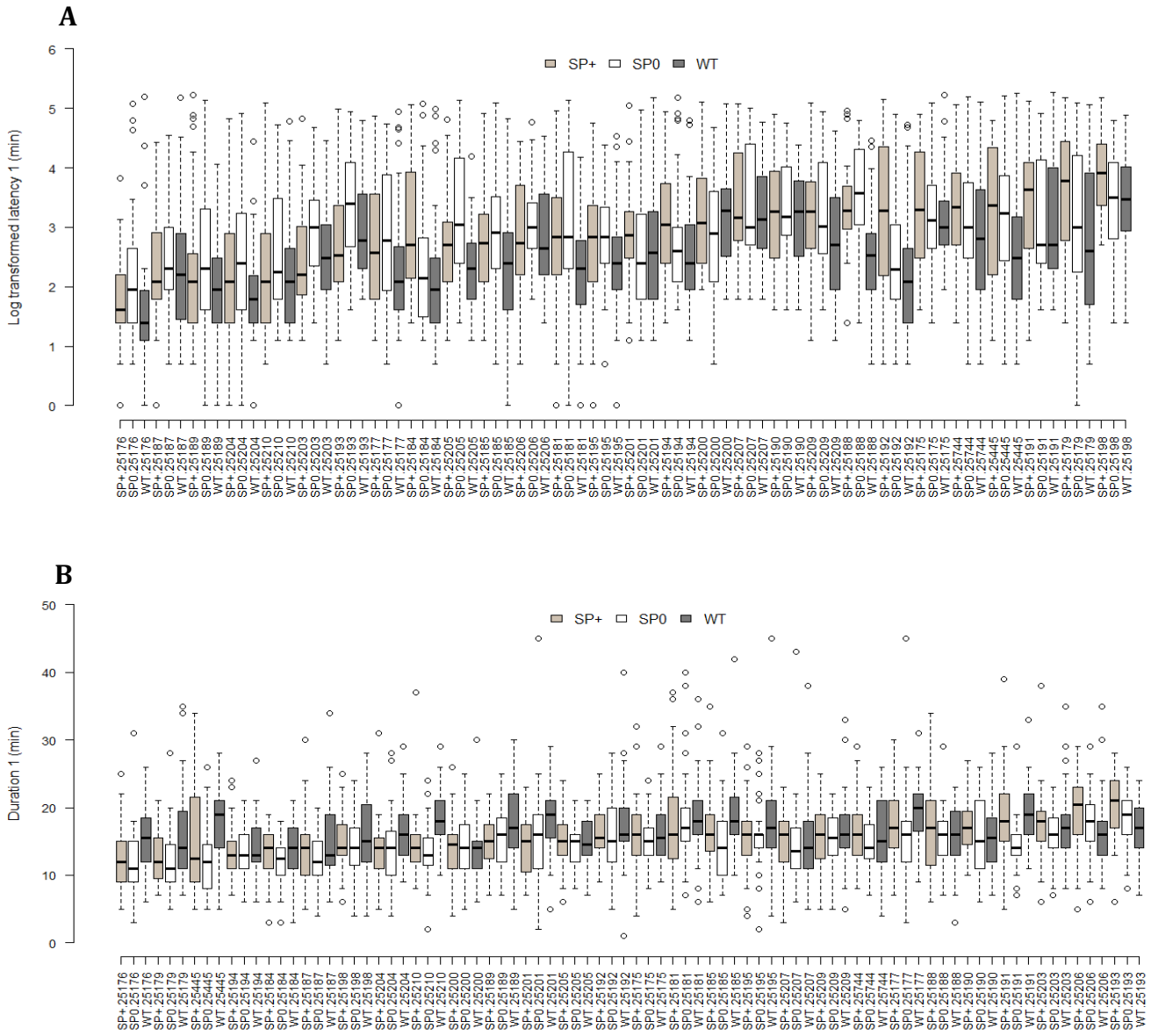
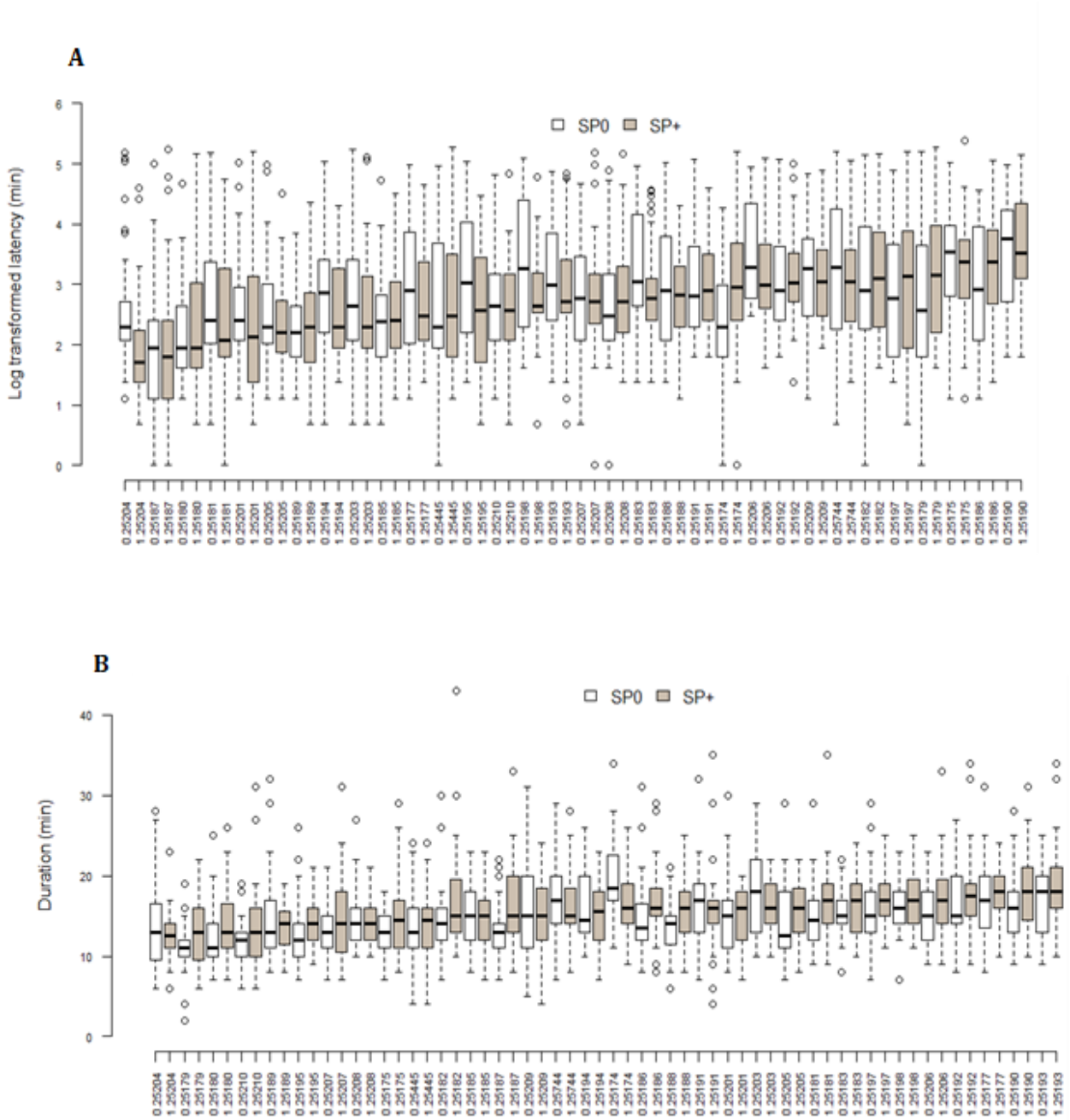


Figure 1: Mating latency and mating duration of females from 30 DGRP lines, when mated to SP⁺, SP⁰ and WT males. (A) Boxplots of mating latency (plotted on logarithmic scale) for DGRP females when mated to either SP⁰, SP⁺ or WT males. (B) Boxplots of mating duration of DGRP females mated to either SP⁰, SP⁺ or WT males. Median represented by horizontal line within box, with box representing the interquartile range (IQR) and whiskers the highest/lowest value within. Outliers represented by points.

Egg laying: mating latency and duration



<u>Figure 2:</u> Mating latency and duration of females from 32 DGRP lines when mated to either SP$^+$ and SP^0 males. (A) Boxplots of mating latency (plotted on logarithmic scale) when DGRP females mated either SP^0 or SP$^+$ males. (B) Boxplots of mating duration of DGRP females mated either SP^0 or SP$^+$ males. Median represented by horizontal line within box, with the box representing the interquartile range (IQR) and whiskers the highest/lowest value within. Outliers represented by points.

Table 1: Parameter estimates from models of remating and egg laying when females were mated to either SP^+ or SP^0 males.

Remating

Final model	Estimate	SE	z value	P
Intercept	-1.1522	0.2573	-4.479	7.51E-06
Male SP0	3.0452	0.2101	14.493	< 2e-16
Random effect				
Groups	**Name**	**Variance**	**SD**	
Date.Lines	MaleSP+	0.34324	0.5859	
	MaleSP0	0.19928	0.4464	
Date.Lines.1	Intercept	0.21627	0.465	
Date	MaleSP+	0.2397	0.4896	
	MaleSP0	0.02909	0.1706	

Family: binomial (logit)

Egg laying

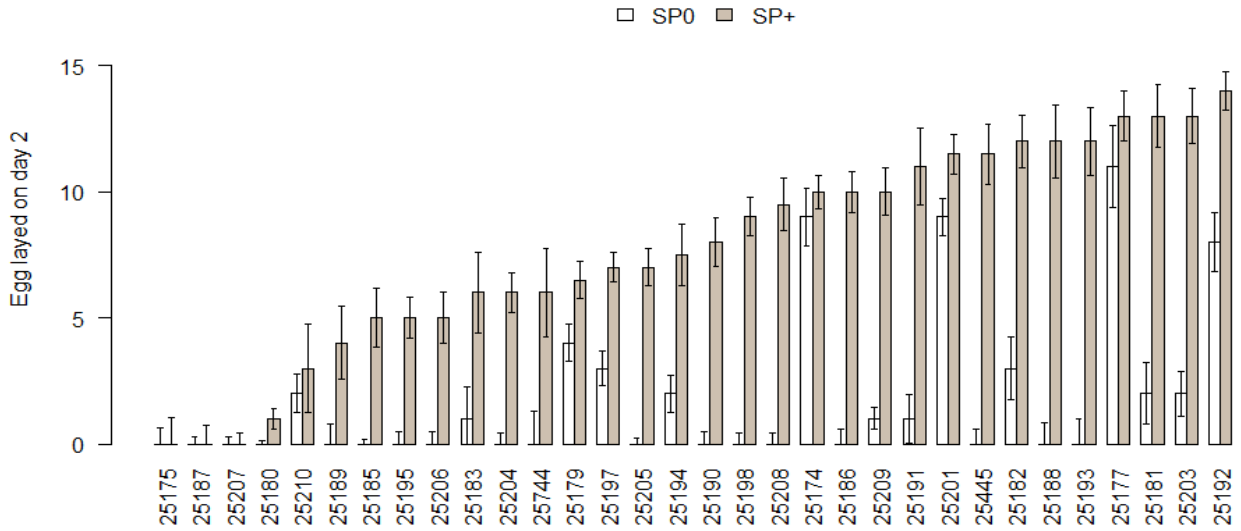
Final model	Estimate	SE	z value	P
Intercept	1.06009	0.33405	3.173	0.00151
SP	0.49308	0.04569	10.793	< 2e-16
weight	1.07145	0.2683	3.994	6.51E-05
Random effect				
Groups	**Name**	**Variance**	**SD**	
lines	SP	0.02676	0.1636	
lines.1	Intercept	0.05941	0.2437	
Date	Intercept	0.02635	0.1623	

Family: nbinom2 (log)

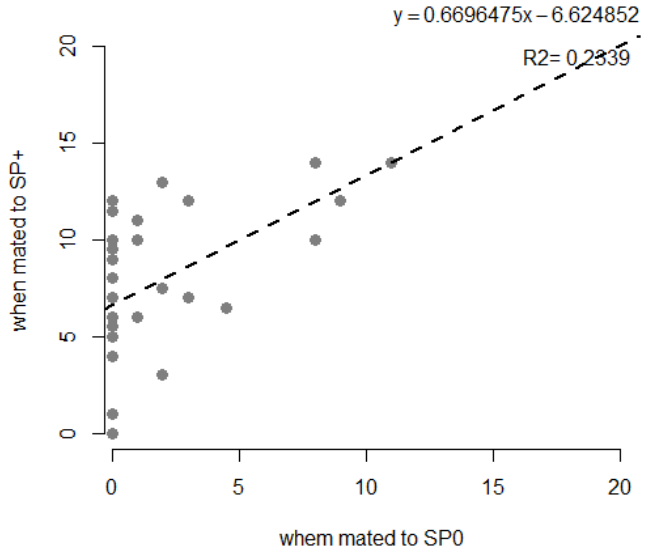
Table 2: GeneMANIA list of functional networks weighted by their ability to connect re-mating candidate genes to related genes, with their occurrence in the sample and in the genome, as analysed by GeneMANIA (Montojo *et al.*, 2010; Warde-Farley *et al.*, 2010). Coverage is the ratio of the number of annotated genes in the displayed network vs the number of genes with that annotation in the genome. The *q*-value is estimated using the Benjamini-Hochberg procedure. Categories are displayed up to a *q*-value cut-off of 0.1.

Description	q-value	Occurrences in Sample	Occurrences in Genome
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	9.19E-10	8	66
regulation of ERBB signaling pathway	0.002896	4	43
regulation of epidermal growth factor receptor signaling pathway	0.002896	4	43
ERBB signaling pathway	0.011836	4	69
epidermal growth factor receptor signaling pathway	0.011836	4	69
monocarboxylic acid biosynthetic process	0.026235	3	29
fatty acid biosynthetic process	0.026235	3	28
carboxylic acid biosynthetic process	0.118636	3	57
positive regulation of ERBB signaling pathway	0.118636	2	10
transmembrane receptor protein tyrosine kinase signaling pathway	0.118636	4	147

Egg laying day 2



<u>Figure 3</u>: Number of eggs laid by females of the DGRP lines on day 2 (24-48 hours) after mating to SP⁺ or SP⁰ males. Bar plot representing the median egg number laid by DGRP females in the period between 24 and 48 hours after mating to SP⁰ or SP⁺ males. Error bars indicate s.e.m.



<u>Figure 4</u>: Correlation of the number of eggs laid by females of the DGRP lines between 24-48 hours after mating to SP⁰ or SP⁺ males. Scatter plot representing a significant correlation (*P=0.005041*) between the number of eggs laid by DGRP females when mated either to SP⁺ males (Y axis) or SP⁰ males (X axis).

Egg laying day 1

Table 3: Functional annotation of top candidate genes from the increase of egg laying GWAS, according to the functional annotation analysis, using DAVID (Huang *et al*., 2009).

Gene	Annotation	Gene ontology
Axin	FBgn0026597	Phagocytosis, imaginal disc pattern formation, eye-antennal disc morphogenesis, imaginal disc-derived wing morphogenesis, heart development, regulation of cell death, Wnt signaling pathway, negative regulation of Wnt signaling pathway, somatic stem cell population maintenance, response to starvation, oogenesis
CG17121	FBgn0039043	General function prediction only, oxidoreductase activity
CG2253	FBgn0029992	Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay, neuromuscular synaptic transmission, RNA interference, gene silencing by miRNA, synapse organization
CG32677	FBgn0052677	Protein localization to membrane
CG34383	FBgn0085412	Sensory perception of pain
CG42260	FBgn0259145	Cation transport, cyclic-nucleotide-mediated signaling, regulation of membrane potential, transmembrane transport
CG43143	FBgn0262617	Protein phosphorylation
CG7906	FBgn0036417	Kazal domain
CG9993	FBgn0034553	Metabolic process
CenB1A	FBgn0039056	Cell morphogenesis, phagocytosis, regulation of GTPase activity
CCAP	FBgn0039007	Neuropeptide signaling pathway, positive regulation of heart rate, positive regulation of heart contraction
Nf-YA	FBgn0035993	Phagocytosis, R7 cell differentiation, positive regulation of transcription, DNA-templated, lateral inhibition, regulation of cell cycle
Tom40	FBgn0016041	Protein targeting to mitochondrion, ion transport, protein import into mitochondrial matrix, transmembrane transport, cellular response to hypoxia
mfr	FBgn0266757	Egg activation, fertilization, synaptic vesicle exocytosis, chorion-containing eggshell pattern formation, sperm plasma membrane disassembly, regulation of embryonic development, sperm capacitation
tin	FBgn0004110	Cell fate specification, regulation of transcription, DNA-templated, pole cell migration, ventral cord development, salivary gland morphogenesis, mesoderm development, mesodermal cell fate specification, gonadal mesoderm development, heart development, cardioblast cell fate determination, metamorphosis, germ cell migration, gonad development, cardioblast differentiation, embryonic heart tube development, cardiocyte differentiation, negative regulation of transcription, DNA-templated, positive regulation of transcription, DNA-templated, positive regulation of transcription from RNA polymerase II promoter, lymph gland development, embryonic anterior midgut (ectodermal) morphogenesis, cardiac muscle cell differentiation, neuroendocrine cell differentiation, pericardial nephrocyte differentiation, determination of digestive tract left/right asymmetry

Table 4: Functional enrichment table derived from the DAVID algorithm for the candidate genes generated by the GWAS, from the increase of egg laying in response to receipt of Sex Peptide. % is the percentage of total genes assigned to term; Count is the number of genes in analysis assigned to each term.

Category	Term	Count	%	P-Value	Benjamini
UP KEYWORDS	Coiled coil	6	42.9	1.10E-02	2.90E-01
INTERPRO	Pleckstrin homology-like domain	3	21.4	1.30E-02	4.10E-01
GOTERM_BP_DIRECT	Phagocytosis	3	21.4	1.80E-02	6.60E-01
GOTERM_BP_DIRECT	Heart development	2	14.3	5.70E-02	8.30E-01
INTERPRO	Pleckstrin homology domain	2	14.3	8.20E-02	8.20E-01
GOTERM_MF_DIRECT	GTPase activator activity	2	14.3	9.00E-02	9.00E-01
SMART	PH	2	14.3	9.80E-02	7.10E-01

Table 5: Functional annotation of top candidate genes from the egg relative change GWAS, according to the functional annotation analysis, using DAVID (Huang *et al*., 2009).

Gene	Annotation	Gene ontology
Acp54A1	FBgn0083936	Multicellular organism reproduction
AstA-R1	FBgn0266429	G-protein coupled receptor signaling pathway, phospholipase C-activating G-protein coupled receptor signaling pathway, neuropeptide signaling pathway, sensory perception of pain
CG11041	FBgn0034481	Calcium ion binding
CG11170	FBgn0034705	Regulation of signal transduction
Liprin-gamma	FBgn0034720	Axon target recognition, negative regulation of synaptic growth at neuromuscular junction
CG11836	FBgn0039272	Proteolysis
c12.2	FBgn0040234	ATP binding, ATPase activity
CG12535	FBgn0029657	Integral component of membrane
Sp1	FBgn0020378	Regulation of transcription, DNA-templated, imaginal disc-derived leg morphogenesis
lectin-21Cb	FBgn0040106	Carbohydrate binding
CG13722	FBgn0035553	Complete proteome, Reference proteome, Signal
CG13921	FBgn0035267	Integral component of membrane
CG14227	FBgn0031058	Proteolysis
CG14528	FBgn0039611	Posttranslational modification, protein turnover, chaperones
CG14720	FBgn0037940	Protein of unknown function DM4/12
CG15537	FBgn0039770	Hormone metabolic process
CG15765	FBgn0029814	Nervous system development
resilin	FBgn0034157	Chitin-based cuticle development
CG18088	FBgn0032082	Metabolic process, sensory perception of pain, multicellular organism reproduction
CG30456	FBgn0050456	Signal transduction mechanisms
CG30460	FBgn0050460	Zinc finger C2HC domain-containing protein
CG31191	FBgn0051191	Coiled coil
CG31427	FBgn0051427	Proteolysis
CG32115	FBgn0052115	Complete proteome, Reference proteome
CG32264	FBgn0052264	Actin cytoskeleton reorganization
CG32365	FBgn0052365	Complete proteome, Reference proteome
CG33203	FBgn0053203	Lateral inhibition
CG33519	FBgn0053519	Protein phosphorylation, adult somatic muscle development, regulation of Rho protein signal transduction, sarcomere organization
CG34043	FBgn0054043	Extracellular space
CG34347	FBgn0085376	Sensory perception of pain, actomyosin structure organization
CG34371	FBgn0085400	Sensory perception of pain
CG42238	FBgn0250867	Complete proteome, Reference proteome, Signal
CG42265	FBgn0259150	Integral component of membrane
prage	FBgn0261548	Integral component of membrane
CG42747	FBgn0261801	Integral component of membrane
CG43143	FBgn0262617	Protein phosphorylation
CG43347	FBgn0263072	Transcription, DNA-templated
CG43693	FBgn0263776	Amino acid transmembrane transport
CG43921	FBgn0264542	Regulation of establishment of planar polarity
CG4622	FBgn0035021	Nucleic acid binding, zinc ion binding
CG7029	FBgn0039026	Protein phosphatase 1 binding
CG7337	FBgn0031374	Mitotic spindle organization, regulation of mitotic cell cycle
par-1	FBgn0260934	Microtubule cytoskeleton organization, establishment of imaginal disc-derived wing hair orientation, negative regulation of protein phosphorylation, protein phosphorylation, actin filament organization, germarium-derived oocyte fate determination, border follicle cell migration, oocyte anterior/posterior axis specification, regulation of pole plasm oskar mRNA localization, pole plasm protein localization, regulation of cell shape, axis specification, anterior/posterior axis specification, oocyte differentiation, oocyte microtubule cytoskeleton organization, antimicrobial humoral response, establishment of cell polarity, regulation of Wnt signaling pathway, ovarian follicle cell development, border follicle cell delamination, regulation of polarized epithelial cell differentiation, positive regulation of hippo signaling, maintenance of protein location, pole plasm oskar mRNA localization, synaptic growth at neuromuscular junction, oocyte nucleus localization involved in oocyte dorsal/ventral axis specification, microtubule cytoskeleton organization involved in establishment of planar polarity,
CG9990	FBgn0039594	Defense mechanisms, Energy production and conversion / Inorganic ion transport and metabolism
CadN	FBgn0015609	Homophilic cell adhesion via plasma membrane adhesion molecules, axon guidance, axon target recognition, axonal fasciculation, ommatidial rotation, calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules, sensory perception of pain, retinal ganglion cell axon guidance, cell-cell adhesion mediated by cadherin, R8 cell development, R7 cell development, axon extension, regulation of dendrite morphogenesis, regulation of axon extension involved in axon guidance, axon extension involved in axon guidance, negative regulation of dendrite morphogenesis
chinmo	FBgn0086758	Regulation of transcription from RNA polymerase II promoter, imaginal disc-derived wing morphogenesis, mushroom body development, male somatic sex determination
Doa	FBgn0265998	MAPK cascade, regulation of alternative mRNA splicing, via spliceosome, startle response, protein phosphorylation, blastoderm segmentation, nervous system development, sex differentiation, visual perception, protein secretion, karyosome formation, locomotion involved in locomotory behavior, negative regulation of MyD88-dependent toll-like receptor signaling pathway, salivary gland cell autophagic cell death, compound eye photoreceptor development, protein autophosphorylation, autophagic cell death, oogenesis, compound eye development, brain morphogenesis, negative regulation of male germ cell proliferation
Dscam2	FBgn0265296	Homophilic cell adhesion via plasma membrane adhesion molecules, neuron projection morphogenesis
Fur1	FBgn0004509	Postsynaptic membrane organization, proteolysis, synaptic target recognition, protein processing, presynaptic membrane organization, glutamate receptor clustering
Fur2	FBgn0004598	Proteolysis, regulation of glucose metabolic process, protein processing, negative regulation of secretion
GstS1	FBgn0010226	Glutathione metabolic process, response to oxidative stress, metabolic process
HmgD	FBgn0004362	Chromatin organization, muscle organ development, dendrite morphogenesis
IA-2	FBgn0031294	Protein dephosphorylation, digestive tract development, regulation of secretion
LRP1	FBgn0053087	Cytosol, integral component of membrane

