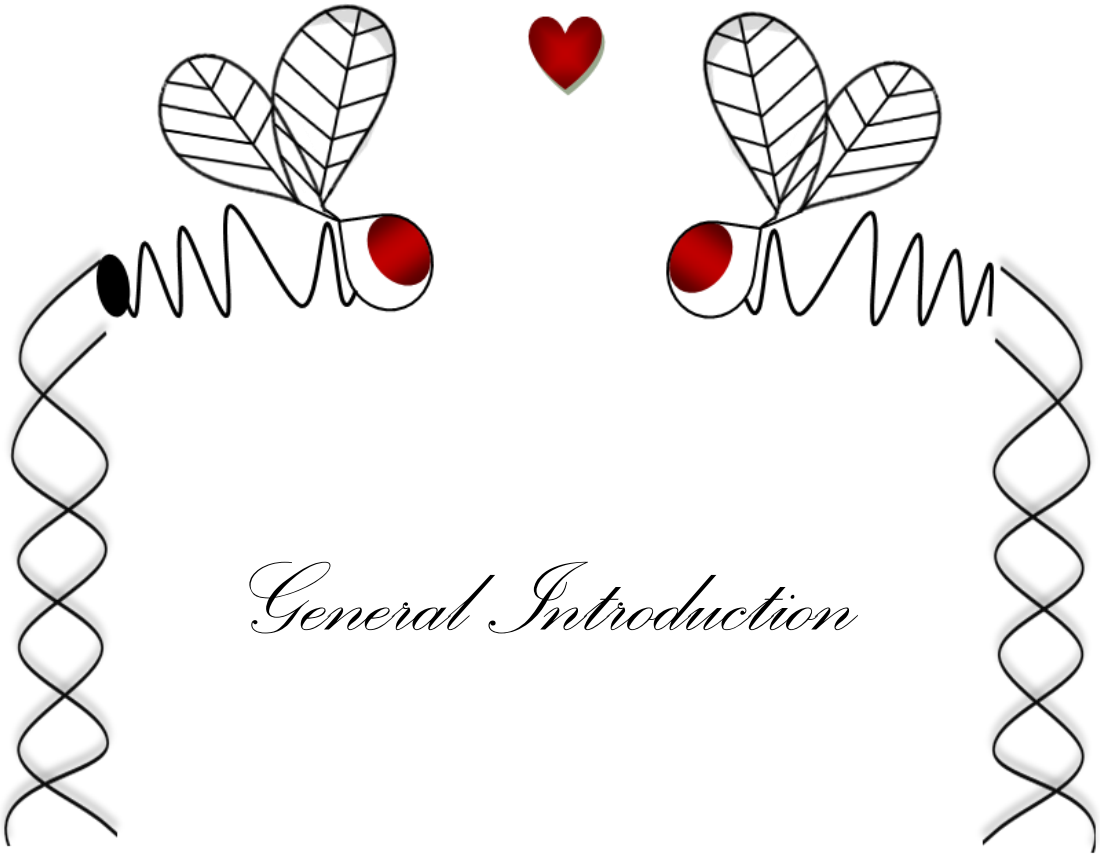
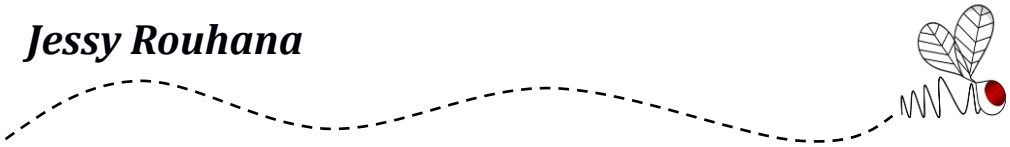
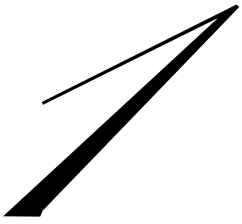


General Introduction

Jessy Rouhana





Introduction

Living organisms compete for survival and reproduction, whereupon the fittest live and thrive and the weakest fail and some cases even die. This battle for life acts 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. These occur because there are differences in the evolutionary interests of the sexes over, for example, optimal reproductive rate, gamete size and parental investments. This has led to the evolution of different strategies to alter or overcome the manipulation of one sex by other, while maintaining a base line level of cooperation sufficient to ensure successful reproduction. This sexual conflict is an important evolutionary process as it can drive rapid evolutionary change.