MED14	FBgn0035145	Regulation of transcription from RNA polymerase II promoter, transcription from RNA polymerase II promoter, transcription initiation from RNA polymerase II promoter, intracellular steroid hormone receptor signaling pathway
mgl	FBgn0261260	Regulation of endocytosis, chitin-based cuticle development, regulation of adult chitin-containing cuticle pigmentation
MtnC	FBgn0038790	Response to metal ion, metal ion homeostasis
Msp300	FBgn0261836	Cell division and chromosome partitioning, DNA replication, recombination, and repair
Nlg2	FBgn0031866	Lipid metabolism
Np	FBgn0265011	Proteolysis
Pde6	FBgn0038237	Signal transduction, negative regulation of nucleobase-containing compound transport, cGMP metabolic process
Pde8	FBgn0266377	Signal transduction, mesoderm development, sensory perception of pain, cAMP metabolic process
Raf	FBgn0003079	Instar larval development, signal transduction, epidermal growth factor receptor signaling pathway, spermatogenesis, border follicle cell migration, terminal region determination, gastrulation, primary branching, open tracheal system, wing disc morphogenesis, imaginal disc-derived wing morphogenesis, metamorphosis, dorsal/ventral axis specification, ovarian follicular epithelium, positive regulation of cell proliferation, torso signaling pathway, imaginal disc-derived wing vein morphogenesis, cellular response to starvation, negative regulation of macroautophagy, hemopoiesis, regulation of cellular pH, lamellocyte differentiation, wing and notum subfield formation, intracellular signal transduction, regulation of multicellular organism growth, hemocyte differentiation, positive regulation of photoreceptor cell differentiation, positive regulation of Ras protein signal transduction, protein autophosphorylation, regulation of cell cycle, positive regulation of ERK1 and ERK2 cascade, negative regulation of apoptotic signaling pathway
SNF4Agamma	FBgn0264357	Lipid metabolic process, autophagy, cellular response to starvation, regulation of glucose metabolic process, sequestering of triglyceride, behavioral response to starvation, cholesterol homeostasis, positive regulation of cell cycle, regulation of response to DNA damage stimulus
Swim	FBgn0034709	Proteolysis, immune response, cell adhesion, positive regulation of Wnt signaling pathway, sleep, positive regulation of Wnt signaling pathway by establishment of Wnt protein localization to extracellular region
Sobp	FBgn0033654	Compound eye development
Sply	FBgn0010591	Amino acid transport and metabolism
Spn	FBgn0010905	Inter-male aggressive behavior, actin filament organization, calcium-mediated signaling, neuron projection development, olfactory behavior
Vps35	FBgn0034708	Intracellular protein transport, endocytosis, sensory perception of pain, positive regulation of Wnt signaling pathway, regulation of protein stability, retrograde transport, endosome to Golgi, positive regulation of Wnt protein secretion, response to rotenone
arc	FBgn0000008	Compound eye development
bun	FBgn0259176	Compound eye photoreceptor cell differentiation, positive regulation of neuroblast proliferation, transcription, DNA-templated, regulation of transcription, DNA-templated, ovarian follicle cell migration, chorion-containing eggshell formation, peripheral nervous system development, imaginal disc-derived wing morphogenesis, decapentaplegic signaling pathway, positive regulation of cell proliferation, determination of adult lifespan, negative regulation of cell fate specification, mushroom body development, positive regulation of cell growth, sleep, ovarian follicle cell development, salivary gland cell autophagic cell death, segmentation, intestinal stem cell homeostasis, tissue regeneration, negative regulation of apoptotic process, negative regulation of Notch signaling pathway, dorsal appendage formation, autophagic cell death
cic	FBgn0262582	Negative regulation of transcription from RNA polymerase II promoter, transcription, DNA-templated, regulation of transcription, DNA-templated, terminal region determination, imaginal disc-derived wing vein specification, negative regulation of cell growth, negative regulation of transcription, DNA-templated, dorsal appendage formation, wing disc dorsal/ventral pattern formation, eye morphogenesis
dpr8	FBgn0052600	Sensory perception of chemical stimulus, synapse organization
forked	FBgn0262111	Sensory perception of sound, chaeta morphogenesis, epidermal cell differentiation, cuticle pattern formation, imaginal disc-derived wing hair organization, antennal morphogenesis, actin filament bundle assembly
Kst	FBgn0004167	Mitotic cytokinesis, endocytosis, plasma membrane organization, regulation of multivesicular body size involved in endosome transport, ovarian follicle cell development, endosome transport via multivesicular body sorting pathway, wound healing, zonula adherens assembly
mei-P26	FBgn0026206	Meiotic nuclear division, gamete generation, germ cell development, protein ubiquitination
mRpL28	FBgn0031660	Mitochondrial translation
nonC	FBgn0263968	Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay, DNA repair, response to stress, neuromuscular synaptic transmission, RNA interference, phosphorylation, peptidyl-serine phosphorylation, gene silencing by miRNA, protein autophosphorylation, synaptic vesicle transport, synapse organization
nub	FBgn0085424	Negative regulation of antibacterial peptide biosynthetic process, transcription, DNA-templated, regulation of transcription from RNA polymerase II promoter, pattern specification process, ganglion mother cell fate determination, ventral cord development, wing disc development, limb joint morphogenesis, dendrite morphogenesis
px	FBgn0003175	Inter-male aggressive behavior, imaginal disc-derived wing vein morphogenesis,
PH4alphaNE1	FBgn0039780	Oxidation-reduction process
svp	FBgn0003651	Compound eye photoreceptor fate commitment, transcription, DNA-templated, regulation of transcription, DNA-templated, neuron-neuron synaptic transmission, ventral cord development, R1/R6 cell fate commitment, R3/R4 cell fate commitment, R7 cell fate commitment, fat body development, heart development, cardioblast cell fate determination, visual perception, regulation of glucose metabolic process, neuroblast development, glial cell development, phototaxis, steroid hormone mediated signaling pathway, photoreceptor cell differentiation, epithelial cell proliferation involved in Malpighian tubule morphogenesis
sog	FBgn0003463	Multicellular organism development, maternal specification of dorsal/ventral axis, oocyte, soma encoded, zygotic determination of anterior/posterior axis, embryo, terminal region determination, amnioserosa formation, ectoderm development, torso signaling pathway, imaginal disc-derived wing vein morphogenesis, BMP signaling pathway, regulation of BMP signaling pathway, positive regulation of transforming growth factor beta receptor signaling pathway, negative regulation of transforming growth factor beta receptor signaling pathway, ring gland development, regulation of growth, posterior Malpighian tubule development
spg	FBgn0264324	Small GTPase mediated signal transduction, central nervous system development, R7 cell differentiation, activation of GTPase activity
sdt	FBgn0261873	Morphogenesis of a polarized epithelium, morphogenesis of an epithelium, cell-cell junction assembly, establishment or maintenance of cell polarity, establishment or maintenance of polarity of embryonic epithelium, chitin-based cuticle development, photoreceptor cell development, apical protein localization, zonula adherens assembly, establishment or maintenance of neuroblast polarity, establishment or maintenance of epithelial cell apical/basal polarity, lateral inhibition, bicellular tight junction assembly, establishment of epithelial cell polarity
tai	FBgn0041092	Transcription, DNA-templated, regulation of transcription, DNA-templated, border follicle cell migration, intracellular receptor signaling pathway, cellular response to hormone stimulus, positive regulation of growth, positive regulation of transcription from RNA polymerase II promoter, axon extension, germ-line stem-cell niche homeostasis
ths	FBgn0033652	Mesoderm development, larval visceral muscle development, larval somatic muscle development, fibroblast growth factor receptor signaling pathway, glial cell differentiation, glial cell development, myoblast migration
tutl	FBgn0010473	Axon guidance, axonal defasciculation, flight behavior, mechanosensory behavior, synaptic target recognition, adult locomotory behavior, axon midline choice point recognition, sensory perception of pain, larval behavior, R7 cell development, lateral inhibition, regulation of dendrite morphogenesis, dendrite self-avoidance
unk	FBgn0004395	Larval development, imaginal disc-derived wing morphogenesis, chaeta morphogenesis, neuron differentiation, compound eye development, regulation of neurogenesis
vfl	FBgn0259789	Mitotic cell cycle, cellularization, regulation of imaginal disc-derived wing size, positive regulation of transcription, DNA-templated

Table 6: Functional enrichment table derived from the DAVID algorithm for the candidate genes generated by the GWAS, from the egg laying ratio (increase over SP^0 level) as an effect of Sex Peptide receipt. % is the percentage of total genes assigned to each term; Count is the number of genes in analysis assigned to each term.

Category	Term	Count	%	P-Value	Benjamini
UP_KEYWORDS	Coiled coil	30	33	9.20E-08	7.30E-06
UP_SEQ_FEATURE	splice variant	14	15.4	4.80E-05	7.80E-03
INTERPRO	Insulin-like growth factor binding protein, N-terminal	5	5.5	1.00E-04	2.00E-02
GOTERM_CC_DIRECT	apical plasma membrane	5	5.5	8.10E-04	5.20E-02
UP_KEYWORDS	Alternative splicing	12	13.2	2.20E-03	8.50E-02
INTERPRO	Immunoglobulin subtype 2	5	5.5	7.50E-03	5.20E-01
INTERPRO	Immunoglobulin subtype	5	5.5	7.90E-03	4.10E-01
GOTERM_CC_DIRECT	plasma membrane	12	13.2	9.10E-03	2.60E-01
UP_KEYWORDS	Disulfide bond	11	12.1	1.00E-02	2.40E-01
INTERPRO	Fibronectin, type III	4	4.4	1.10E-02	4.10E-01
GOTERM_BP_DIRECT	terminal region determination	3	3.3	1.20E-02	9.80E-01
GOTERM_BP_DIRECT	protein autophosphorylation	3	3.3	1.20E-02	9.80E-01
GOTERM_MF_DIRECT	cytoskeletal protein binding	3	3.3	1.30E-02	7.00E-01
INTERPRO	Src homology-3 domain	4	4.4	1.30E-02	4.10E-01
SMART	FN3	4	4.4	1.50E-02	6.30E-01
INTERPRO	Immunoglobulin-like domain	5	5.5	1.50E-02	4.00E-01
INTERPRO	Epidermal growth factor-like domain	4	4.4	1.60E-02	3.60E-01
GOTERM_MF_DIRECT	Rho guanyl-nucleotide exchange factor activity	3	3.3	1.60E-02	5.30E-01
INTERPRO	EGF-type aspartate/asparagine hydroxylation site	3	3.3	1.90E-02	3.80E-01
INTERPRO	Peptidase S8, subtilisin, Asp-active site	2	2.2	2.10E-02	3.80E-01
INTERPRO	Proprotein convertase, P	2	2.2	2.10E-02	3.80E-01
INTERPRO	EGF-like calcium-binding	3	3.3	2.70E-02	4.20E-01
INTERPRO	Immunoglobulin V-set	3	3.3	2.80E-02	4.00E-01
INTERPRO	Six-bladed beta-propeller, TolB-like	3	3.3	3.00E-02	3.90E-01
GOTERM_MF_DIRECT	protein serine/threonine kinase activity	5	5.5	3.00E-02	6.10E-01
SMART	IGc2	5	5.5	3.10E-02	6.30E-01
GOTERM_BP_DIRECT	proteolysis	8	8.8	3.10E-02	9.90E-01
SMART	IG	5	5.5	3.20E-02	5.10E-01
UP_KEYWORDS	EGF-like domain	3	3.3	3.30E-02	4.90E-01
SMART	EGF	4	4.4	3.50E-02	4.40E-01
INTERPRO	Peptidase S8, subtilisin, Ser-active site	2	2.2	3.50E-02	4.20E-01
INTERPRO	Peptidase S8/S53 domain	2	2.2	3.50E-02	4.20E-01
INTERPRO	Peptidase S8, subtilisin-related	2	2.2	3.50E-02	4.20E-01
INTERPRO	Peptidase S8, subtilisin, His-active site	2	2.2	3.50E-02	4.20E-01
UP_KEYWORDS	Immunoglobulin domain	3	3.3	3.60E-02	4.50E-01
UP_KEYWORDS	Zinc-finger	7	7.7	3.60E-02	3.90E-01
INTERPRO	Immunoglobulin-like fold	5	5.5	3.70E-02	4.10E-01
GOTERM_CC_DIRECT	adherens junction	3	3.3	3.90E-02	5.90E-01
INTERPRO	3'5'-cyclic nucleotide phosphodiesterase	2	2.2	4.20E-02	4.30E-01
INTERPRO	Furin-like repeat	2	2.2	4.20E-02	4.30E-01
INTERPRO	3'5'-cyclic nucleotide phosphodiesterase, catalytic domain	2	2.2	4.20E-02	4.30E-01
GOTERM_MF_DIRECT	kinase activity	3	3.3	4.30E-02	6.40E-01
GOTERM_MF_DIRECT	3',5'-cyclic-nucleotide phosphodiesterase activity	2	2.2	4.40E-02	5.70E-01
GOTERM_BP_DIRECT	imaginal disc-derived wing vein morphogenesis	3	3.3	4.50E-02	9.90E-01
GOTERM_BP_DIRECT	compound eye development	4	4.4	4.70E-02	9.80E-01
INTERPRO	HD/PDEase domain	2	2.2	4.90E-02	4.60E-01
GOTERM_MF_DIRECT	actin binding	4	4.4	5.20E-02	5.60E-01
GOTERM_BP_DIRECT	synapse organization	3	3.3	5.30E-02	9.70E-01
UP_SEQ_FEATURE	compositionally biased region:Ser-rich	4	4.4	5.40E-02	9.90E-01
GOTERM_BP_DIRECT	inter-male aggressive behavior	3	3.3	5.40E-02	9.50E-01
GOTERM_BP_DIRECT	larval visceral muscle development	2	2.2	5.60E-02	9.20E-01
INTERPRO	Protein kinase-like domain	6	6.6	5.80E-02	5.00E-01
SMART	EGF_CA	3	3.3	5.80E-02	5.40E-01
GOTERM_BP_DIRECT	zonula adherens assembly	2	2.2	6.20E-02	9.20E-01
UP_SEQ_FEATURE	domain:Ig-like C2-type 5	2	2.2	6.20E-02	9.70E-01
UP_SEQ_FEATURE	DNA-binding region:HMG box	2	2.2	6.20E-02	9.70E-01
INTERPRO	Actinin-type, actin-binding, conserved site	2	2.2	6.20E-02	5.10E-01
INTERPRO	Spectrin/alpha-actinin	2	2.2	6.20E-02	5.10E-01
INTERPRO	Spectrin repeat	2	2.2	6.20E-02	5.10E-01
GOTERM_BP_DIRECT	mesoderm development	3	3.3	6.30E-02	9.00E-01
SMART	FU	2	2.2	6.40E-02	5.10E-01
UP_KEYWORDS	Serine protease	4	4.4	6.80E-02	5.50E-01
INTERPRO	LDLR class B repeat	2	2.2	6.90E-02	5.20E-01
INTERPRO	EGF-like, conserved site	3	3.3	7.00E-02	5.10E-01
INTERPRO	Protein kinase, catalytic domain	5	5.5	7.30E-02	5.10E-01
SMART	HDc	2	2.2	7.40E-02	5.10E-01
UP_SEQ_FEATURE	domain:Ig-like C2-type 4	2	2.2	7.50E-02	9.60E-01
INTERPRO	Immunoglobulin I-set	3	3.3	8.00E-02	5.30E-01
INTERPRO	PDZ domain	3	3.3	8.40E-02	5.30E-01
INTERPRO	Serine/threonine-protein kinase, active site	4	4.4	8.70E-02	5.30E-01
GOTERM_BP_DIRECT	sensory perception of pain	8	8.8	8.70E-02	9.40E-01
UP_KEYWORDS	Zinc	8	8.8	8.80E-02	6.00E-01
UP_SEQ_FEATURE	domain:Ig-like C2-type 3	2	2.2	8.80E-02	9.50E-01
GOTERM_MF_DIRECT	protein homodimerization activity	4	4.4	9.00E-02	7.20E-01
GOTERM_BP_DIRECT	transcription, DNA-templated	6	6.6	9.10E-02	9.30E-01
GOTERM_BP_DIRECT	axon target recognition	2	2.2	9.10E-02	9.20E-01
INTERPRO	Pleckstrin homology-like domain	4	4.4	9.20E-02	5.30E-01
SMART	SPEC	2	2.2	9.40E-02	5.50E-01
GOTERM_BP_DIRECT	adult somatic muscle development	2	2.2	9.70E-02	9.20E-01
GOTERM_BP_DIRECT	actin filament organization	3	3.3	1.00E-01	9.10E-01

Table 7: GeneMANIA list of functional networks weighted by their ability to connect candidate genes to other genes, with their occurrence in the sample and in the genome, as analysed by GeneMANIA (Montojo *et al.*, 2010; Warde-Farley *et al.*, 2010). Coverage is the ratio of the number of annotated genes in the displayed network vs the number of genes with that annotation in the genome. The *q*-value is estimated using the Benjamini-Hochberg procedure. Categories are displayed up to a *q*-value cut-off of 0.1.

Increase in egg laying due to Sex Peptide

Description	q-value	Occurrences in Sample	Occurrences in Genome
Organelle outer membrane	9.30E-04	4	20
Anion channel activity	9.30E-04	4	22
Mitochondrial outer membrane	9.30E-04	4	19
Outer membrane	9.30E-04	4	21
Voltage-gated channel activity	0.005441374	4	37
Voltage-gated ion channel activity	0.005441374	4	37
Mitochondrial transport	0.018593586	4	52
Gated channel activity	0.060338924	5	146
Ion channel activity	0.261354451	5	204
Substrate-specific channel activity	0.263329367	5	209

Fold change in egg laying due to Sex Peptide

Description	q-value	Occurrences in Sample	Occurrences in Genome
Phosphoric diester hydrolase activity	0.084329157	4	16
Plasma membrane region	0.085432007	6	70
Apical plasma membrane	0.085432007	5	42
Membrane region	0.153625891	7	120
Photoreceptor cell differentiation	0.190728946	8	174
Cyclic nucleotide metabolic process	0.28488655	4	33
Cell growth	0.45161132	6	110
Imaginal disc-derived wing vein morphogenesis	0.606372986	4	43
Regulation of cell growth	0.824426622	4	48
Apical part of cell	0.839628066	5	87

Table 8: The clustering of the candidate genes based on their peak of expression during the embryonic stages based on Flybase (Gramates *et al.*, 2017) and FlyAtlas (Chintapalli, Wang and Dow, 2007). Highlighted are the genes generated by the GWAS, on the increase of egg laying data as an effect of Sex Peptide; and the remaining are the genes generated by the GWAS for the fold change in egg laying as effect of Sex Peptide.

Embryonic stages							
0-6h		6-12h		12-18h		18-24h	
Axin	FBgn0026597	**CG2253**	FBgn0029992	**CG17121**	FBgn0039043	**CG17121**	FBgn0039043
CG2253	FBgn0029992	**CG34383**	FBgn0085412	**CG32677**	FBgn0052677	**CG42260**	FBgn0259145
CG34383	FBgn0085412	**Nf-YA**	FBgn0035993	**CG34383**	FBgn0085412	**CG34383**	FBgn0085412
CenB1A	FBgn0039056	**Tom40**	FBgn0016041	**CG42260**	FBgn0259145	**CG9993**	FBgn0034553
Tom40	FBgn0016041	**HmgD**	FBgn0004362	**Tom40**	FBgn0016041	**Tom40**	FBgn0016041
tin	FBgn0004110	**Sp1**	FBgn0020378	**CG11041**	FBgn0034481	**CG11041**	FBgn0034481
HmgD	FBgn0004362	**CG13921**	FBgn0035267	**chinmo**	FBgn0086758	**Liprin-gamma**	FBgn0034720
Fur1	FBgn0004509	**CG14528**	FBgn0039611	**CG11836**	FBgn0039272	**chinmo**	FBgn0086758
Fur2	FBgn0004598	**CG14720**	FBgn0037940	**c12.2**	FBgn0040234	**c12.2**	FBgn0040234
CG13921	FBgn0035267	**CG18088**	FBgn0032082	**Sp1**	FBgn0020378	**IA-2**	FBgn0031294
CG14227	FBgn0031058	**CG34371**	FBgn0085400	**Fur1**	FBgn0004509	**Fur1**	FBgn0004509
CG34371	FBgn0085400	**CG42238**	FBgn0250867	**Fur2**	FBgn0004598	**Fur2**	FBgn0004598
CG42238	FBgn0250867	**CG4622**	FBgn0035021	**CG14528**	FBgn0039611	**CG14528**	FBgn0039611
CG4622	FBgn0035021	**CG7337**	FBgn0031374	**CG14720**	FBgn0037940	**CG15537**	FBgn0039770
CG7337	FBgn0031374	**MED14**	FBgn0035145	**CG34347**	FBgn0085376	**CG15765**	FBgn0029814
MED14	FBgn0035145	**LRP1**	FBgn0053087	**CG34371**	FBgn0085400	**CG31191**	FBgn0051191
LRP1	FBgn0053087	**mgl**	FBgn0261260	**CG42265**	FBgn0259150	**CG42265**	FBgn0259150
Raf	FBgn0003079	**Raf**	FBgn0003079	**CG7029**	FBgn0039026	**CG7337**	FBgn0031374
Spn	FBgn0010905	**Sobp**	FBgn0033654	**CG7337**	FBgn0031374	**CG9990**	FBgn0039594
Vps35	FBgn0034708	**svp**	FBgn0003651	**CG9990**	FBgn0039594	**Pde6**	FBgn0038237
sog	FBgn0003463	**tutl**	FBgn0010473	**mgl**	FBgn0261260	**Swim**	FBgn0034709
ths	FBgn0033652	**unk**	FBgn0004395	**Pde6**	FBgn0038237	**Sobp**	FBgn0033654
unk	FBgn0004395	**vfl**	FBgn0259789	**Sobp**	FBgn0033654	**dpr8**	FBgn0052600
vfl	FBgn0259789	**CG32264**	FBgn0052264	**svp**	FBgn0003651	**svp**	FBgn0003651
CG32365	FBgn0052365	**tai**	FBgn0041092	**ths**	FBgn0033652	**tutl**	FBgn0010473
px	FBgn0003175	**px**	FBgn0003175	**tutl**	FBgn0010473	**unk**	FBgn0004395
par-1	FBgn0260934	**par-1**	FBgn0260934	**unk**	FBgn0004395	**CG32264**	FBgn0052264
CadN	FBgn0015609	**arc**	FBgn0000008	**CG32264**	FBgn0052264	**CG32365**	FBgn0052365
arc	FBgn0000008	**nub**	FBgn0085424	**CG32365**	FBgn0052365	**tai**	FBgn0041092
bun	FBgn0259176	**mRpL28**	FBgn0031660	**tai**	FBgn0041092	**px**	FBgn0003175
nub	FBgn0085424			**px**	FBgn0003175	**par-1**	FBgn0260934
mei-P26	FBgn0026206			**par-1**	FBgn0260934	**CadN**	FBgn0015609
mRpL28	FBgn0031660			**arc**	FBgn0000008	**bun**	FBgn0259176
				bun	FBgn0259176	**Kst**	FBgn0004167
				Kst	FBgn0004167	**mRpL28**	FBgn0031660
				mRpL28	FBgn0031660		

Table 9: The clustering of the GWAS candidate genes based Flybase information highlighted are the genes generated by the GWAS, on the increase of egg laying data as an effect of Sex Peptide. The remainder are genes generated by the GWAS for the fold change in egg laying as effect of Sex Peptide.

Developmental		Neural/ signaling pathway		Protein metabolism molecular function		Reproduction		Unknown	
Axin	FBgn0026597	**CG34383**	FBgn0085412	**CG17121**	FBgn0039043	**CG18088**	FBgn0032082	**CG7906**	FBgn0036417
CenB1A	FBgn0039056	**CCAP**	FBgn0039007	**CG2253**	FBgn0029992	**Acp54A1**	FBgn0083936	**CG11041**	FBgn0034481
mfr	FBgn0266757	**AstA-R1**	FBgn0266429	**CG32677**	FBgn0052677	**prage**	FBgn0261548	**c12.2**	FBgn0040234
tin	FBgn0004110	**CG11170**	FBgn0034705	**CG42260**	FBgn0259145			**CG12535**	FBgn0029657
Liprin-gamma	FBgn0034720	**CG34371**	FBgn0085400	**CG43143**	FBgn0262617			**lectin-21Cb**	FBgn0040106
Sp1	FBgn0020378	**CG9990**	FBgn0039594	**CG9993**	FBgn0034553			**CG13722**	FBgn0035553
CG15765	FBgn0029814	**CadN**	FBgn0015609	**Nf-YA**	FBgn0035993			**CG13921**	FBgn0035267
resilin	FBgn0034157	**GstS1**	FBgn0010226	**Tom40**	FBgn0016041			**CG14720**	FBgn0037940
CG30456	FBgn0050456	**MED14**	FBgn0035145	**CG11836**	FBgn0039272			**CG30460**	FBgn0050460
CG43921	FBgn0264542	**Pde6**	FBgn0038237	**CG14227**	FBgn0031058			**CG31191**	FBgn0051191
chinmo	FBgn0086758	**Pde8**	FBgn0266377	**CG14528**	FBgn0039611			**CG34043**	FBgn0054043
Doa	FBgn0265998	**Swim**	FBgn0034709	**CG42747**	FBgn0261801			**CG42238**	FBgn0250867
Dscam2	FBgn0265296	**dpr8**	FBgn0052600	**CG43143**	FBgn0262617			**CG34347**	FBgn0085376
Fur1	FBgn0004509	**nonC**	FBgn0263968	**CG43347**	FBgn0263072			**CG7029**	FBgn0039026
IA-2	FBgn0031294			**CG43693**	FBgn0263776			**CG31427**	FBgn0051427
mgl	FBgn0261260			**CG42265**	FBgn0259150			**CG32115**	FBgn0052115
Msp300	FBgn0261836			**CG4622**	FBgn0035021			**CG32365**	FBgn0052365
Nlg2	FBgn0031866			**CG7337**	FBgn0031374			**CG33203**	FBgn0053203
Sply	FBgn0010591			**Fur2**	FBgn0004598				
Sobp	FBgn0033654			**HmgD**	FBgn0004362				
Spn	FBgn0010905			**LRP1**	FBgn0053087				
a	FBgn0000008			**MtnC**	FBgn0038790				
bun	FBgn0259176			**Np**	FBgn0265011				
cic	FBgn0262582			**SNF4Agamma**	FBgn0264357				
forked	FBgn0262111			**Vps35**	FBgn0034708				
Kst	FBgn0004167			**mRpL28**	FBgn0031660				
mei-P26	FBgn0026206			**PH4alphaNE1**	FBgn0039780				
nub	FBgn0085424			**CG15537**	FBgn0039770				
px	FBgn0003175			**CG32264**	FBgn0052264				
svp	FBgn0003651								
sog	FBgn0003463								
spg	FBgn0264324								
sdt	FBgn0261873								
tai	FBgn0041092								
ths	FBgn0033652								
tutl	FBgn0010473								
unk	FBgn0004395								
par-1	FBgn0260934								
Raf	FBgn0003079								
CG33519	FBgn0053519								
vfl	FBgn0259789								

Table 10: GeneMANIA list of functional networks weighted by their ability to connect starvation lifespan candidate genes to related genes, with their occurrence in the sample and in the genome, as analysed by GeneMANIA (Montojo *et al.*, 2010; Warde-Farley *et al.*, 2010). Coverage is the ratio of the number of annotated genes in the displayed network vs the number of genes with that annotation in the genome. The *q*-value is estimated using the Benjamini-Hochberg procedure. Categories are displayed up to a *q*-value cut-off of 0.1.

Description	q-value	Occurrences in Sample	Occurrences in Genome
Transforming growth factor beta receptor signaling pathway	8.44E-11	7	30
Response to transforming growth factor beta	8.44E-11	7	32
Cytokine receptor binding	8.44E-11	6	13
Cellular response to transforming growth factor beta stimulus	8.44E-11	7	32
Response to growth factor	1.64E-09	7	50
Cellular response to growth factor stimulus	1.64E-09	7	49
Transmembrane receptor protein serine/threonine kinase signaling pathway	1.66E-08	7	70
Cellular response to endogenous stimulus	1.75E-07	7	99
Cellular response to organic substance	1.71E-06	7	141
Response to endogenous stimulus	1.71E-06	7	139
Receptor binding	7.06E-06	7	175
Response to organic substance	8.32E-06	8	292
Cellular response to chemical stimulus	2.16E-05	7	213
Enzyme linked receptor protein signaling pathway	2.16E-05	7	212
BMP signaling pathway	1.23E-04	4	31
Chemotaxis	0.0015086	6	252
Embryonic morphogenesis	0.0162117	5	224
Neuron projection guidance	0.0216025	5	241
Morphogenesis of embryonic epithelium	0.0216025	4	118
Renal tubule morphogenesis	0.0240414	3	43
Imaginal disc-derived wing vein morphogenesis	0.0240414	3	43
Malpighian tubule morphogenesis	0.0240414	3	43
Formation of organ boundary	0.0263882	3	45
Embryonic hindgut morphogenesis	0.0284459	3	47
Morphogenesis of an epithelium	0.0284459	5	273
Formation of anatomical boundary	0.0360997	3	52
Organ formation	0.036252	3	54
Hindgut morphogenesis	0.036252	3	54
Hindgut development	0.036252	3	54
Eye-antennal disc development	0.0456981	3	59
Regulation of organ formation	0.0489341	2	11
Malpighian tubule development	0.0489341	3	63
Positive regulation of transmembrane receptor protein serine/threonine kinase signaling Pathway	0.0489341	2	11
Digestive tract morphogenesis	0.0489341	3	63
Renal tubule development	0.0489341	3	63
Renal system development	0.0643586	3	71
Urogenital system development	0.0643586	3	71
Chaperone binding	0.0724536	2	14
Ovarian follicle cell stalk formation	0.0724536	2	14
Dendrite morphogenesis	0.0736291	4	196
Dendrite development	0.0761352	4	199
Regulation of muscle organ development	0.0885508	2	16

Discussion

Jessy Rouhana

5

The fundamental key to fitness is reproduction, whereby males and female cooperate to successfully producing offspring. Even though this cooperation is fruitful for both, it often masks sexual conflicts, whereby males and females have optimised different strategies to maximise their reproductive success, potentially at the cost of the other sex. These conflicts can lead to evolutionary arm races between the sexes over a number of traits, such as the control of reproduction, parental investment and resources (Parker, 2006). Consequently, the results of this conflict can be cycles of adaptation followed by counteradaptation between the two sexes (Chapman *et al.*, 2003).

Across taxa, males release both sperm and seminal fluid proteins inside the female's body during mating. The effects of seminal fluid proteins can benefit both sperm and egg functions, but intriguingly they can also favour the interests of males whilst generating costs in females, resulting in sexual conflict (Chapman *et al.*, 1995; Wedell *et al.*, 2002; Kelly and Jennions, 2011; Liberti, Baer and Boomsma, 2018). In *Drosophila melanogaster*, the female body can represent the arena for the battlefield of sexual conflict, as seminal fluid proteins exert their effects in females after mating. This manipulation by males through molecular interactions can inflict substantial physical and physiological costs of mating in females. One enigmatic seminal fluid protein the 'Sex Peptide', generates strikingly diverse changes in female physiological and reproductive behaviour (Liu and Kubli, 2003; Wigby and Chapman, 2005; Gioti *et al.*, 2012). Sex Peptide triggers remarkable female post mating responses including altered fertility, immunity, libido, eating and sleep patterns, by the activation of diverse sets of genes (Chen *et al.*, 1988; Chapman *et al.*, 2000; Heifetz *et al.*, 2000; Lung *et al.*, 2002; Ram *et al.*, 2005; Carvalho *et al.*, 2006; Avila and Wolfner, 2009; Isaac *et al.*, 2010).

In many studies of the molecular mechanisms of female manipulation via the effects of Sex Peptide, genetic variation is minimised to clearly delineate biological functions. However, to understand the evolutionary processes and dynamics that characterise Sex Peptide mediated interactions between males and females, it is important to study this genetic variation. With high-throughput sequencing technologies that have provided resources such as >200 fully sequenced DGRP lines (*Drosophila* Genome Reference Panel)(MacKay *et al.*, 2012; Mackay and Huang, 2018), we traced the impact of the enigmatic Sex Peptide on the fruitfly genome.

In this thesis I performed an in-depth investigation of the phenotypic and genomic differences among a core set of 30-32 DGRP lines, with respect to male release of, and female responses to,

Sex Peptide. I measured phenotypic variation for Sex Peptide release in males; and in females the phenotypic variation in immune responses, egg laying, receptivity and starvation lifespan in response to Sex Peptide receipt. I compared these phenotypic post-mating responses to those of females that mated to males with a null-allele for Sex Peptide, to distinguish the specific response to Sex Peptide. I mapped these phenotypes to genomic variation using Genome Wide Association Studies and conducted functional characterizations on the genomic variation identified.

In chapter 2, we developed and successfully employed a novel quantification method, the immuno-Q-PCR. Using this, we detected significant variation among 31 DGRP lines in Sex Peptide release in males to wild type Dahomey females during mating. Our study showed no significant variation in mating latency or mating duration between males from 31 DGRP lines, indicating this variation in Sex Peptide transfer was not mediated by differences in mating behaviour among the lines. To search for genetic variations that were associated with variation in Sex Peptide release, we conducted a GWAS. This analysis yielded significant associations between Sex Peptide release and a set of 54 candidate genes. An extensive gene ontology search revealed that these top candidate genes clustered within the following functional categories: development, membrane, protein and RNA processing and reproduction. A literature search on the reproductive gene cluster showed that four of these genes were seminal fluid proteins. Some have yet unidentified functions; two are cyclic nucleotide phosphodiesterase that seem to be involved in male fertility and female mating behaviour; some are involved in germ cell development in males and/or in females; others are uniquely expressed in male testis and/or accessory gland protein but have unknown molecular and biological functions. Our study highlighted new candidate genes not detected by any other methods and that might show novel associations with Sex Peptide in determining reproduction and post-mating gene expression in females.

Chapter 3 revealed that mating and the transfer of Sex Peptide can induce the expression of several AMP genes in females, and that there was significant phenotypic variation in these responses among lines. The induction of, and variation in, AMP gene expression was recorded in isogenic lines of two different *D. melanogaster* populations (French and DGRP). The lines differed both in whether or not they induced the expression of AMPs after mating, and the

extent to which they did so after receipt of Sex Peptide. Immune gene expression was not always upregulated in response to Sex Peptide. For some lines it was even down-regulated in females mated to SP^+ compared to virgin and/or female mated to SP^0 males. In other lines Sex Peptide had no effect at all, or none in addition to the response to mating itself. Furthermore, there were also differences among the three immune genes tested in detail, with those being regulated by the Imd pathway (*Dpt-B, Mtk*) being more responsive to Sex Peptide than the gene (*IM1*) under the regulatory control of the Toll pathway. The GWAS performed on the variation in expression of the antimicrobial AMPs in response to Sex Peptide in the DGRP population identified 13 candidate genes for *Mtk* (Toll and Imd pathway), 51 candidate genes for *Dpt-b* (Imd pathway) and 38 candidate genes for *IM1* (Toll pathway). The network analysis indicated that the majority of these genes are part of different networks, which suggests that most have several different functions in the organism, one role of which could be direct or indirect modulation of the immune response. For all these candidate genes, genetic variation was significantly associated with variation in the expression of AMPs after mating or Sex Peptide receipt. The functional annotation revealed that 8 of these candidate genes code for immunoglobulin superfamily proteins, and 8 modulate the Imd immune pathway, with 6 of these showing negative regulation.

Chapter 4 showed that across the tested DGRP lines, the transfer of Sex Peptide had a clear overall effect to significantly reduce of re-mating, increase egg laying and increase in lifespan. However, the extent of these effects varied significantly across lines. This phenotypic variation in response to Sex Peptide was tracked through GWAS, revealing a set of genes involved for each of these phenotypes. For receptivity, 2 candidate genes were identified by the GWAS, of which one regulates the JAK/Stat pathway. There is, however, no clear link to how these genes may interfere in reducing the receptivity. For egg laying a total of 104 candidate were identified by the GWAS, where by 13 of these genes show direct involvement in the development and the regulation of egg laying, and half of the rest are highly expressed in early embryonic stages. Finally, the GWAS performed on the starvation survival hazard ratio revealed 2 candidate genes, of which *daw* is known to determine adult lifespan. These results confirm the pleiotropic effect of Sex Peptide in influencing female post mating responses. In single matings Sex Peptide receipt extended starvation survival, showing the the

effects of Sex Peptide on lifespan depend upon ther frequency of Sex Peptide receipt and the nutritional status of the female.

Male "Sex Peptide" variation

Studies of sexual conflict have been important in providing direct evidence on how males can potentially influence female reproductive processes and this has led to the identification of several mechanisms involved in sperm competition and post-mating sexual selection (Parker, 1970). Sexually antagonistic co-evolution between males and females has occurred over time in which each sex tries to maximize their reproductive success. Sexual conflict occurs over the sexually antagonistic effects of seminal fluid proteins in *D. melanogaster.* These male made proteins are transferred to females during mating and influence female sexual behavior (Sirot *et al.*, 2015). One of these male-derived molecules, Sex Peptide, represents a "master regulator" of female physiology and reproduction (Liu and Kubli, 2003; Chapman *et al.*, 2003). To understand the pace, dynamics and trajectory of co-evolution arising from this potential manipulation of gene expression in one sex by the other, I started characterising the variation in the males' release of Sex Peptide to females during mating, by first quantifing the amount of Sex Peptide that males produce and transfer to females during mating, and then associating this to genomic variation. Aiming for more detailed understanding of the male variation in Sex Peptide's release to females, and identification of candidate genes that are putatively involved in the regulation of this variation (**chapter 2**).

Genetic variation within the Sex Peptide gene and in the upstream region has been previously identified (Cirera and Aguade, 1997), this could represent variation in regulatory *cis*-regions influenceing Sex Peptide, which could result in altered levels of production of Sex Peptide. Additionally, male *D. melanogaster* vary in Sex Peptide gene expression level has been shown (Smith *et al.*, 2009). However, in the GWAS that I performed on the variation in male transfer of Sex Peptide to females, Sex Peptide itself was not among the candidate genes. This might suggest that the variation in Sex Peptide transfer is not primarily due to variation in production *per se*, but to variation in the allocation or transfer of the produced Sex Peptide to females. To test whether males genetically vary in how much Sex Peptide they produce, I would have needed to also measure and compare the Sex Peptide in unmated males. However, as production and accumulation of Sex Peptide is known to be plastic (e.g. dependent on social

context (Wigby *et al.*, 2009)) and may vary with maturation and aging (Bonduriansky, 2014), such assays would need to be performed under stringently controlled conditions and preferably across a time course and/or various conditions. With the technique that I developed, this may now be feasible.

With the new protein quantification methods developed in chapter 2, I was able to detect significant variation in Sex Peptide release among 31 DGRP lines. Through a GWAS, this phenotypic variation in Sex Peptide release was associated to sequence variations in several genes. Of these genes, we identified 12 candidates with putative direct links to Sex Peptide that are highly expressed in male reproductive tissue. Four of these genes are seminal fluid proteins; two are cyclic nucleotide phosphodiesterase that seem to be involved in male fertility and female mating behaviour; some are involved in germ cell development in males; and others are uniquely expressed in male testis and/or accessory gland protein but have unknown molecular and biological function. These results reveal potentially new genes that might regulate Sex Peptide transfer and determine the extent of its post-mating responses in females. Intriguingly, a functional homologue of Sex Peptide has been isolated, Dup99B (Ductus ejaculatorious peptide cytological region 99B) (Saudan *et al.*, 2002), which has arisen from a gene duplication (Kubli, 2003). Both Sex Peptide and Dup99B genes contain an intron at the exact same site. In addition, Sex Peptide and Dup99B show a high sequence similarity in the C-terminal part, where each have a cyclic C-terminal part (a disulfide bridge between amino acids in position 24 and 36 and 19 and 31, respectively), and where 10 out of 12 amino acid are identical (Figure 1). The C-terminal parts is needed for the binding of Dup99B and Sex Peptide to specific sites in the nervous system and in the genital tract of female and are essential for eliciting the post-mating responses once transferred to mated females (Schmidt *et al.*, 1993; Saudan *et al.*, 2002; Kubli, 2003). Both proteins increase oviposition and egg laying and reduce receptivity to future mating (Ding *et al.*, 2003). In our assay the antibody was raised against a specific region of Sex Peptide not shared by Dup99B (WPWNRKPTKF), therefore we could only detect Sex peptide. Our research revealed the variation in Sex Peptide transfer might also involve other genes, although more research is needed to confirm a role of these genes in regulating the Sex Peptide transfer. We could speculate that the same genes may also be involved in regulating the transfer of Dup99B, or alternatively, focus on the differences between Dup99B and Sex Peptide (in the N-terminal part).

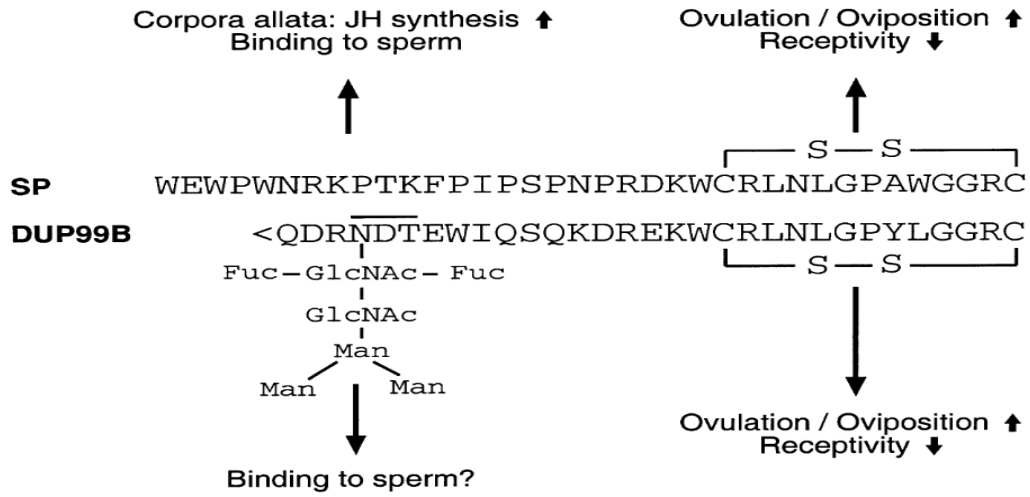


Figure 1: Compilation of known and assumed functions of DUP99B and SP. Results from in vitro and in vivo experiments. Some of the functions may be shared by the two peptides but based on different structures, some may be performed by both peptides with almost identical structures and some functions are unique to Sex Peptide. After Saudan *et al*., 2002.

Female variation in response to "Sex Peptide"

Sex Peptide is a key component in the male strategy for sexual conflict in *D. melanogaster*. Once in females, Sex Peptide manipulates female physiology and reproductive behavior, including increased egg laying, increased food intake, slowed intestinal transit and water balance, altered immunity, reduced sleep patterns, reduced sexual receptivity to re-mating and increased aggression (Manning, 1967; Chen et al., 1988; Liu and Kubli, 2003; Carvalho et al., 2006; Barnes et al., 2008; Isaac et al., 2010; Ribeiro and Dickson, 2010; Isaac, Kim and Audsley, 2014; Bath et al., 2017). The study of female post-mating responses to Sex Peptide has often been constraint to one line or strain, in order to accurately measure the effects of Sex Peptide. However, by doing so, the evolution of, and variation in, female resistance to the Sex Peptide has not/never been systematically measured. This leaves a gap in our understanding, as it is becoming increasingly clear that sexually antagonistic selection generated by males' manipulative trait, such as Sex Peptide, has shaped female resistance and generated a striking phenotypic variation and genetic response in retaliation.

This wide variation in female response to Sex Peptide has been shown in **chapter 3 and chapter 4.** When it comes to Sex Peptide effect on immunity **(chapter 3)**, a significant phenotypic variation in the induction of the immune responses among lines was shown. Sex Peptide did not always result in the changed transcription of AMP genes. Therefore, the canonical assumption that Sex Peptide always activates the innate immune response in *D. melanogaster* is incorrect. In some lines, neither mating nor the receipt of Sex Peptide induced the expression of AMPs, while in other lines, mating without the receipt of Sex Peptide induced an equally strong activation of immune responses as mating with the receipt of Sex Peptide. Similar phenotypic variations were observed in response to Sex peptide, for female receptivity, egg laying and starvation lifespan (**chapter 4**). Overall, Sex Peptide significantly reduced female re-mating, increased female egg laying and increased female lifespan, but the extent of these effects varied significantly across lines. For the receptivity, females who received Sex Peptide had lower to very low re-mating rates compared to females that did not, with the re-mating rates ranging from 3% to 80%. The variation in the increased egg laying in response to receipt of Sex Peptide ranged from, on average, 5 eggs/day to 30 eggs/day. The magnitude of the effects of Sex Peptide receipt on lifespan also varied greatly in the tested DGRP lines, resulting in no effects, reduced or higher survival. The general increase in starvation longevity after receipt of Sex Peptide in single matings contrasts with the decrease in longevity seen after continual Sex Peptide receipt during the whole lifetime and under ad libitum food conditions (Wigby and Chapman, 2005; Mueller *et al*., 2007). Overall, this shows that the effect of Sex Peptide on lifespan is likely to depend upon the level of Sex Peptide receibed and the nutitional status of the female.

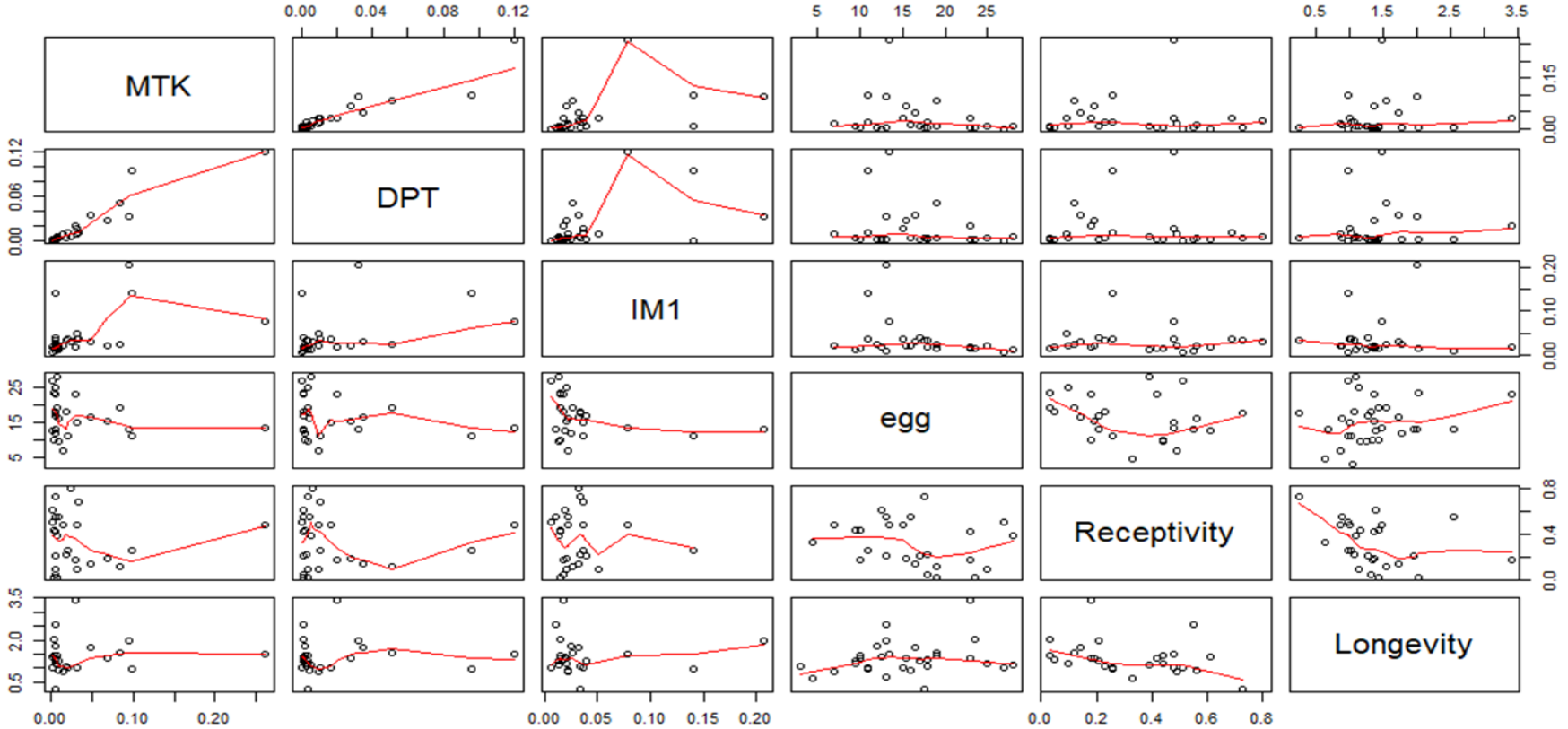
My findings show striking variation in the effects of Sex Peptide on various post-mating responses in females. A fascinating question is whether the various post-mating responses are correlated, either positively or negatively. Is it the same genotypes that strongly respond to, or "resist", the effects of Sex Peptide in the various phenotypic traits? Additionally, or alternatively, are there trade-offs, such that the genotypes with a strong response in one post-mating aspect tend to respond less in another post-mating response? To analyse this, I tested for correlations between the variation in different phenotypic responses to the receipt of Sex Peptide (Figure 2). Only within the immune gene expression, there were significant positive correlations, with a significant correlation in the expression of *Mtk* and *Dpt-B* (*F= 185,*

P=7.338e-14), *Mtk* and *IM1* (*F= 7.361, P=0.01127*) and *Dpt-B* and *IM1* (*F=7.447, P= 0.01085*), following Sex Peptide receipt. Thus, the lines that responded most strongly to Sex Peptide in the expression of one AMP also responded strongly in the other AMPs, while lines that had weak AMP expression following Sex Peptide receipt were consistently weak in the responses for all the AMPs tested. This indicated that some lines were immune-responsive overall to Sex Peptide, contributing to the significant phenotypic variation observed. However, for the remaining phenotypic traits, there were no significant correlations detected in the effects of Sex Peptide receipt. The uncoupling of the effects of Sex Peptide receipt on different post-mating traits might indicate that female responses could be subject to several different, or divergent, selection forces, or that Sex Peptide acts through different molecular pathways to generate different outcomes.

To better understand the mechanisms underlying the phenotypic and genetic variation in the post-mating responses to Sex Peptide, I performed GWAS analyses to generate a list of candidate genes that show polymorphisms correlated with the phenotypic differences for each of the phenotypic traits. Overall, the GWAS identified 104 genes that were associated with an effect of Sex Peptide on egg laying, 94 genes that were associated with the effect of Sex Peptide on immune gene expression 2 genes for Receptivity and 2 genes as effect of Sex Peptide on starvation lifespan (Figure 3). When combined, only 4 genes (*dpr8, mgl, bun* and *Dscam2*) were shared among different candidate gene lists: these 4 were associated with the phenotypic variation in the response to Sex Peptide on both eggs laying and immune gene expression. This overlap is larger than what you expect from chance, based on the number of genes in both gene lists and the total number of genes in the *D. melanogaster* genome (hypergeometric test, *P* = 0.001). At this stage, it is unknown whether and how these genes may be involved in the phenotypic variation in egg laying and immune gene expression, and how they may be affected following Sex Peptide receipt. Importantly, though, this analysis also reveals that many different candidate genes are associated with the various phenotypic responses to Sex Peptide.

I also performed a gene network mapping, using the GeneMANIA app in Cytoscape (Montojo *et al.*, 2010; Warde-Farley *et al.*, 2010), combining all the 203 genes identified by the GWASs for the variation in egg laying, AMP expression, re-mating and starvation lifespan. This analysis reveals that many of these genes might be part of one big gene interaction network.

Most of the gene-gene associations in the network were based on databases that catalogue patterns of co-expression (64.26%), while some of the gene-gene interactions reflect predictions based on orthologs in other species (15.13%), reported physical interaction between proteins (10.71%), co-localisation of the proteins (4.56%), genetic interactions (2.90%) and shared protein domains (2.44%) (Figure 4). The predictions of this gene network could suggest that many of the GWAS-identified genes in this thesis are connected through different types of interaction. Interestingly, Sex Peptide and the known Sex Peptide Receptor (SPR) do not feature in this predicted gene interaction network. The GWAS detects variation in SNPs between the DGRP lines tested. The fact that we didn't detect Sex Peptide or SPR as candidate SNPs in our analyses could arise due to several reasons (i) these two genes are genetically conserved in the tested DGRP lines, but their expression is not, (ii) the alleles that regulate the expression of these loci show up in the GWAS but not the target loci themselves, (iii) or the expression of Sex Peptide and SPR are regulated by non coding RNA, that recent study have shown there involvement in reproduction (Fricke *et al.*, 2014).



<u>Figure 2:</u> Correlation matrix of the post-mating responses to Sex Peptide. This matrix correlates the phenotypic responses to Sex Peptide receipt in the expression of 3 immune genes (*Mtk, Dpt-B, IM1*), egg laying, receptivity and starvation lifespan in 30-32 tested DGRP lines.

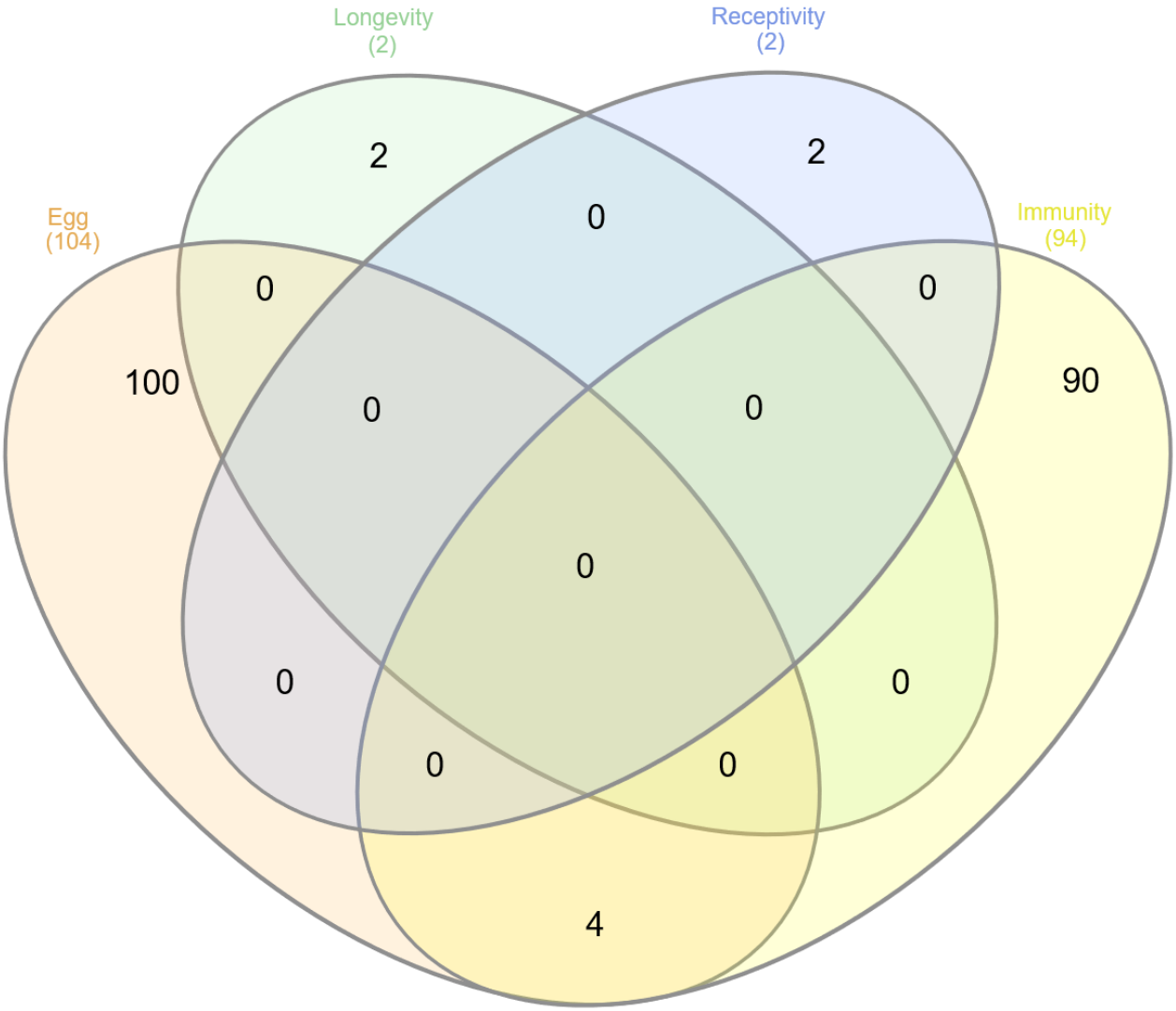
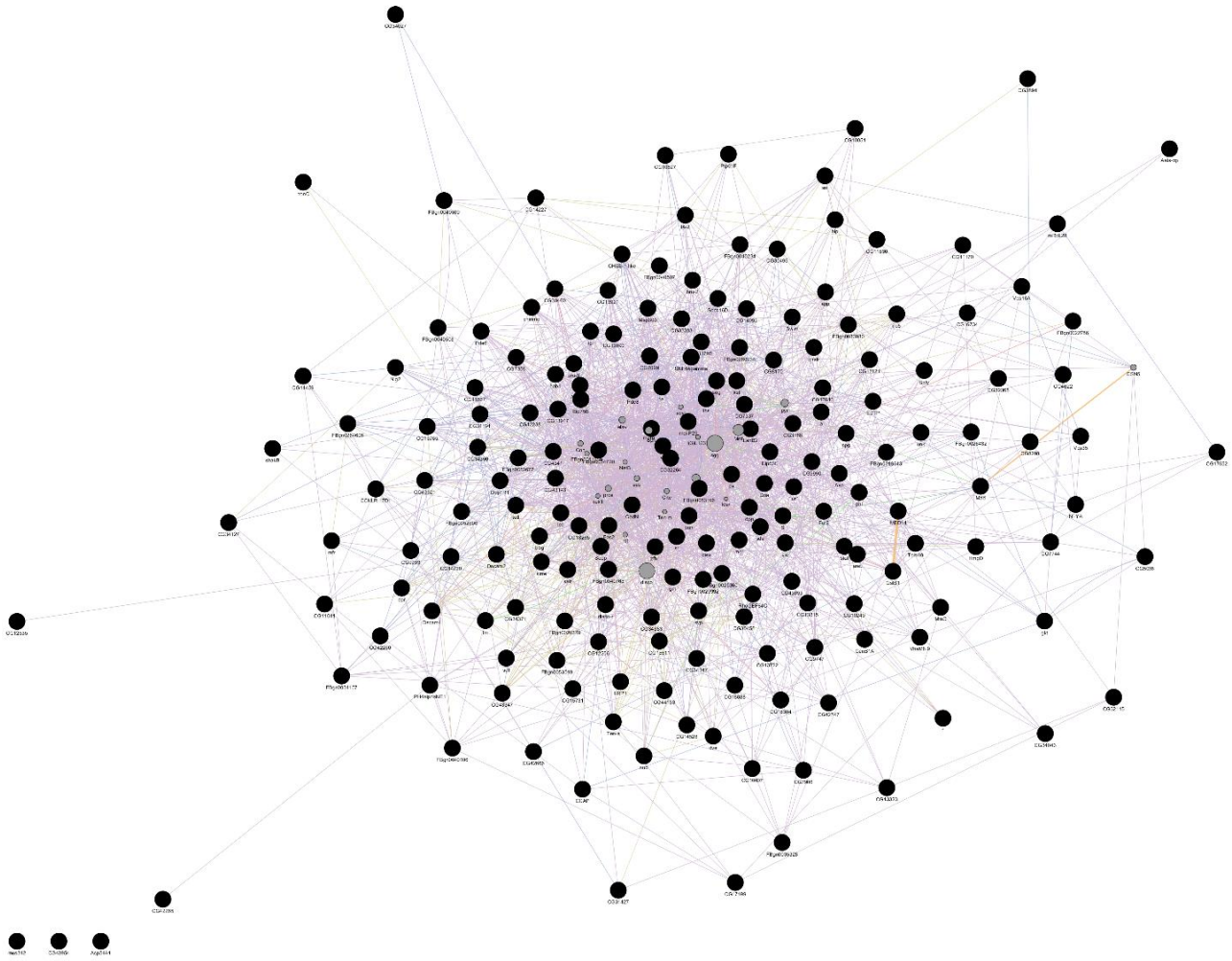


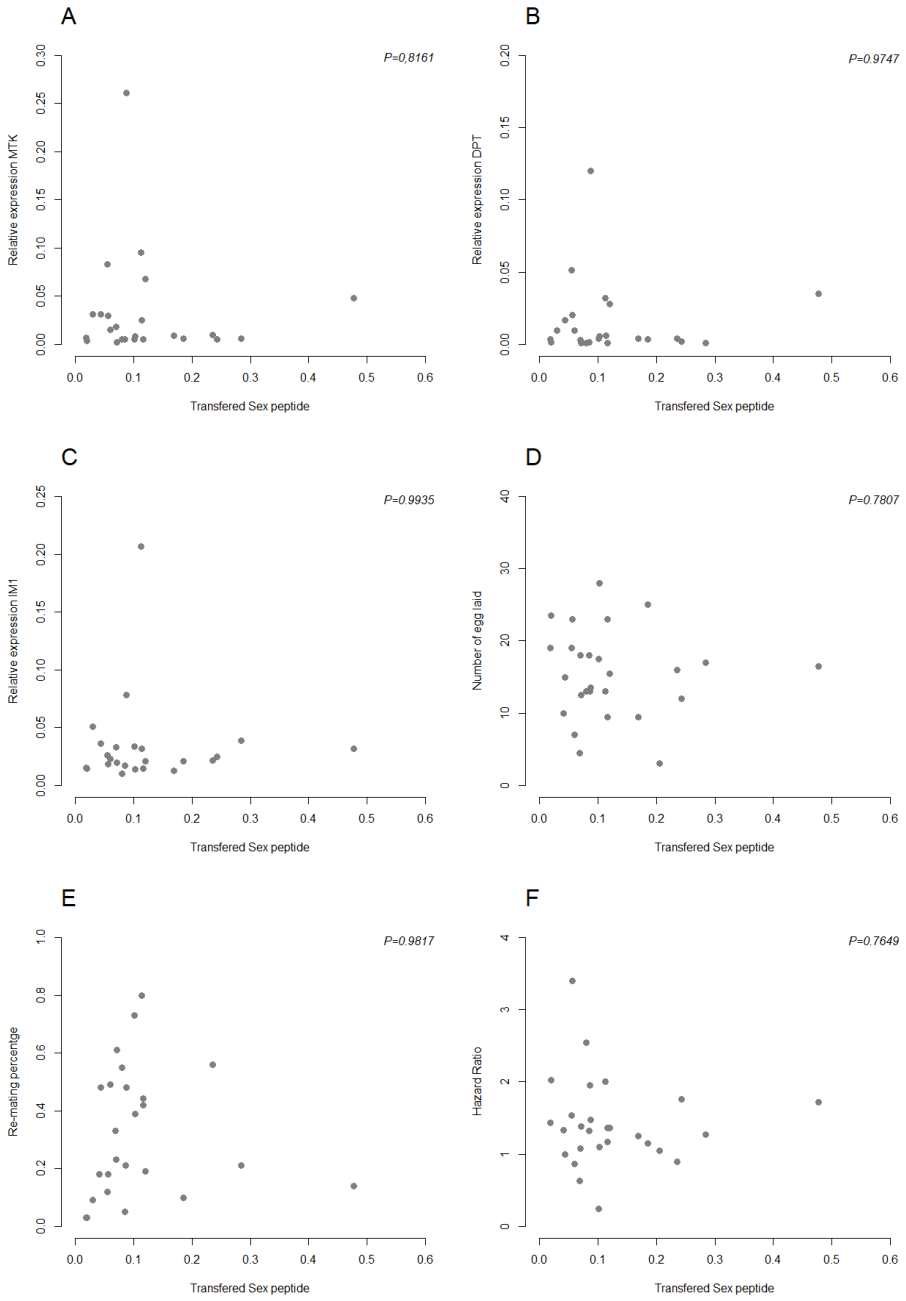
Figure 3: Venn diagram representing the candidate genes that were identified in GWASs on the variation in immune gene expression, egg laying, receptivity and starvation lifespan in response to Sex Peptide release. The Venn diagram was drawn using interactivenn (Heberle *et al.*, 2015).



<u>Figure 4:</u> Interaction network showing the relationships between the candidate genes resulted by GWASs, for egg laying, immunity, receptivity and starvation lifespan responses to Sex Peptide. Interaction networks of candidate genes identified by the GWAS for egg laying, immunity, receptivity and starvation lifespan, when females were mated to SP^+ males. Black nodes depict candidate genes generated by the GWAS with significant SNPs from the DGRP analysis (Query genes). Grey nodes are other genes that are related to a set of input candidate genes (Non-query genes). The links representing the networks in this case are based 64.26% on co-expression, 15.13% predicted, 10.71% physical interactions, 4.56% co-localisations, 2.90% genetic interactions and 2.44% shared protein domains.

Male Sex Peptide transfer and female responses

The variation in Sex Peptide transfer by males was not correlated to the extent of any of the female post-mating responses (Figure 5). This could suggest that females may show variation in their responses, or even resistance, to Sex Peptide, but that this is not reflecting the variation in the amount of Sex Peptide they would typically receive from males within their line, or a strong genetic correlation between Sex Peptide transfer in males and post-mating responses to Sex Peptide in females. The quantification of Sex Peptide transfer occurred immediately after mating: females were instantly frozen in order to make the most precise measurement on male Sex Peptide transfer. However, by doing so we may have removed the potential for females to also exert some form of control on the amount of Sex Peptide they retain or utilize. Before Sex Peptide can act on the female's reproductive behaviour and physiology, females eject a great number of sperm and seminal fluid proteins, along with the mating plug (Laturney, 2016). The remaining sperm are stored in the spermathecae, and then the Sex Peptide is cleaved and released in female circulatory system over a period of time (Peng *et al.*, 2005). Thus, our measurements reflect accurately how much the males transfer in their first mating, and we showed that the DGRP lines significantly varied in the amount of Sex Peptide they transferred. Yet, this does not capture how much the females retain or utilize, nor does it correlate with the female's post mating responses. The latter was perhaps also not to be expected, as the DGRP lines were generated by setting up a large collection of isofemale lines and making them isogenic by repeated sib crossings; this provides a snap-shot of the available genetic variation across DGRP lines but would leave little scope for sexual selection within DGRP lines to modulate the male or female strategies.



<u>Figure 5:</u> Correlation between male Sex Peptide transfer and female phenotypic responses to the receipt of Sex Peptide in the DGRP lines. For each DGRP line, the amount of Sex Peptide that males transferred to females and the effect on various female post-mating responses (when mated to SP^+ and SP^0 males) were determined. These plots reflect the correlation within DGRP lines between the male and female traits. Correlation between the variation among DGRP lines in male transfer of Sex Peptide, and female responses after the receipt of Sex Peptide for (A) *Mtk* relative expression; (B) *DPT* relative expression; (C) *IM1* relative expression; (D) the increase in numbers of egg laid; (E) re-mating rate after 24 hours; and (F) the hazard ratio under starvation conditions.

Conclusion

After years of matchmaking, counting, collecting, mating and scrutinizing every aspect of the reproductive life of *Drosophila melanogaster*, we now know more than ever the importance of sexual selection and the evolution co-associations that have emerged from the tug of war between males and females. At the same time, in most of these studies, the variation between the costs and benefits of antagonistic interactions has been overlooked. Yet, this variation is important for the dynamics and trajectory of co-evolution that arise from the potential manipulation one sex by the other. For the first time, I studied this variation in a sexually antagonistic trait, in males and females, and my results are reported in this thesis.

The results of this thesis suggest there is more variation in female responses and male transfer of Sex Peptide then previously realised. The magnitude of the Sex Peptide effect on females post-mating traits (immunity, egg laying, receptivity and lifespan) varied greatly across the tested DGRP lines. Similar variation was also detected in male Sex Peptide transfer. This offers evidence that there is no overall consistent pattern, for both males and females, there is scope for evolution, as there is both genetic variation and several different opportunities for selection. Moreover, the absence of a correlation between male Sex Peptide transfer and female post-mating behaviours in response to Sex Peptide shows that these are not inherently genetically correlated. Importantly, even though Sex Peptide has major effects on the females post-mating behaviour, reproduction and physiology, female differ largely in how strong this effect of Sex Peptide is on different aspects of their reproductive lives, perhaps reflecting varying levels of sensitivity or resistance to Sex Peptide. All these findings emphasise the importance of studying different populations and different genetic backgrounds, to better understand the selection pressures that shape reproductive traits in males and females.

Our results also highlighted many new candidate genes that may be involved in the production, release and/or transfer of Sex Peptide, and in the female responses to Sex Peptide. More work is now needed to understand the exact role and the involvement of these genes in the Sex Peptide pathway.

Bibliography

Aigaki, T. *et al.* (1991) 'Ectopic expression of sex peptide alters reproductive behavior of female D. melanogaster', *Neuron*, 7(4), pp. 557–563. doi: 10.1016/0896-6273(91)90368-A.

Alberts, S. C., Watts, H. E. and Altmann, J. (2003) 'Queuing and queue-jumping: Long-term patterns of reproductive skew in male savannah baboons, Papio cynocephalus', *Animal Behaviour*, 65(4), pp. 821–840. doi: 10.1006/anbe.2003.2106.

Alcock, J. (2005) *Animal behavior: An evolutionary approach, 8th ed.*, *Animal behavior: An evolutionary approach, 8th ed.* Sunderland, MA, US: Sinauer Associates.

Andersson and W. Simmons, L. (2006) 'Sexual selection and mate choice', *Trends in Ecology and Evolution*, 21. Available at: https://ac.els-cdn.com/S0169534706001029/1-s2.0-S0169534706001029-main.pdf?_tid=edd23d02-e130-433f-8d76-110ed6174a83&acdnat=1520256696_727b803f52230f7f4093b8c8b0472c3e.

Andersson, J., Borg-Karlson, A.-K. and Wiklund, C. (2000) 'Sexual cooperation and conflict in butterflies: a male-transferred anti-aphrodisiac reduces harassment of recently mated females', *Proceedings of the Royal Society B: Biological Sciences*, 267(1450), pp. 1271–1275. doi: 10.1098/rspb.2000.1138.

Andersson, M. (1994) *Sexual Selection*, *Princeton University Press*. Princeton University Press.

Andersson, M. and Iwasa, Y. (1996) 'Sexual selection', *Trends in Ecology and Evolution*, pp. 53–58. doi: 10.1016/0169-5347(96)81042-1.

Andolfatto, P. (2005) 'Adaptive evolution of non-coding DNA in Drosophila', *Nature*, 437(7062), pp. 1149–1152. doi: 10.1038/nature04107.

Arbouzova, N. I. (2006) 'JAK/STAT signalling in Drosophila: insights into conserved regulatory and cellular functions', *Development*, 133(14), pp. 2605–2616. doi: 10.1242/dev.02411.

Arnqvist, G. (1998) 'Comparative evidence for the evolution of genitalia by sexual selection', *Nature*, 393(6687), pp. 784–786. doi: 10.1038/31689.

Arnqvist, G. and Nilsson, T. (2000) 'The evolution of polyandry: Multiple mating and female fitness in insects', *Animal Behaviour*, 60(2), pp. 145–164. doi: 10.1006/anbe.2000.1446.

Arnqvist, G. and Rowe, L. (2002) 'Antagonistic coevolution between the sexes in a group of insects', *Nature*, 415(6873), pp. 787–789. doi: 10.1038/415787a.

Avila, F. W. *et al.* (2010) 'Sex Peptide is Required for the Efficient Release of Stored Sperm in Mated Drosophila Females', *Genetics*, p. genetics.110.119735. doi: 10.1534/genetics.110.119735.

Avila, F. W. *et al.* (2011) 'Insect Seminal Fluid Proteins: Identification and Function', *Annual Review of Entomology*, 56(1), pp. 21–40. doi: 10.1146/annurev-ento-120709-144823.

Avila, F. W., Wong, A., *et al.* (2015) 'Don't pull the plug! the Drosophila mating plug preserves fertility', *Fly*. doi: 10.1080/19336934.2015.1120931.

Avila, F. W., Cohen, A. B., *et al.* (2015) 'Retention of ejaculate by Drosophila melanogaster females requires the male-derived mating plug protein PEBme', *Genetics*, 200(4), pp. 1171–1179. doi: 10.1534/genetics.115.176669.

Avila, F. W., Mattei, A. L. and Wolfner, M. F. (2015) 'Sex peptide receptor is required for the release of stored sperm by mated Drosophila melanogaster females', *Journal of Insect Physiology*, 76, pp. 1–6. doi: 10.1016/j.jinsphys.2015.03.006.

Avila, F. W. and Wolfner, M. F. (2009) 'Acp36DE is required for uterine conformational changes in mated Drosophila females', *Proceedings of the National Academy of Sciences*, 106(37), pp. 15796–15800. doi: 10.1073/pnas.0904029106.

Baer, B. *et al.* (2009) 'The seminal fluid proteome of the honeybee Apis mellifera', *Proteomics*. doi: 10.1002/pmic.200800708.

Bai, H. *et al.* (2013) 'Activin Signaling Targeted by Insulin/dFOXO Regulates Aging and Muscle Proteostasis in Drosophila', *PLoS Genetics*, 9(11). doi: 10.1371/journal.pgen.1003941.

Barnes, A. I. *et al.* (2008) 'Feeding, fecundity and lifespan in female Drosophila melanogaster', *Proc Biol Sci*, 275(1643), pp. 1675–1683. doi: 10.1098/rspb.2008.0139.

Barribeau, S. M. and Schmid-Hempel, P. (2017) 'Sexual healing: mating induces a protective immune response in bumblebees', *Journal of Evolutionary Biology*, 30(1), pp. 202–209. doi: 10.1111/jeb.12964.

Bastock, M. and Manning, A. (1955) 'The Courtship of Drosophila Melanogaster', *Behaviour*, 8(1), pp. 85–110. doi: 10.1163/156853955X00184.

Bateman, A. J. (1948) 'Intra-sexual selection in Drosophila', *Heredity*, 2(3), pp. 349–368. doi: 10.1038/hdy.1948.21.

Bates, D. *et al.* (2014) 'Fitting Linear Mixed-Effects Models using lme4', 67(1). doi: 10.18637/jss.v067.i01.

Bath, E. *et al.* (2017) 'Sperm and sex peptide stimulate aggression in female Drosophila', *Nature Ecology & Evolution*. Macmillan Publishers Limited, part of Springer Nature., 1(6), p. 0154. doi: 10.1038/s41559-017-0154.

Baumann, U. and Angst, J. (1975) *Methodological development of the AMP system, EXCERPTA MEDICA, AMSTERDAM*. Available at: http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L6176973%5Cnhttp://sfx.library.uu.nl/utrecht?sid=EMBASE&issn=&id=doi:&atitle=Methodological+development+of+the+AMP+system&stitle=EXCERPTA+MEDICA%2C+AMSTERDAM&title=&volume=ICS+No.+35.

Becalska, A. N. and Gavis, E. R. (2009) 'Lighting up mRNA localization in Drosophila oogenesis', *Development*, 136(15), pp. 2493–2503. doi: 10.1242/dev.032391.

Beccari, S., Teixeira, L. and Rørth, P. (2002) 'The JAK/STAT pathway is required for border cell migration during Drosophila oogenesis', *Mechanisms of Development*, 111(1–2), pp. 115–123. doi: 10.1016/S0925-4773(01)00615-3.

Beckage, N. E. (2008) *Insect Immunology*. San Diego: Academic Press. doi: https://doi.org/10.1016/B978-012373976-6.50001-X.

Bergman, C. M. and Haddrill, P. R. (2015) ‘Strain-specific and pooled genome sequences for populations of Drosophila melanogaster from three continents . [v1 ; ref status : indexed , http://f1000r.es/515] Referee Status ’:, 31, pp. 1–5. doi: 10.12688/f1000research.6090.1.

Berkey, C. D., Blow, N. and Watnick, P. I. (2009) ‘Genetic analysis of Drosophila melanogaster susceptibility to intestinal Vibrio cholerae infection’, *Cellular Microbiology*, 11(3), pp. 461–474. doi: 10.1111/j.1462-5822.2008.01267.x.

Bertram, M. J. *et al.* (1992) ‘Cell type-specific gene expression in the Drosophila melanogaster male accessory gland.’, *Mechanisms of development*, 38, pp. 33–40. doi: 10.1016/0925-4773(92)90036-J.

Bertram, M. J., Neubaum, D. M. and Wolfner, M. F. (1996) ‘Localization of the Drosophila male accessory gland protein Acp36DE in the mated female suggests a role in sperm storage’, *Insect Biochemistry and Molecular Biology*, 26(8–9), pp. 971–980. doi: 10.1016/S0965-1748(96)00064-1.

Bertrand, P. V. *et al.* (2006) ‘Akaike Information Criterion Statistics.’, *Journal of the Royal Statistical Society. Series A (Statistics in Society)*. doi: 10.2307/2983028.

Bess, C. *et al.* (2012) ‘The Drosophila melanogaster’, pp. 3–8. doi: 10.1038/nature10811.

Billeter, J.-C. and Levine, J. D. (2015) ‘The role of cVA and the Odorant binding protein Lush in social and sexual behavior in Drosophila melanogaster’, *Frontiers in Ecology and Evolution*. doi: 10.3389/fevo.2015.00075.

Billeter, J. *et al.* (2012) ‘Drosophila melanogaster females change mating behaviour and offspring production based on social context Drosophila melanogaster females change mating behaviour and offspring production based on social context’, (February). doi: 10.1098/rspb.2011.2676.

Billeter, J. C. and Wolfner, M. F. (2018) ‘Chemical cues that guide female reproduction in *Drosophila melanogaster*’, *Journal of Chemical Ecology*. Journal of Chemical Ecology, pp. 1–20. doi: 10.1007/s10886-018-0947-z.

Birkhead, T. R. *et al.* (1999) ‘Sperm mobility determines the outcome of sperm competition in the domestic fowl Sperm mobility determines the outcome of sperm competition in the domestic fowl’, (September). doi: 10.1098/rspb.1999.0843.

Bolker, B. M. (2016) ‘Tools for general maximum likelihood estimation’, *Cran R Project*. Available at: https://cran.r-project.org/package=bbmle.

Bonduriansky, R. (2014) ‘Sexual conflict, life span, and aging’, *Cold Spring Harbor Perspectives in Biology*. doi: 10.1101/cshperspect.a017566.

Bonnay, F. *et al.* (2013) ‘big bang gene modulates gut immune tolerance in Drosophila’, *Proceedings of the National Academy of Sciences*, 110(8), pp. 2957–2962. doi: 10.1073/pnas.1221910110.

Boorman, E. and Parker, G. A. (1976) ‘reproductive value of females to males in relation to female age and mating status’, pp. 145–155.

Brand, A. H. and Perrimon, N. (1994) ‘Raf acts downstream of the EGF receptor to determine

dorsoventral polarity during Drosophila oogenesis', *Genes and Development*, 8(5), pp. 629–639. doi: 10.1101/gad.8.5.629.

Bretman, A. *et al.* (2011) 'Males use multiple, redundant cues to detect mating rivals', *Current Biology*, 21(7), pp. 617–622. doi: 10.1016/j.cub.2011.03.008.

Bretman, A., Fricke, C. and Chapman, T. (2009) 'Plastic responses of male Drosophila melanogaster to the level of sperm competition increase male reproductive fitness', *Proceedings of the Royal Society B: Biological Sciences*, 276(1662), pp. 1705–1711. doi: 10.1098/rspb.2008.1878.

Brieger, G. and Butterworth, F. M. (1970) 'Drosophila melanogaster: Identity of male lipid in reproductive system', *Science*, 167(3922), p. 1262. doi: 10.1126/science.167.3922.1262.

Brooks, M. E. *et al.* (2017) 'glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling', *R Journal*, 9(2), pp. 378–400. doi: 10.3929/ETHZ-B-000240890.

Broughton, S. J. *et al.* (2005) 'Longer lifespan, altered metabolism, and stress resistance in Drosophila from ablation of cells making insulin-like ligands', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.0405775102.

Bryantsev, A. L. *et al.* (2012) 'Extradenticle and Homothorax Control Adult Muscle Fiber Identity in Drosophila', *Developmental Cell*, 23(3), pp. 664–673. doi: 10.1016/j.devcel.2012.08.004.

Bulmer, M. G. (1983) 'Models for the evolution of protandry in insects', *Theoretical Population Biology*, 23(3), pp. 314–322. doi: 10.1016/0040-5809(83)90021-7.

Carneiro, K. *et al.* (2006) 'Graded maternal short gastrulation protein contributes to embryonic dorsal-ventral patterning by delayed induction', *Developmental Biology*, 296(1), pp. 203–218. doi: 10.1016/j.ydbio.2006.04.453.

Carreira-rosario, A. *et al.* (2016) 'Repression of Pumilio Protein Expression by Rbfox1 Promotes Germ Cell Differentiation', *Developmental Cell*. Elsevier Inc., 36(5), pp. 562–571. doi: 10.1016/j.devcel.2016.02.010.

Carvalho, G. B. *et al.* (2006) 'Allocrine Modulation of Feeding Behavior by the Sex Peptide of Drosophila', *Current Biology*, 16(7), pp. 692–696. doi: 10.1016/j.cub.2006.02.064.

Chambers, J. M. (1992) 'Linear Models', *Statistical Models in S*, pp. 153–193. doi: 10.1111/j.1751-0813.2008.00380.x.

Chang, L., Li, J. and Wang, L. (2016) 'Immuno-PCR: An ultrasensitive immunoassay for biomolecular detection', *Analytica Chimica Acta*. Elsevier Ltd, 910, pp. 12–24. doi: 10.1016/j.aca.2015.12.039.

Chapman, T. *et al.* (1995) 'Cost of mating in Drosophila melanogaster females is mediated by male accessory gland products.', *Nature*, pp. 241–244. doi: 10.1038/373241a0.

Chapman, T. *et al.* (2000) 'The role of male accessory gland protein Acp36DE in sperm competition in Drosophila melanogaster', *Proceedings of the Royal Society B: Biological Sciences*, 267(1448), pp. 1097–1105. doi: 10.1098/rspb.2000.1114.

Chapman, T. (2001) ‘Seminal fluid-mediated fitness traits in Drosophila’, *Heredity*, pp. 511–521. doi: 10.1046/j.1365-2540.2001.00961.x.

Chapman, T. *et al.* (2001) ‘The Acp26Aa seminal fluid protein is a modulator of early egg hatchability in Drosophila melanogaster’, *Proceedings of the Royal Society B: Biological Sciences*, 268(1477), pp. 1647–1654. doi: 10.1098/rspb.2001.1684.

Chapman, T *et al.* (2003) ‘Sexual conflict’, *Trends in Ecology & Evolution*, 18(41–47).

Chapman, T. *et al.* (2003) ‘The sex peptide of Drosophila melanogaster: Female post-mating responses analyzed by using RNA interference’, *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.1631635100.

Chapman, T. and Davies, S. J. (2004) ‘Functions and analysis of the seminal fluid proteins of male Drosophila melanogaster fruit flies’, *Peptides*, pp. 1477–1490. doi: 10.1016/j.peptides.2003.10.023.

Chen, P. S. *et al.* (1988) ‘A male accessory gland peptide that regulates reproductive behavior of female D. melanogaster’, *Cell*, 54(3), pp. 291–298. doi: 10.1016/0092-8674(88)90192-4.

Chen, S. (1984) ‘The functional morphology and biochemistry of insect male accessory glands and their secretions’, *Annual review of entomology*, (29), pp. 233–255.

Chen, S. *et al.* (2002) ‘Fighting fruit flies: A model system for the study of aggression’, *Proceedings of the National Academy of Sciences*, 99(8), pp. 5664–5668. doi: 10.1073/pnas.082102599.

Chintapalli, V. R., Wang, J. and Dow, J. A. T. (2007) ‘Using FlyAtlas to identify better Drosophila melanogaster models of human disease’, *Nature Genetics*, 39(6), pp. 715–720. doi: 10.1038/ng2049.

Chow, C. Y., Wolfner, M. F. and Clark, A. G. (2010) ‘The genetic basis for male x female interactions underlying variation in reproductive phenotypes of drosophila’, *Genetics*, 186(4), pp. 1355–1365. doi: 10.1534/genetics.110.123174.

Chow, C. Y., Wolfner, M. F. and Clark, A. G. (2013) ‘Large neurological component to genetic differences underlying biased sperm use in rosophila’, in *Genetics*. doi: 10.1534/genetics.112.146357.

Cirera, S and Aguade, M. (1997) ‘Evolutionary History of the Sex-Peptide (Aq70A) Gene Region in Drosophila melanogaster’, 197, pp. 189–197.

Cirera, S. (1997) ‘Evolutionary history of the sex-peptide (Acp70A) gene region in Drosophila melanogaster’, 197, pp. 189–197.

Civetta, A. and Singh, R. S. (1995) ‘High divergence of reproductive tract proteins and their association with postzygotic reproductive isolation in Drosophila melanogaster and Drosophila virilis group species’, *Journal of Molecular Evolution*, 41(6), pp. 1085–1095. doi: 10.1007/BF00173190.

Clark, A. G. *et al.* (1995) ‘Variation i n Sperm Displacement and Its Association with Accessory Gland Protein Loci i n’.

Clark, R. I. *et al.* (2011) ‘Multiple TGF-β superfamily signals modulate the adult drosophila

immune response', *Current Biology*, 21(19), pp. 1672–1677. doi: 10.1016/j.cub.2011.08.048.

Clutton-Brock, T. H. and Parker, G. A. (1995) 'Sexual coercion in animal societies', *Animal Behaviour*, 49(5), pp. 1345–1365. doi: 10.1006/anbe.1995.0166.

Coleman, S. *et al.* (1995) 'A Drosophila male accessory gland protein that is a member of the serpin superfamily of proteinase inhibitors is transferred to females during mating', *Insect Biochemistry and Molecular Biology*. doi: 10.1016/0965-1748(94)00055-M.

Consortium, G. (2000) 'The genome sequence of Drosophila melanogaster', *Science*, 287(5461), pp. 2185–2195. doi: 10.1126/science.287.5461.2185.

Cooper, D. N. (2010) 'Functional intronic polymorphisms: Buried treasure awaiting discovery within our genes.', *Human genomics*, pp. 284–288. doi: 10.1186/1479-7364-4-5-284.

Corderos, A. and Miller, P. L. (1992) 'Sperm transfer, displacement and precedence in Ischnura graellsii (Odonata : Coenagrionidae)', *Behavioral Ecology and Sociobiology*, 30(3–4), pp. 261–267. doi: 10.1007/BF00166711.

Cox, D. N. *et al.* (2001) 'Drosophila par-1 is required for oocyte differentiation and microtubule organization', pp. 75–87.

Crawley, J. M. (2013) *The R Book Second Edition*, *Cran*. doi: 10.1002/9780470515075.

Crook, J. R. and Shields, W. M. (1985) 'Sexually selected infanticide by adult male barn swallows', *Animal Behaviour*, 33(3), pp. 754–761. doi: 10.1016/S0003-3472(85)80007-5.

Daborn, P. J. *et al.* (2007) 'Evaluating the insecticide resistance potential of eight Drosophila melanogaster cytochrome P450 genes by transgenic over-expression', *Insect Biochemistry and Molecular Biology*, 37(5), pp. 512–519. doi: 10.1016/j.ibmb.2007.02.008.

Day, J. P. *et al.* (2005) 'Cyclic nucleotide phosphodiesterases in Drosophila melanogaster.', *The Biochemical journal*, 388, pp. 333–342. doi: 10.1042/BJ20050057.

Dean, M. D. *et al.* (2009) 'Proteomics and comparative genomic investigations reveal heterogeneity in evolutionary rate of male reproductive proteins in mice (Mus domesticus)', *Molecular Biology and Evolution*. doi: 10.1093/molbev/msp094.

DelBarco-Trillo, J. and Ferkin, M. H. (2004) 'Male mammals respond to a risk of sperm competition conveyed by odours of conspecific males', *Nature*, 431(7007), pp. 446–449. doi: 10.1038/nature02845.

Dickinson, J. L. and Rutowski, R. L. (1989) 'The function of the mating plug in the chalcedon checkerspot butterfly', *Animal Behaviour*, 38(1), pp. 154–162. doi: 10.1016/S0003-3472(89)80074-0.

Ding, Z. *et al.* (2003) 'Sex-peptides bind to two molecularly different targets in Drosophila melanogaster females', *Journal of Neurobiology*. doi: 10.1002/neu.10218.

Dobens, L. *et al.* (2005) 'Bunched sets a boundary for Notch signaling to pattern anterior eggshell structures during Drosophila oogenesis', *Developmental Biology*, 287(2), pp. 425–437. doi: 10.1016/j.ydbio.2005.09.019.

Dobens, L. L. and Raftery, L. A. (2000) ‘A PEER REVIEWED FORUM Integration of Epithelial Patterning and Morphogenesis in’, 93(February), pp. 80–93.

Doerflinger, H. *et al.* (2006) ‘Report Drosophila Anterior-Posterior Polarity Requires Actin-Dependent PAR-1 Recruitment to the Oocyte Posterior’, pp. 1090–1095. doi: 10.1016/j.cub.2006.04.001.

Domanitskaya, E. V. *et al.* (2007) ‘The hydroxyproline motif of male sex peptide elicits the innate immune response in Drosophila females’, *FEBS Journal*, 274(21), pp. 5659–5668. doi: 10.1111/j.1742-4658.2007.06088.x.

Dong, Y., Taylor, H. E. and Dimopoulos, G. (2006) ‘AgDscam, a hypervariable immunoglobulin domain-containing receptor of the Anopheles gambiae innate immune system’, *PLoS Biology*, 4(7), pp. 1137–1146. doi: 10.1371/journal.pbio.0040229.

Döring, F. *et al.* (2006) ‘Novel Drosophila two-pore domain K+ channels: Rescue of channel function by heteromeric assembly’, *European Journal of Neuroscience*, 24(8), pp. 2264–2274. doi: 10.1111/j.1460-9568.2006.05102.x.

Dow, M. A. and Schilcher, F. Von (1975) ‘Aggression and mating success in Drosophila melanogaster’, *Nature*, 254(5500), pp. 511–512. doi: 10.1038/254511a0.

Eleftherianos, I. *et al.* (2007) ‘The immunoglobulin family protein Hemolin mediates cellular immune responses to bacteria in the insect Manduca sexta’, *Cellular Microbiology*, 9(5), pp. 1137–1147. doi: 10.1111/j.1462-5822.2006.00855.x.

Elias, D. O. *et al.* (2014) ‘Mate-guarding courtship behaviour: Tactics in a changing world’, *Animal Behaviour*. Elsevier Ltd, 97, pp. 25–33. doi: 10.1016/j.anbehav.2014.08.007.

Ellen, M. *et al.* (1984) ‘The Effect of Experimental Design on Female Receptivity to Remating and its Impact on Reproductive Success in Drosophila melanogaster’, *Evolution*, 38(386), pp. 1261–1272. doi: 10.2307/2408633.

Emmons, S., Phan, H., Calley, J., Chen, W. and James, B. (1995) ‘ca puccino’, pp. 2482–2494.

Emmons, S., Phan, H., Calley, J., Chen, W., James, B., *et al.* (1995) ‘cappuccino, a Drosophila maternal effect gene required for polarity of the egg and embryo, is related to the vertebrate limb deformity locus’, *Genes and Development*, 9(20), pp. 2482–2494. doi: 10.1101/gad.9.20.2482.

Ertürk-Hasdemir, D. *et al.* (2009) ‘Two roles for the Drosophila IKK complex in the activation of Relish and the induction of antimicrobial peptide genes.’, *Proceedings of the National Academy of Sciences of the United States of America*, 106(24), pp. 9779–84. doi: 10.1073/pnas.0812022106.

Feder, M. E. and Mitchell-Olds, T. (2003) ‘Evolutionary and ecological functional genomics’, *Nature Reviews Genetics*, 4(8), pp. 649–655. doi: 10.1038/nrg1128.

Ferveur, J. F. (2005) ‘Cuticular hydrocarbons: Their evolution and roles in Drosophila pheromonal communication’, *Behavior Genetics*, pp. 279–295. doi: 10.1007/s10519-005-3220-5.

Findlay, G. D. *et al.* (2008) ‘Proteomics reveals novel *Drosophila* seminal fluid proteins transferred at mating’, *PLoS Biology*, 6(7), p. e178. doi: 10.1371/journal.pbio.0060178.

Findlay, G. D. *et al.* (2014) 'Evolutionary Rate Covariation Identifies New Members of a Protein Network Required for Drosophila melanogaster Female Post-Mating Responses', 10(1). doi: 10.1371/journal.pgen.1004108.

Findlay, G. D., MacCoss, M. J. and Swanson, W. J. (2009) 'Proteomic discovery of previously unannotated, rapidly evolving seminal fluid genes in Drosophila', *Genome Research*, 19(5), pp. 886–895. doi: 10.1101/gr.089391.108.

Finley, D. (2009) 'Recognition and Processing of Ubiquitin-Protein Conjugates by the Proteasome', *Annual Review of Biochemistry*, 78(1), pp. 477–513. doi: 10.1146/annurev.biochem.78.081507.101607.

Fisher, R. A. (1915) 'The evolution of sexual preference.', *The Eugenics review*, 7(3), pp. 184–192.

Fisher, R. A. (1930) *The genetical theory of natural selection.*, *The genetical theory of natural selection.* Oxford, England: Clarendon Press. doi: 10.5962/bhl.title.27468.

FlyBase Curators, Swiss-Prot Project Members, I. P. M. (2004) 'Gene Ontology annotation in FlyBase through association of InterPro records with GO terms.', *Personal communication to Flybase.*

Foellmer, M. W. and Fairbairn, D. J. (2003) 'Spontaneous male death during copulation in an orb-weaving spider', *Proceedings of the Royal Society B: Biological Sciences*, 270(Suppl_2), pp. S183–S185. doi: 10.1098/rsbl.2003.0042.

Fowler, G. L. (1973) 'Some aspects of the reproductive biology of Drosophila: Sperm transfer, sperm storage, and sperm utilization', *Advances in Genetics*, 17(C), pp. 293–360. doi: 10.1016/S0065-2660(08)60173-X.

Fowler, K. and Partridge, L. (1989) 'A cost of mating in female fruitflies', *Nature*, 338(6218), pp. 760–761. doi: 10.1038/338760a0.

Frandsen, J. L. *et al.* (2008) 'Salmonella pathogenesis reveals that BMP signaling regulates blood cell homeostasis and immune responses in Drosophila.', *Proceedings of the National Academy of Sciences of the United States of America*, 105(39), pp. 14952–14957. doi: 10.1073/pnas.0808208105.

Franklin, A. M. and Stuart-Fox, D. (2017) 'Single and multiple mating reduces longevity of female dumpling squid (Euprymna tasmanica)', *Journal of Evolutionary Biology*, 30(5), pp. 977–984. doi: 10.1111/jeb.13063.

Fricke, C. *et al.* (2014) 'MicroRNAs influence reproductive responses by females to male sex peptide in drosophila melanogaster', *Genetics*. doi: 10.1534/genetics.114.167320.

Fricke, C., Bretman, A. and Chapman, T. (2010) 'Female nutritional status determines the magnitude and sign of responses to a male ejaculate signal in Drosophila melanogaster', 23, pp. 157–165. doi: 10.1111/j.1420-9101.2009.01882.x.

Futuyma, D. J. (2009) 'Evolution, Second Edition. Douglas J. Futuyma.', *Integrative and Comparative Biology*, 49(6), pp. 722–723. doi: 10.1093/icb/icp095.

Gage, M. (2004) ‘Evolution: Sexual arms races’, *Current Biology*, 14(10), pp. 378–380. doi: 10.1016/j.cub.2004.05.010.

Ganguly, A. and Lee, D. (2013) ‘Suppression of inhibitory GABAergic transmission by cAMP signaling pathway: Alterations in learning and memory mutants’, *European Journal of Neuroscience*, 37(9), pp. 1383–1393. doi: 10.1111/ejn.12144.

Garbaczewska, M., Billeter, J. and Levine, J. D. (2013) ‘Drosophila melanogaster males increase the number of sperm in their ejaculate when perceiving rival males’, *Journal of Insect Physiology*, 59(3), pp. 306–310. doi: 10.1016/j.jinsphys.2012.08.016.

Gehring, W. J. (1992) ‘The homeobox in perspective’, *Trends in Biochemical Sciences*, 17(8), pp. 277–280. doi: 10.1016/0968-0004(92)90434-B.

Gilchrist, A. S. and Partridge, L. (2000) ‘Why it is difficult to model sperm displacement in Drosophilamelanogaster: The relation between sperm transfer andcopulation duration’, *Evolution*, 54(2), pp. 534–542. Available at: Journal.

Gillespie, J. P., Kanost, M. R. and Trenczek, T. (1997) ‘Biological mediators of insect immunity’, *Annu. Rev. Entomol*, 42, pp. 611–43. doi: 10.1146/annurev.ento.42.1.611.

Gillott, C. (2003) ‘Male accessory Glands Secretions: Modulators of Female Reproductive Physiology and Behavior’. doi: 10.1146/annurev.ento.48.091801.112657.

Gioti, A. *et al.* (2012) ‘Sex peptide of Drosophila melanogaster males is a global regulator of reproductive processes in females Sex peptide of Drosophila melanogaster males is a global regulator of reproductive processes in females’. doi: 10.1098/rspb.2012.1634.

Gomendio, M. and Roldan, E. R. S. (1991) ‘Sperm competition influences sperm size in mammals’, *Proceedings of the Royal Society B: Biological Sciences*, 243(1308), pp. 181–185. doi: 10.1098/rspb.1991.0029.

Gorter, J. A. . (2018) ‘The nature and nurture of female receptivity’.

Gramates, L. S. *et al.* (2017) ‘FlyBase at 25: Looking to the future’, *Nucleic Acids Research*, 45(D1), pp. D663–D671. doi: 10.1093/nar/gkw1016.

Gromko, MH., DG. Gilbert, and R. R. (1984) ‘Sperm transfer and use in the multiple mating system of Drosophila.’, in *Sperm competition and the evolution of animal mating systems*, pp. 371–426.

Gromko, M., Newport, M. and Kortier, M. (1984) ‘Sperm Dependence of Female Receptivity to Remating in Drosophila melanogaster.’, *Evolution*, 38(6), pp. 1273–1282. doi: 10.2307/2408634.

Gwynne, D. T. (1991) ‘Sexual competition among females: What causes courtship-role reversal?’, *Trends in Ecology and Evolution*, pp. 118–121. doi: 10.1016/0169-5347(91)90089-G.

Halpern, M. D. *et al.* (2014) ‘Simple objective detection of human lyme disease infection using immuno-PCR and a single recombinant hybrid antigen’, *Clinical and Vaccine Immunology*, 21(8), pp. 1094–1105. doi: 10.1128/CVI.00245-14.

Hartl, D. L. and Clark, A. G. (2007) *Principles of Population Genetics*, *4th Edition Sinauer*

Associates. doi: 10.2980/1195-6860(2007)14[544b:popg]2.0.co;2.

Häsemeyer, M. *et al.* (2009) 'Sensory Neurons in the Drosophila Genital Tract Regulate Female Reproductive Behavior', *Neuron*, 61(4), pp. 511–518. doi: 10.1016/j.neuron.2009.01.009.

Haussmann, I. U. *et al.* (2013) 'Multiple pathways mediate the sex-peptide-regulated switch in female Drosophila reproductive behaviours Multiple pathways mediate the sex- peptide-regulated switch in female Drosophila reproductive behaviours', (October).

Hayashi, S. and Scott, M. P. (1990) 'What determines the specificity of action of Drosophila homeodomain proteins?', *Cell*, 63(5), pp. 883–894. doi: 10.1016/0092-8674(90)90492-W.

Heberle, H. *et al.* (2015) 'InteractiVenn: A web-based tool for the analysis of sets through Venn diagrams', *BMC Bioinformatics*, 16(1), pp. 1–7. doi: 10.1186/s12859-015-0611-3.

Heifetz, Y. *et al.* (2000) 'The Drosophila seminal fluid protein Acp26Aa stimulates release of oocytes by the ovary', *Current Biology*, 10(2), pp. 99–102. doi: 10.1016/S0960-9822(00)00288-8.

Herndon, L. A. and Wolfner, M. F. (1995) 'A Drosophila seminal fluid protein, Acp26Aa, stimulates egg laying in females for 1 day after mating.', *Proceedings of the National Academy of Sciences*, 92(22), pp. 10114–10118. doi: 10.1073/pnas.92.22.10114.

Higham, J. P., Heistermann, M. and Maestripieri, D. (2011) 'The energetics of male-male endurance rivalry in free-ranging rhesus macaques, Macaca mulatta', *Animal Behaviour*, 81(5), pp. 1001–1007. doi: 10.1016/j.anbehav.2011.02.001.

Hoffmann, J. A. (2003) 'The immune response of Drosophila', *Nature*, 426(6962), pp. 33–38. doi: 10.1038/nature02021.

Hombría, J. C. and Brown, S. (2002) 'The Fertile Field of Drosophila JAK / STAT Signalling', *Current Biology*, 12(02), pp. 569–575.

Hooper, S. D. *et al.* (2007) 'Identification of tightly regulated groups of genes during Drosophila melanogaster embryogenesis', *Molecular Systems Biology*, 3(72), pp. 1–11. doi: 10.1038/msb4100112.

Horváth, B. and Kalinka, A. T. (2018) 'The genetics of egg retention and fertilization success in Drosophila: One step closer to understanding the transition from facultative to obligate viviparity', *Evolution*, 72(2), pp. 318–336. doi: 10.1111/evo.13411.

Huang, D. W., Lempicki, R. a and Sherman, B. T. (2009) 'Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.', *Nature Protocols*, 4(1), pp. 44–57. doi: 10.1038/nprot.2008.211.

Huang, D. W., Sherman, B. T. and Lempicki, R. A. (2009) 'Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists', *Nucleic Acids Research*, 37(1), pp. 1–13. doi: 10.1093/nar/gkn923.

Huang, W., Massouras, A. and Inoue, Y. (2014) 'Natural variation in genome architecture among 205 Drosophila melanogaster Genetic Reference Panel lines Natural Variation in Genome Architecture Among 205 Drosophila melanogaster Genetic Reference Panel Lines', pp. 1193–1208.

doi: 10.1101/gr.171546.113.

Hull-Thompson, J. *et al.* (2009) 'Control of metabolic homeostasis by stress signaling is mediated by the Lipocalin NLaz', *PLoS Genetics*, 5(4). doi: 10.1371/journal.pgen.1000460.

Hunter, F. M. and Birkhead, T. R. (2002) 'Sperm viability and sperm competition in insects', *Current Biology*, 12(2), pp. 121–123. doi: 10.1016/S0960-9822(01)00647-9.

Huxley, J. S. (1938) 'Darwin's Theory of Sexual Selection and the Data Subsumed by it, in the Light of Recent Research', *The American Naturalist*, 72(742), pp. 416–433. doi: 10.1086/280795.

Imhof, M. *et al.* (1998) 'Multiple mating in wild Drosophila melanogaster revisited by microsatellite analysis', *Molecular Ecology*, 7(7), pp. 915–917. doi: 10.1046/j.1365-294x.1998.00382.x.

Innocenti, P. and Morrow, E. H. (2009) 'Immunogenic males: A genome-wide analysis of reproduction and the cost of mating in Drosophila melanogaster females', *Journal of Evolutionary Biology*, 22(5), pp. 964–973. doi: 10.1111/j.1420-9101.2009.01708.x.

Insco, M. L. *et al.* (2012) 'A self-limiting switch based on translational control regulates the transition from proliferation to differentiation in an adult stem cell lineage', *Cell Stem Cell*. Elsevier Inc., 11(5), pp. 689–700. doi: 10.1016/j.stem.2012.08.012.

Isaac, R. E. *et al.* (2010) 'Drosophila male sex peptide inhibits siesta sleep and promotes locomotor activity in the post-mated female', *Proceedings of the Royal Society B: Biological Sciences*, 277(1678), pp. 65–70. doi: 10.1098/rspb.2009.1236.

Isaac, R. E., Kim, Y. and Audsley, N. (2014) 'Peptides The degradome and the evolution of Drosophila sex peptide as a ligand for the MIP receptor', *Peptides*. Elsevier Inc., 53, pp. 258–264. doi: 10.1016/j.peptides.2013.12.016.

Jallon, J. M. (1984) 'A few chemical words exchanged by Drosophila during courtship and mating', *Behavior Genetics*, 14(5), pp. 441–478. doi: 10.1007/BF01065444.

Jarrige, A. *et al.* (2016) 'Recently mated males of a lek-mating insect intensify precopulatory mate guarding under male competition', *Animal Behaviour*, 117, pp. 21–34. doi: 10.1016/j.anbehav.2016.04.012.

Jeffs, P. S., Holmes, E. C. and Ashburner, M. (1994) 'The molecular evolution of the alcohol dehydrogenase and alcohol dehydrogenase-related genes in the Drosophila melanogaster species subgroup.', *Molecular biology and evolution*, 11(2), pp. 287–304. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8170369.

Johnson, A. N. *et al.* (2011) 'JAK/Stat signaling regulates heart precursor diversification in Drosophila', *Development*, 138(21), pp. 4627–4638. doi: 10.1242/dev.071464.

Kalb, J. M., DiBenedetto, A. J. and Wolfner, M. F. (1993) 'Probing the function of Drosophila melanogaster accessory glands by directed cell ablation.', *Proceedings of the National Academy of Sciences of the United States of America*, 90, pp. 8093–8097. doi: 10.1073/pnas.90.17.8093.

Kelly, C. D. and Jennions, M. D. (2011) 'Sexual selection and sperm quantity: Meta-analyses of

strategic ejaculation', *Biological Reviews*. doi: 10.1111/j.1469-185X.2011.00175.x.

Kern, A. D., Jones, C. D. and Begun, D. J. (2004) 'Molecular population genetics of male accessory gland proteins in the Drosophila simulans complex', *Genetics*, 167(2), pp. 725–735. doi: 10.1534/genetics.103.020883.

Kim, W. J., Jan, L. Y. and Jan, Y. N. (2013) 'A PDF/NPF neuropeptide signaling circuitry of male Drosophila melanogaster controls rival-induced prolonged mating', *Neuron*, 80(5), pp. 1190–1205. doi: 10.1016/j.neuron.2013.09.034.

Kim, Y.-J. *et al.* (2010) 'MIPs are ancestral ligands for the sex peptide receptor', *Proceedings of the National Academy of Sciences*, 107(14), pp. 6520–6525. doi: 10.1073/pnas.0914764107.

Kimura, M. (1980) 'A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences', *Journal of Molecular Evolution*, 16(2), pp. 111–120. doi: 10.1007/BF01731581.

Kleino, A. *et al.* (2005) 'Inhibitor of apoptosis 2 and TAK1-binding protein are components of the Drosophila Imd pathway', *EMBO Journal*, 24(19), pp. 3423–3434. doi: 10.1038/sj.emboj.7600807.

Knell, R. J. and Webberley, K. M. (2004) 'Sexually transmitted diseases of insects: Distribution, evolution, ecology and host behaviour', *Biological Reviews of the Cambridge Philosophical Society*, pp. 557–581. doi: 10.1017/S1464793103006365.

König, A. *et al.* (2011) 'Ecdysteroids affect Drosophila ovarian stem cell niche formation and early germline differentiation', *EMBO Journal*, 30(8), pp. 1549–1562. doi: 10.1038/emboj.2011.73.

Kubli, E. (2003) 'Sex-peptides: Seminal peptides of the Drosophila male', *Cellular and Molecular Life Sciences*, pp. 1689–1704. doi: 10.1007/s00018-003-3052.

Kubli, E. and Bopp, D. (2012) 'Sexual behavior: How sex peptide flips the postmating switch of female flies', *Current Biology*. Elsevier, 22(13), pp. R520–R522. doi: 10.1016/j.cub.2012.04.058.

Kubrak, O. I. *et al.* (2016) 'Characterization of reproductive dormancy in male Drosophila melanogaster', *Frontiers in Physiology*, 7(NOV), pp. 1–13. doi: 10.3389/fphys.2016.00572.

Kurtovic, A., Widmer, A. and Dickson, B. J. (2007) 'A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone', *Nature*, 446(7135), pp. 542–546. doi: 10.1038/nature05672.

Kurtz, J. and Armitage, S. A. O. (2006) 'Alternative adaptive immunity in invertebrates', 27(11), pp. 9–12. doi: 10.1016/j.it.2006.09.001.

LaFlamme, B. A., Ravi Ram, K. and Wolfner, M. F. (2012) 'The Drosophila melanogaster seminal fluid protease "Seminase" regulates proteolytic and post-mating reproductive processes', *PLoS Genetics*, 8(1). doi: 10.1371/journal.pgen.1002435.

Larracuente, A. M. *et al.* (2008) 'Evolution of protein-coding genes in Drosophila', *Trends in Genetics*, 24(3), pp. 114–123. doi: 10.1016/j.tig.2007.12.001.

Laturney, M. (2016) *The second sex: Functions and mechanisms of sperm manipulation in female Drosophila melanogaster*.

Laturney, M. and Billeter, J. C. (2014) ‘Neurogenetics of female reproductive behaviors in drosophila melanogaster’, *Advances in Genetics*, 85, pp. 1–108. doi: 10.1016/B978-0-12-800271-1.00001-9.

Lefrve, G. and Jonssom, U. B. (1962) ‘Sperm transfer, storage, displacement, and utilization in Drosophila melanogaster.’, *Genetics*, 47, pp. 1719–1736.

Lemaitre, B. and Hoffmann, J. (2007) ‘The Host Defense of Drosophila melanogaster’. doi: 10.1146/annurev.immunol.25.022106.141615.

Liberti, J., Baer, B. and Boomsma, J. J. (2018) ‘Rival seminal fluid induces enhanced sperm motility in a polyandrous ant’, *BMC Evolutionary Biology*. doi: 10.1186/s12862-018-1144-y.

Lindley, D. V and Smith, A. F. M. (1972) ‘Bayes Estimates for the Linear Model’, *Journal of the Royal Statistical Society. Series B (Methodological)*, 34(1), pp. 1–41. doi: 10.2307/2985048.

Liu, H. and Kubli, E. (2003) ‘Sex-peptide is the molecular basis of the sperm effect in Drosophila melanogaster’, 100(17), pp. 9929–9933.

Liu, N. and Lasko, P. (2015) ‘Analysis of RNA Interference Lines Identifies New Functions of Maternally-Expressed Genes Involved in Embryonic Patterning in Drosophila melanogaster’, *G3: Genes|Genomes|Genetics*, 5(6), pp. 1025–1034. doi: 10.1534/g3.115.017517.

Liu, Y. H. *et al.* (2009) ‘A Systematic Analysis of Tinman Function Reveals Eya and JAK-STAT Signaling as Essential Regulators of Muscle Development’, *Developmental Cell*. Elsevier Ltd, 16(2), pp. 280–291. doi: 10.1016/j.devcel.2009.01.006.

Lung, O. *et al.* (2002) ‘The Drosophila melanogaster seminal fluid protein Acp62F is a protease inhibitor that is toxic upon ectopic expression’, *Genetics*, 160(1), pp. 211–224.

Lung, O., Kuo, L. and Wolfner, M. F. (2001) ‘Drosophila males transfer antibacterial proteins from their accessory gland and ejaculatory duct to their mates’, 47, pp. 617–622.

Lung, O. and Wolfner, M. F. (1999) ‘Drosophila seminal fluid proteins enter the circulatory system of the mated female fly by crossing the posterior vaginal wall’, *Insect Biochemistry and Molecular Biology*, 29(12), pp. 1043–1052. doi: 10.1016/S0965-1748(99)00078-8.

Lung, O. and Wolfner, M. F. (2001) ‘Identification and characterization of the major Drosophila melanogaster mating plug protein’, 31, pp. 543–551.

Lüpold, S. *et al.* (2011) ‘Male Drosophila melanogaster adjust ejaculate size based on female mating status, fecundity, and age’, *Behavioral Ecology*, 22(1), pp. 185–191. doi: 10.1093/beheco/arq193.

MacBean, I. T. and Parsons, P. A. (1967) ‘Directional selection for duration of copulation in Drosophila melanogaster.’, *Genetics*.

MacKay, T. F. C. *et al.* (2012) ‘The Drosophila melanogaster Genetic Reference Panel’, *Nature*. doi: 10.1038/nature10811.

Mackay, T. F. C. and Huang, W. (2018) ‘Charting the genotype–phenotype map: lessons from the Drosophila melanogaster Genetic Reference Panel’, *Wiley Interdisciplinary Reviews:*

Developmental Biology, 7(1), pp. 1–18. doi: 10.1002/wdev.289.

Mandrioli, M., Monti, M. and Tedeschi, R. (2015) 'Presence and conservation of the immunoglobulin superfamily in insects: current perspective and future challenges', *Invertebrate Survival Journal*, 12, pp. 188–194. Available at: https://doaj.org/article/a757b629a0f345caaa36739e463a7f78.

Mane, S. D., Tompkins, L. and Richmond, R. C. (1983) 'Male esterase 6 catalyzes the synthesis of a sex pheromone in Drosophila melanogaster females', *Science*, 222(4622), pp. 419–421. doi: 10.1126/science.222.4622.419.

Manier, M. K. *et al.* (2010) 'Resolving mechanisms of competitive fertilization success in drosophila Melanogaster', *Science*, 328(5976), pp. 354–357. doi: 10.1126/science.1187096.

Manning, A. (1962) 'A sperm factor affecting the receptivity of Drosophila melanogaster females', *Nature*, 194(4825), pp. 252–253. doi: 10.1038/194252a0.

Manning, A. (1967) 'The control of sexual receptivity in female', *Animal Behaviour*, 15(2–3), pp. 239–250.

Manoli, D. S. *et al.* (2005) 'Male-specific fruitless specifies the neural substrates of Drosophila courtship behaviour', *Nature*, 436(7049), pp. 395–400. doi: 10.1038/nature03859.

Markow, T. A. (1987) 'Behavioral and sensory basis of courtship success in Drosophila melanogaster', *Proceedings of the National Academy of Sciences of the United States of America*, 84(September), pp. 6200–6204. doi: VL - 84.

Markow, T. A. and Manning, M. (1980) 'Mating success of photoreceptor mutants of Drosophila melanogaster', *Behavioral and Neural Biology*, 29(2), pp. 276–280. doi: 10.1016/S0163-1047(80)90612-3.

Markow, T. a. and O'Grady, P. (2006) '[Book] Drosophila: A Guide to Species Identification and Use', *Elsevier*. doi: 10.1017/CBO9781107415324.004.

Markow, T. a (2000) 'Forced Matings in Natural Populations of Drosophila', *American Naturalist*, 156(1), pp. 155–156. doi: 10.1258/002367770781036508.

Marshall, D. L. and Ellstrand, N. C. (1986) 'Sexual Selection in Raphanus sativus: Experimental Data on Nonrandom Fertilization, Maternal Choice, and Consequences of Multiple Paternity', *The American Naturalist*, 127(4), pp. 446–461. doi: 10.1086/284494.

Martan, J. and Shepherd, B. A. (1976) 'The role of the copulatory plug in reproduction of the guinea pig', *Journal of Experimental Zoology*. doi: 10.1002/jez.1401960108.

Mathieu, J. *et al.* (2007) 'A sensitized PiggyBac-based screen for regulators of border cell migration in drosophila', *Genetics*, 176(3), pp. 1579–1590. doi: 10.1534/genetics.107.071282.

McCullagh, P. and Nelder, J. A. (1989) *Generalized Linear Models*, *Generalized Linear Models*. doi: 10.1007/978-1-4899-3242-6.

McGraw, L. A. *et al.* (2004) 'Genes Regulated by Mating , Sperm , or Seminal Proteins in Mated Female Drosophila melanogaster', 14, pp. 1509–1514. doi: 10.1016/j.

McGraw, L. A., Clark, A. G. and Wolfner, M. F. (2008) 'Post-mating gene expression profiles of female Drosophila melanogaster in response to time and to four male accessory gland proteins', *Genetics*, 179(3), pp. 1395–1408. doi: 10.1534/genetics.108.086934.

McKie, A. *et al.* (2002) 'A quantitative immuno-PCR assay for the detection of mumps-specific IgG', *Journal of Immunological Methods*, 270(1), pp. 135–141. doi: 10.1016/S0022-1759(02)00325-3.

Mehta, P. K. *et al.* (2017) 'Immuno-PCR, a new technique for the serodiagnosis of tuberculosis', *Journal of Microbiological Methods*. Elsevier, 139(May), pp. 218–229. doi: 10.1016/j.mimet.2017.05.009.

Merle, J. (1968) 'Fonctionnement ovarien et réceptivité sexuelle de Drosophila melanogaster après implantation de fragments de l'appareil génital mâle', *Journal of Insect Physiology*, 14(8), pp. 1159–1168. doi: 10.1016/0022-1910(68)90055-3.

Merrell, D. J. (1949) 'Selective mating in Drosophila melanogaster.', *Genetics*, 34(4), pp. 370–389.

Miest, T. S. and Bloch-Qazi, M. C. (2008) 'Sick of mating sexual transmission of a pathogenic bacterium in drosophila melanogaster', *Fly*, 2(4), pp. 215–219. doi: 10.4161/fly.6726.

Monahan, A. J. and Starz-Gaiano, M. (2013) 'Socs36E attenuates STAT signaling to optimize motile cell specification in the Drosophila ovary', *Developmental Biology*. doi: 10.1016/j.ydbio.2013.03.022.

Monsma, S. A., Harada, H. A. and Wolfner, M. F. (1990) 'Synthesis of two Drosophila male accessory gland proteins and their fate after transfer to the female during mating', *Developmental Biology*, 142(2), pp. 465–475. doi: 10.1016/0012-1606(90)90368-S.

Monsma, S. a and Wolfner, M. F. (1988) 'Structure and expression of a Drosopbila male accessory gland gene whose product resembles a peptide pheromone precursor', *Genes and Development*, 2, pp. 1063–1073. doi: 10.1101/gad.2.9.1063.

Montojo, J. *et al.* (2010) 'GeneMANIA cytoscape plugin: Fast gene function predictions on the desktop', *Bioinformatics*, 26(22), pp. 2927–2928. doi: 10.1093/bioinformatics/btq562.

Moore, L. A. *et al.* (1998) 'Identification of genes controlling germ cell migration and embryonic gonad formation in Drosophila', *Development (Cambridge, England)*, 125(4), pp. 667–678. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9435287.

Morbey, Y. E. and Ydenberg, R. C. (2001) 'Protandrous arrival timing to breeding areas: A review', *Ecology Letters*, pp. 663–673. doi: 10.1046/j.1461-0248.2001.00265.x.

Morris, J. Z., Navarro, C. and Lehmann, R. (2003) 'Identification and analysis of mutations in bob, Doa and eight new genes required for oocyte specification and development in Drosophila melanogaster', *Genetics*, 164(4), pp. 1435–1446.

Morrow, E. H. and Innocenti, P. (2012) 'Female postmating immune responses, immune system evolution and immunogenic males', *Biological Reviews*, 87(3), pp. 631–638. doi: 10.1111/j.1469-185X.2011.00214.x.

Morton, D. B. *et al.* (2010) 'Infertility and male mating behavior deficits associated with Pde1c in Drosophila melanogaster', *Genetics*, 186(1), pp. 159–165. doi: 10.1534/genetics.110.118018.

Moshitzky, P. *et al.* (1996) 'Sex-peptide activates juvenile hormone biosynthesis in theDrosophila melanogaster corpus allatum', *Archives of Insect Biochemistry and Physiology*, 32(3–4), pp. 363–374. doi: 10.1002/(SICI)1520-6327(1996)32:3/4<363::AID-ARCH9>3.0.CO;2-T.

Mostafavi, S. *et al.* (2008) 'GeneMANIA: A real-time multiple association network integration algorithm for predicting gene function', *Genome Biology*. doi: 10.1186/gb-2008-9-s1-s4.

Mueller, J. L. *et al.* (2008) 'Targeted gene deletion and phenotypic analysis of the Drosophila melanogaster seminal fluid protease inhibitor Acp62F', *Genetics*. doi: 10.1534/genetics.107.083766.

Mueller, J. L., Page, J. L. and Wolfner, M. F. (2007) 'An ectopic expression screen reveals the protective and toxic effects of Drosophila seminal fluid proteins', *Genetics*, 175(2), pp. 777–783. doi: 10.1534/genetics.106.065318.

Muller H. J and Settles. F (1927) 'The non-functioning of the genes in spermatozoa.', *Molecular and General Genetics*, 43.1, pp. 285–312.

Myllymäki, H. and Rämet, M. (2013) 'Transcription factor zfh1 downregulates Drosophila Imd pathway', *Developmental and Comparative Immunology*, 39(3), pp. 188–197. doi: 10.1016/j.dci.2012.10.007.

Myllymäki, H. and Rämet, M. (2014) 'JAK/STAT Pathway in Drosophila Immunity', *Scandinavian Journal of Immunology*, 79(6), pp. 377–385. doi: 10.1111/sji.12170.

Myllymäki, H., Valanne, S. and Rämet, M. (2014) 'The Drosophila Imd Signaling Pathway', *J Immunol*, 192:3455–3(doi: 10.4049/jimmunol.1303309), pp. 3455–3462. doi: 10.4049/jimmunol.1303309.

Nagao, T. *et al.* (2000) 'Patterning defects in the primary axonal scaffolds caused by the mutations of the extradenticle and homothorax genes in the embryonic Drosophila brain.', *Development genes and evolution*, 210(6), pp. 289–299. doi: 10.1007/s004270050316.

Neubaum, D. M. and Wolfner, M. F. (1999) 'Mated Drosophila melanogaster females require a seminal fluid protein, Acp36DE, to store sperm efficiently', *Genetics*, 153(2), pp. 845–857.

Niemeyer, C. M., Adler, M. and Wacker, R. (2007) 'Detecting antigens by quantitative immuno-PCR', *Nature Protocols*, 2(8), pp. 1918–1930. doi: 10.1038/nprot.2007.267.

Nonidez, J. F. (1920) 'The internal phenomena of reproduction in Drosophila', *The Biological Bulletin*, 39.4, pp. 207–230.

Obbard, D. J. *et al.* (2009) 'Quantifying adaptive evolution in the Drosophila immune system', *PLoS Genetics*, 5(10). doi: 10.1371/journal.pgen.1000698.

Özkan, E. *et al.* (2013) 'XAn extracellular interactome of immunoglobulin and LRR proteins reveals receptor-ligand networks', *Cell*, 154(1). doi: 10.1016/j.cell.2013.06.006.

Page, S. L. *et al.* (2000) 'Genetic studies of mei-P26 reveal a link between the processes that control

germ cell proliferation in both sexes and those that control meiotic exchange in Drosophila', *Genetics*, 155(4), pp. 1757–1772.

Panel, T. D. melanogaster G. R. (2012) 'The Drosophila melanogaster Genetic Reference Panel.', *Nature*, 482(7384), pp. 173–178. doi: 10.1038/nature10811.The.

Park, W. J., Liu, J. and Adler, P. N. (1994) 'The frizzled gene of Drosophila encodes a membrane protein with an odd number of transmembrane domains', *Mechanisms of Development*, 45(2), pp. 127–137. doi: 10.1016/0925-4773(94)90026-4.

Parker, G. A. (1970) 'Sperm competition and its evolutionary on copula duration in the fly effect', 16.

Parker, G. A. (1974) 'Courtship Persistence and Female-Guarding as Male Time Investment Strategies', *Behaviour*, 48(1–4), pp. 157–183. doi: 10.1163/156853974X00327.

Parker, G. A. (1979) 'Sexual selection and sexual conflict', in *Sexual Selection and Reproductive Competition in Insects*, pp. 123–166. doi: 10.1016/B978-0-12-108750-0.50010-0.

Parker, G. A. (2006) 'Sexual conflict over mating and fertilization: An overview', in *Philosophical Transactions of the Royal Society B: Biological Sciences*. doi: 10.1098/rstb.2005.1785.

Parker, G. A., Baker, R. R. and Smith, V. G. F. (1972) 'The origin and evolution of gamete dimorphism and the male-female phenomenon', *Journal of Theoretical Biology*, 36(3), pp. 529–553. doi: 10.1016/0022-5193(72)90007-0.

Partridge, L., Ewing, A. and Chandler, A. (1987) 'Male size and mating success in Drosophila melanogaster: the roles of male and female behaviour', *Animal Behaviour*, 35(2), pp. 555–562. doi: 10.1016/S0003-3472(87)80281-6.

Penalva, L. O. F. *et al.* (2000) 'The Drosophila fl(2)d gene, required for female-specific splicing of Sxl and tra pre-mRNAs, encodes a novel nuclear protein with a HQ-rich domain', *Genetics*, 155(1), pp. 129–139.

Peng, J. *et al.* (2005) 'Gradual Release of Sperm Bound Sex-Peptide Controls Female Postmating Behavior in Drosophila', 15, pp. 207–213. doi: 10.1016/j.

Peng, J., Zipperlen, P. and Kubli, E. (2005) 'Drosophila sex-peptide stimulates female innate immune system after mating via the toll and Imd pathways', *Current Biology*, 15(18), pp. 1690–1694. doi: 10.1016/j.cub.2005.08.048.

Pilch, B. and Mann, M. (2006) 'Large-scale and high-confidence proteomic analysis of human seminal plasma', *Genome Biology*. doi: 10.1186/gb-2006-7-5-r40.

Pilpel, N. *et al.* (2008) 'Mating-increases trypsin in female Drosophila hemolymph'. doi: 10.1016/j.ibmb.2007.11.010.

Pinheiro, J. *et al.* (2018) 'nlme: Linear and Nonlinear Mixed Effects Models.', *R package*.

Pischedda, A. *et al.* (2011) 'Male genotype influences female reproductive investment in Drosophila melanogaster', *Proceedings of the Royal Society B: Biological Sciences*, 278(1715), pp. 2165–2172. doi: 10.1098/rspb.2010.2272.

Polak, M. *et al.* (2001) ‘Function of the mating plug in Drosophila hibisci Bock’, *Behavioral Ecology and Sociobiology*, 49(2–3), pp. 196–205. doi: 10.1007/s002650000281.

Powell, J. R. (1997) *Progress and Prospects in Evolutionary Biology: The Drosophila Model*, *Oxford University Press, New York*. doi: 10.1017/CBO9781107415324.004.

Price, C. S. C., Dyer, K. A. and Coyne, J. A. (1999) ‘Sperm competition between Drosophila males involves both displacement and incapacitation’, *Nature*, 400(6743), pp. 449–452. doi: 10.1038/22755.

Priest, N. K., Galloway, L. F. and Roach, D. a (2008) ‘Mating frequency and inclusive fitness in Drosophila melanogaster.’, *The American naturalist*. doi: 10.1086/523944.

Qazi, M. C. B. and Wolfner, M. F. (2003) ‘An early role for the Drosophila melanogaster male seminal protein Acp36DE in female sperm storage’, *Journal of Experimental Biology*, 206(19), pp. 3521–3528. doi: 10.1242/jeb.00585.

Ram, K. R., Ji, S. and Wolfner, M. F. Ã. (2005) ‘Fates and targets of male accessory gland proteins in mated female Drosophila melanogaster’. doi: 10.1016/j.ibmb.2005.05.001.

Ram, K. R. and Wolfner, M. F. (2007) ‘Seminal influences: Drosophila Acps and the molecular interplay between males and females during reproduction’, *Integrative and Comparative Biology*, 47(3), pp. 427–445. doi: 10.1093/icb/icm046.

Ram, K. R. and Wolfner, M. F. (2009) ‘A network of interactions among seminal proteins underlies the long-term postmating response in Drosophila’, *Proceedings of the National Academy of Sciences*, 106(36), pp. 15384–15389. doi: 10.1073/pnas.0902923106.

Ram, R. K., Ji, S. and Wolfner, M. F. (2005) ‘Fates and targets of male accessory gland proteins in mated female Drosophila melanogaster’, *Insect Biochemistry and Molecular Biology*, 35(9), pp. 1059–1071. doi: 10.1016/j.ibmb.2005.05.001.

Ramakers, C. *et al.* (2003) ‘Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data’, 339, pp. 62–66. doi: 10.1016/S0304-3940(02)01423-4.

Rawlings, J. S. *et al.* (2004) ‘Two Drosophila suppressors of cytokine signaling (SOCS) differentially regulate JAK and EGFR pathway activities’, *BMC Cell Biology*. doi: 10.1186/1471-2121-5-38.

Ribeiro, C. and Dickson, B. J. (2010) ‘Sex peptide receptor and neuronal TOR/S6K signaling modulate nutrient balancing in Drosophila’, *Current Biology*. Elsevier Ltd, 20(11), pp. 1000–1005. doi: 10.1016/j.cub.2010.03.061.

Rice, W. R. (1996) ‘Sexually antagonistic male adaptation triggered by experimental arrest of female evolution’, *Nature*, pp. 232–234. doi: 10.1038/381232a0.

Robertson, S. A. *et al.* (2014) ‘Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring’, *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.1305609111.

Rogers, D. W. *et al.* (2009) ‘Transglutaminase-mediated semen coagulation controls sperm storage

in the malaria mosquito', *PLoS Biology*. doi: 10.1371/journal.pbio.1000272.

Roy, S. *et al.* (2010) 'Identification of functional elements and regulatory circuits by *Drosophila* modENCODE.', *Science (New York, N.Y.)*, 330(6012), pp. 1787–97. doi: 10.1126/science.1198374.

RStudio (2016) 'RStudio: Integrated development for R', *[Online] RStudio, Inc., Boston, MA URL http://www. rstudio. com*, p. RStudio, Inc., Boston, MA. doi: 10.1007/978-81-322-2340-5.

Rubin, G. M. and Lewis, E. B. (2000) 'A brief history of Drosophila's contributions to genome research', *Science*, pp. 2216–2218. doi: 10.1126/science.287.5461.2216.

Ruijter, J. M. *et al.* (2009) 'Amplification efficiency : linking baseline and bias in the analysis of quantitative PCR data', 37(6). doi: 10.1093/nar/gkp045.

Ruiz, M. *et al.* (2011) 'Sex-dependent modulation of longevity by two Drosophila homologues of human Apolipoprotein D, GLaz and NLaz', *Experimental Gerontology*. Elsevier Inc., 46(7), pp. 579–589. doi: 10.1016/j.exger.2011.02.014.

Rus, F. *et al.* (2013) 'Ecdysone triggered PGRP-LC expression controls Drosophila innate immunity', *EMBO Journal*. Nature Publishing Group, 32(11), pp. 1626–1638. doi: 10.1038/emboj.2013.100.

Saudan, P. *et al.* (2002) 'Ductus ejaculatorius peptide 99B (DUP99B), a novel Drosophila melanogaster sex-peptide pheromone', *European Journal of Biochemistry*. doi: 10.1046/j.0014-2956.2001.02733.x.

Schmid-Hempel, P. (2005) 'Evolutionary Ecology of Insect Immune Defenses', *Annual Review of Entomology*, 50(1), pp. 529–551. doi: 10.1146/annurev.ento.50.071803.130420.

Schmidt, T. *et al.* (1993) 'The Drosophila melanogaster sex-peptide: A molecular analysis of structure-function relationships', *Journal of Insect Physiology*, 39(5), pp. 361–368. doi: 10.1016/0022-1910(93)90023-K.

Schnakenberg, S. L., Siegal, M. L. and Bloch Qazi, M. C. (2012) 'Oh, the places they'll go', *Spermatogenesis*, 2(3), pp. 224–235. doi: 10.4161/spmg.21655.

Scott, D. (1986) 'Sexual mimicry regulates the attractiveness of mated Drosophila melanogaster females.', *Proceedings of the National Academy of Sciences of the United States of America*, 83(November), pp. 8429–8433. doi: 10.1073/pnas.83.21.8429.

Scott, D. and Jackson, L. L. (1988) 'Interstrain comparison of male-predominant antiaphrodisiacs in Drosophila melanogaster', *Journal of Insect Physiology*, 34(9), pp. 863–871. doi: 10.1016/0022-1910(88)90120-5.

Scott, D. and Richmond, R. C. (1987) 'Evidence against an antiaphrodisiac role for cis-vaccenyl acetate in Drosophila melanogaster', *Journal of Insect Physiology*, 33(5), pp. 363–369. doi: 10.1016/0022-1910(87)90125-9.

Sepil, I. *et al.* (2018) 'Quantitative proteomics identification of seminal fluid proteins in male Drosophila melanogaster', *bioRxiv*, p. 296491. doi: 10.1101/296491.

Shannon, P. *et al.* (2003) 'Cytoscape: A software Environment for integrated models of

biomolecular interaction networks', *Genome Research*, 13(11), pp. 2498–2504. doi: 10.1101/gr.1239303.

Shorrocks, B. (1972) 'Drosophila.' London: Ginn & Company Ltd., p. 144pp.

Short, S. M. and Lazzaro, B. P. (2010) 'Female and male genetic contributions to post-mating immune defence in female Drosophila melanogaster', *Proceedings of the Royal Society B: Biological Sciences*, 277(1700), pp. 3649–3657. doi: 10.1098/rspb.2010.0937.

Short, S. M. and Lazzaro, B. P. (2013) 'Reproductive Status Alters Transcriptomic Response to Infection in Female *Drosophila melanogaster*', *G3: Genes|Genomes|Genetics*, 3(5), pp. 827–840. doi: 10.1534/g3.112.005306.

Simmons, L. W. (2001) *Sperm Competition and Its Evolutionary Consequences in the Insects*, *book*. doi: 10.1111/j.1469-185X.1970.tb01176.x.

Singh, A. *et al.* (2011) 'Opposing interactions between homothorax and Lobe define the ventral eye margin of Drosophila eye', *Developmental Biology*, 359(2), pp. 199–208. doi: 10.1016/j.ydbio.2011.08.017.

Sirot, L. K. *et al.* (2011) 'Towards a semen proteome of the dengue vector mosquito: Protein identification and potential functions', *PLoS Neglected Tropical Diseases*. doi: 10.1371/journal.pntd.0000989.

Sirot, L. K. *et al.* (2015) 'Sexual conflict and seminal fluid proteins: A dynamic landscape of sexual interactions', *Cold Spring Harbor Perspectives in Biology*, 7(2). doi: 101101/cshperspect.a017533.

Smith, D. T. *et al.* (2009) 'Variation in sex peptide expression in D. melanogaster', *Genetics Research*, 91(4), pp. 237–242. doi: 10.1017/S0016672309000226.

Smith, D. T. *et al.* (2012) 'The consequences of genetic variation in sex peptide expression levels for egg laying and retention in females', *Heredity*. Nature Publishing Group, 109(4), pp. 222–225. doi: 10.1038/hdy.2012.32.

Smith, M. K. and Wakimoto, B. T. (2007) 'Complex regulation and multiple developmental functions of misfire, the Drosophila melanogaster ferlin gene', *BMC Developmental Biology*, 7, pp. 1–15. doi: 10.1186/1471-213X-7-21.

Smith, R. (1979) 'Repeated Copulation and Sperm Precedence: Paternity Assurance for a Male Brooding Water Bug', *Science*.

Smith, R. L. (1984) *Sperm Competition and the Evolution of Animal Mating systems*, *Academic Press, Inc*. doi: 10.1016/B978-0-12-100543-6.50023-4.

Snedden, W. A. (1990) 'Determinants of Male Mating Success in the Temperate Crayfish Orconectes Rusticus: Chela Size and Sperm Competition', *Behaviour*, 115(1–2), pp. 100–113. doi: 10.1163/156853990X00301.

Snook, R. R. (2005) 'Sperm in competition: Not playing by the numbers', *Trends in Ecology and Evolution*, pp. 46–53. doi: 10.1016/j.tree.2004.10.011.

Snook, R. R. and Hosken, D. J. (2004) 'Sperm death and dumping in Drosophila', *Nature*,

428(6986), pp. 939–941. doi: 10.1038/nature02455.

Soller, M., Bownes, M. and Kubli, E. (1997) ‘Mating and sex peptide stimulate the accumulation of yolk in oocytes of Droosophila melanogaster’, *European Journal of Biochemistry*, 243(3), pp. 732–738. doi: 10.1111/j.1432-1033.1997.00732.x.

Soller, M., Bownes, M. and Kubli, E. (1999) ‘Control of oocyte maturation in sexually mature Drosophila females’, *Developmental Biology*, 208(2), pp. 337–351. doi: 10.1006/dbio.1999.9210.

Song, X. (2003) ‘wingless signaling regulates the maintenance of ovarian somatic stem cells in Drosophila’, *Development*, 130(>14), pp. 3259–3268. doi: 10.1242/dev.00524.

Spieth, H. T, and Ringo, J. (1983) ‘Mating behaviour and sexual isolation in Drosophila’, *In The Genetics and Biology of Drosophila*, 3((New York: Academic Press), pp. 223–284), p. 1983.

Spieth, H. T. (1974) ‘Courtship behavior in Drosophila’, *Annual Review of Entomology*, 19, pp. 383–406. doi: 10.1146/annurev.en.19.010174.002125.

Stockley, P. *et al.* (1997) ‘Sperm Competition in Fishes: The Evolution of Testis Size and Ejaculate Characteristics’, *The American Naturalist*, 149(5), pp. 933–954. doi: 10.1086/286031.

Sturtevant, A. H. (1915) ‘Experiments on sex recognition and the problem of sexual selection in Drosoophilia’, *Journal of Animal Behavior*, 5(5), pp. 351–366. doi: 10.1037/h0074109.

Sturtevant, A. H. (1920) ‘Genetic Studies on Drosophila Simulans. I. Introduction. Hybrids With Drosophila Imelanogaster’, *Genetics*, 5, pp. 488–500. doi: 10.1002/jez.b.21128.

Swanson, W. J. and Vacquier, V. D. (2002) ‘The rapid evolution of reproductive proteins’, *Genetics*, 3(February), pp. 137–144. doi: 10.1038/nrg/733.

Tadros, W. *et al.* (2003) ‘Regulation of maternal transcript destabilization during egg activation in drosophila’, *Genetics*, 164(3), pp. 989–1001.

Tastan, O. Y. *et al.* (2010) ‘Drosophila ataxin 2-binding protein 1 marks an intermediate step in the molecular differentiation of female germline cysts.’, *Development (Cambridge, England)*, 137(19), pp. 3167–3176. doi: 10.1242/dev.050575.

Terry, N. A. *et al.* (2006) ‘Novel regulators revealed by profiling Drosophila testis stem cells within their niche’, *Developmental Biology*, 294(1), pp. 246–257. doi: 10.1016/j.ydbio.2006.02.048.

Therneau, M. (2015) ‘Coxme: Mixed effects Cox models.’, (R package version 2.2-5).

Tinsley, M. C., Blanford, S. and Jiggins, F. M. (2006) ‘Genetic variation in Drosophila melanogaster pathogen susceptibility’, *Parasitology*, 132(6), pp. 767–773. doi: 10.1017/S0031182006009929.

Tompkins, L. and Hall, J. C. (1981) ‘The different effects on courtship of volatile compounds from mated and virgin Drosophila females’, *Journal of Insect Physiology*, 27(1), pp. 17–21. doi: 10.1016/0022-1910(81)90026-3.

Tram, U. and Wolfner, M. F. (1999) ‘Male seminal fluid proteins are essential for sperm storage in Drosophila melanogaster.’, *Genetics*, 153(2), pp. 837–844. Available at:

http://www.genetics.org/content/153/2/837.short.

Trivers, R. L. L. (1972) ‘Parental investment and sexual selection’, *Sexual selection and the descent of man*, pp. 136–179. doi: 10.1002/ajpa.1330400226.

Tzou, P. *et al.* (2000) ‘Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia’, *Immunity*, 13(5), pp. 737–748. doi: 10.1016/S1074-7613(00)00072-8.

Valanne, S., Wang, J.-H. and Ramet, M. (2011) ‘The Drosophila Toll Signaling Pathway’, *The Journal of Immunology*, 186(2), pp. 649–656. doi: 10.4049/jimmunol.1002302.

Vander-Meer, R. K. *et al.* (1986) ‘A reevaluation of the role of cis-vaccenyl acetate, cis-vaccenol and esterase 6 in the regulation of mated female sexual attractiveness in Drosophila melanogaster’, *Journal of Insect Physiology*, 32(8), pp. 681–686. doi: 10.1016/0022-1910(86)90109-5.

Verspoor, R. L. and Haddrill, P. R. (2011) ‘Genetic Diversity , Population Structure and Wolbachia Infection Status in a Worldwide Sample of Drosophila melanogaster and D . simulans Populations’, 6(10). doi: 10.1371/journal.pone.0026318.

Vogel, C. (2003) ‘The immunoglobulin superfamily in Drosophila melanogaster and Caenorhabditis elegans and the evolution of complexity’, *Development*, 130(25), pp. 6317–6328. doi: 10.1242/dev.00848.

Waage, J. K. (1979) ‘Dual function of the damselfly penis: Sperm removal and transfer’, *Science*, 203(4383), pp. 916–918. doi: 10.1126/science.203.4383.916.

Walldorf, U. *et al.* (2000) ‘Homeobrain, a novel paired-like homeobox gene is expressed in the Drosophila brain’, *Mechanisms of Development*, 96(1), pp. 141–144. doi: 10.1016/S0925-4773(00)00380-4.

Warde-Farley, D. *et al.* (2010) ‘The GeneMANIA prediction server: Biological network integration for gene prioritization and predicting gene function’, *Nucleic Acids Research*, 38(SUPPL. 2). doi: 10.1093/nar/gkq537.

Watson, F. L. *et al.* (2005) ‘Immunology: Extensive diversity of Ig-superfamily proteins in the immune system of insects’, *Science*, 309(5742), pp. 1874–1878. doi: 10.1126/science.1116887.

Wedell, N. *et al.* (2002) ‘Sperm competition , male prudence and sperm-limited females’, 17(7), pp. 313–320.

Wertheim, B. *et al.* (2006) ‘Effects of aggregation pheromone on individual behaviour and food web interactions: a field study on Drosophila’, *Ecological Entomology*, 31(3), pp. 216–226. doi: 10.1111/j.1365-2311.2006.00757.x.

Wertheim, B., Dicke, M. and Vet, L. E. M. (2001) ‘Behavioural plasticity in support of a benefit for aggregation pheromone use in Drosophila melanogaster’, *Entomologia Experimentalis et Applicata*, 103(1), pp. 61–71. doi: 10.1023/A:1019832101537.

Wigby, S. *et al.* (2008) ‘The effect of mating on immunity can be masked by experimental piercing in female Drosophila melanogaster’, *Journal of Insect Physiology*, 54(2), pp. 414–420. doi: 10.1016/j.jinsphys.2007.10.010.

Wigby, S. *et al.* (2009) ‘Seminal Fluid Protein Allocation and Male Reproductive Success’, *Current Biology*. Elsevier Ltd, 19(9), pp. 751–757. doi: 10.1016/j.cub.2009.03.036.

Wigby, S. and Chapman, T. (2004a) ‘Female resistance to male harm evolves in response to manipulation of sexual conflict’, *Evolution*, 58(5), pp. 1028–1037. doi: 10.1111/j.0014-3820.2004.tb00436.x.

Wigby, S. and Chapman, T. (2004b) ‘Sperm competition’, *Current Biology*, 14(3), pp. R100–R103. doi: 10.1016/j.cub.2004.01.013.

Wigby, S. and Chapman, T. (2005) ‘Sex peptide causes mating costs in female Drosophila melanogaster’, *Current Biology*, 15(4), pp. 316–321. doi: 10.1016/j.cub.2005.01.051.

Wojtowicz, W. M. *et al.* (2007) ‘A Vast Repertoire of Dscam Binding Specificities Arises from Modular Interactions of Variable Ig Domains’, *Cell*, 130(6), pp. 1134–1145. doi: 10.1016/j.cell.2007.08.026.

Wolfner, M. F. *et al.* (1997) ‘New genes for male accessory gland proteins in Drosophila melanogaster.’, *Insect Biochemistry and Molecular Biology*. doi: 10.1016/S0965-1748(97)00056-8.

Wolfner, M. F. (1997) ‘Tokens of love: Functions and regulation of Drosophila male accessory gland products’, *Insect Biochemistry and Molecular Biology*, 27(3), pp. 179–192. doi: 10.1016/S0965-1748(96)00084-7.

Wolfner, M. F. (2007) ‘“S.P.E.R.M.” (seminal proteins (are) essential reproductive modulators): the view from Drosophila.’, *Society of Reproduction and Fertility supplement*.

Xi, R., McGregor, J. R. and Harrison, D. A. (2003) ‘A gradient of JAK pathway activity patterns the anterior-posterior axis of the follicular epithelium’, *Developmental Cell*, 4(2), pp. 167–177. doi: 10.1016/S1534-5807(02)00412-4.

Xiong, X. P. *et al.* (2016) ‘miR-34 Modulates Innate Immunity and Ecdysone Signaling in Drosophila’, *PLoS Pathogens*, 12(11), pp. 1–30. doi: 10.1371/journal.ppat.1006034.

Xu, J. and Wang, Q. (2011) ‘Seminal fluid reduces female longevity and stimulates egg production and sperm trigger oviposition in a moth’, *Journal of Insect Physiology*. Elsevier Ltd, 57(3), pp. 385–390. doi: 10.1016/j.jinsphys.2010.12.006.

Yamamoto, D., Jallon, J. M. and Komatsu, A. (1997) ‘Genetic dissection of sexual behavior in Drosophila melanogaster’, *Annu Rev Entomol*, 42, pp. 551–585. doi: 10.1146/annurev.ento.42.1.551.

Yang, C. hui *et al.* (2009) ‘Control of the Postmating Behavioral Switch in Drosophila Females by Internal Sensory Neurons’, *Neuron*, 61(4), pp. 519–526. doi: 10.1016/j.neuron.2008.12.021.

Yang, S. A. *et al.* (2013) ‘FOXO/Fringe is necessary for maintenance of the germline stem cell niche in response to insulin insufficiency’, *Developmental Biology*. Elsevier, 382(1), pp. 124–135. doi: 10.1016/j.ydbio.2013.07.018.

Yapici, N. *et al.* (2008) ‘A receptor that mediates the post-mating switch in Drosophila reproductive behaviour’, *Nature*, 451(7174), pp. 33–37. doi: 10.1038/nature06483.

Yoo, H. *et al.* (2015) ‘Drosophila Cappuccino alleles provide insight into formin mechanism and role in oogenesis’, *Molecular Biology of the Cell*, 26(10), pp. 1875–1886. doi: 10.1091/mbc.E14-11-1558.

Yoon, W. H., Meinhardt, H. and Montell, D. J. (2011) ‘miRNA-mediated feedback inhibition of JAK/STAT morphogen signalling establishes a cell fate threshold’, *Nature Cell Biology*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 13, p. 1062. Available at: http://dx.doi.org/10.1038/ncb2316.

Yu, J. *et al.* (2006) ‘The KASH domain protein MSP-300 plays an essential role in nuclear anchoring during Drosophila oogenesis’, *Developmental Biology*, 289(2), pp. 336–345. doi: 10.1016/j.ydbio.2005.10.027.

Zahavi, A. (1975) ‘Mate selection-A selection for a handicap’, *Journal of Theoretical Biology*, 53(1), pp. 205–214. doi: 10.1016/0022-5193(75)90111-3.

Zarnescu, D. C. and Thomas, G. H. (1999) ‘Apical spectrin is essential for epithelial morphogenesis but not apicobasal polarity in Drosophila’, *Journal of Cell Biology*, 146(5), pp. 1075–1086. doi: 10.1083/jcb.146.5.1075.

Zhou, H., Fisher, R. J. and Papas, T. S. (1994) ‘Universal immuno-PCR for ultra-sensitive target protein detection’, *Nucleic Acids Research*, p. 892. doi: 10.1093/nar/22.5.892-a.

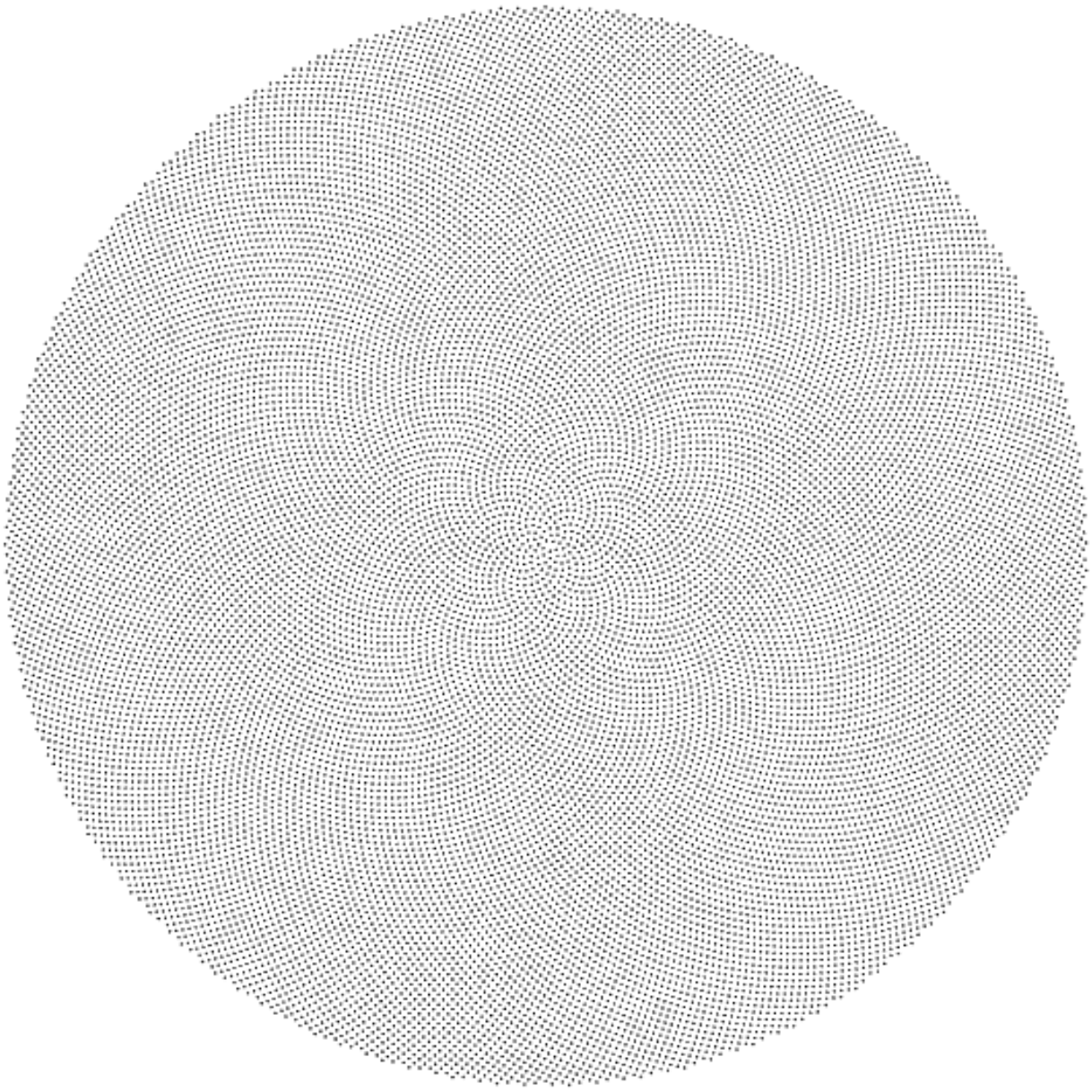
Zinn, K. and Özkan, E. (2017) ‘Neural immunoglobulin superfamily interaction networks’, *Current Opinion in Neurobiology*, 45, pp. 99–105. doi: 10.1016/j.conb.2017.05.010.

Zuckerkandl, E. (1976) ‘Evolutionary processes and evolutionary noise at the molecular level - I. Functional Density in Proteins’, *Journal of Molecular Evolution*, 7(3), pp. 167–183. doi: 10.1007/BF01731487.

Zuk, M., Rotenberry, J. T. and Tinghitella, R. M. (2006) ‘Silent night: Adaptive disappearance of a sexual signal in a parasitized population of field crickets’, *Biology Letters*, 2(4), pp. 521–524. doi: 10.1098/rsbl.2006.0539.

Zuur, A. F. *et al.* (2009) ‘Mixed Effects Models and Extensions in Ecology with R’, *Statistics for Biology and Health*, p. 579 p. doi: 10.1007/978-0-387-87458-6.

Zwaan, B. J., Bijlsma, R. and Hoekstra, R. F. (1991) ‘On the developmental theory of ageing. I. starvation resistance and longevity in drosophila melanogaster in relation to pre-adult breeding conditions’, *Heredity*, 66(1), pp. 29–39. doi: 10.1038/hdy.1991.4.



Representative of the 20000 female flies used in this thesis.