The manipulation of one sex by the other through molecular interactions has been illuminated in studies using the fruit fly *Drosophila melanogaster.* Males tend to maximize their chances at fatherhood by releasing large ejaculates containing numerous sperm inside the female's body. The effects of semen proteins can benefit both sperm and eggs, but intriguingly they can also favour the interests of males whilst generating costs in females. One enigmatic semen protein of the fruit fly, 'Sex Peptide', generates strikingly diverse changes in the behavior, reproductive and immune system of the female. These changes benefit males by increasing their share of paternity but can also result in costs to females.

In most studies of the molecular mechanisms of reproductive manipulation of females by males via the effects of Sex peptide, genetic variation has been minimized in order to clearly delineate Sex Peptide function. 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. In this thesis, I trace the impact of Sex Peptide to explore the consequences of sexual conflict for the evolution of the *Drosophila melanogaster* genome. An in-depth investigation was performed to measure the extent of phenotypic and genomic variation in Sex Peptide transfer among males and responses to it among females, using 32 Drosophila reference panel lines (DGRP). I focused on measuring female post-mating phenotypic traits in response to receipt of Sex Peptide (immunity, egg laying, receptivity to re-mating and longevity). I also developed a novel quantification technique to quantify variation in the amount of Sex Peptide transferred among males from 32 DGRP lines. A genome wide association (GWAS) and functional annotation study of the different phenotypic traits tested revealed significant

genetic variation in a number of genes involved in the transfer and the post-mating response of Sex Peptide. This investigation of natural variation allowed us to map phenotypes to genomic variation, and to determine the 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 the fundamental differences, **anisogamy** in sexual reproduction, in which females generally produce a small number of immobile **macrogametes** (ovules) rich with energy, while males produce a great excess of mobile **microgametes** (spermatozoids)(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 gametes size is proportional to the parental investment between sexes. This concerns both the production of the gametes, and possibly the nourishing of the developing embryos. Additionally, each male can potentially produce more offspring than each female, particularly when they are only minimally involved in caring for the 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 the female's. In some species these so called 'sex roles' roles are even reversed, with males providing nutrition to their mates females and caring for the 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 for 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 engenders **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, the females favour such characters because they benefit their sons through increased attractiveness.

Sexual conflict and mating strategies

An individual's success at reproducing often depends on its behaviour. Hence sexual selection tends to favour animals that are efficient in mate selection, avoiding competition, copulating and parental care. Therefore, sexual conflict occurs 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(1)).

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 heightening the male's vulnerability to predators, as well as the costs of maintaining these traits 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, Watts and Altmann, 2003). A contrasting strategy is called 'scramble', in which males use enhanced speed to find mates before rivals, which may lead males to emerge or even mature and become reproductively active before females (Bulmer, 1983; Morbey and Ydenberg, 2001). An endurance strategy would be 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 that rival males can achieve insemination 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 releases pheromones that act as anti-aphrodisiacs, repelling other males from mating with the labelled female (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 male's chances in fertilizing the females' ova, therefore males have evolved complex genital morphology. The evolution of internal fertilization is thought to reduce the absolute risk of sperm competition (Parker, 1970; Smith, 1984) and provide females with a mechanism of selecting "good" motile sperm (Keller, 1999). Consistent with the idea that sexual selection can drive reproductive trait evolution, it is found that the complexity of the penis morphology is more elaborated in species where 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 sperm from previous males by ejaculating new sperm into the female, thus hindering successful insemination opportunities of the previous male (Xu and Wang, 2010). Infanticide is also considered a type of sexual offensive strategy because it enhances the reproductive success of males. In several species with internal gestation brood or offspring care, males resort to infanticide by inducing abortion or killing offspring to make 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 “role reversal” i.e. when males are the sex investing more time and energy in caring for offspring, females compete to be chosen by males. However, for convenience here we 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). Subject to inter-sexual selection, male traits favoured by females will evolve to exaggerated states if they enhance mating success. The female preferences affecting the male traits could have direct benefits, such that the 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 an honest signal of male quality and are under 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 benefit to either female or her offspring, but contributes only his, presumably high quality, genes. The preferred male may provide genes that increase the survivorship or mating success of the offspring as compared to the genes provided by less desirable males (Kokko *et al.*, 2003). 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 Morgan and his students Sturtevant, Bridges and Muller, that *Drosophila* started to be adopted and 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 where 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* is cheap and easy to culture and maintain in the laboratory, it has a short life cycle and also produces 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).

Drosophila 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* presents a great model system to study sexual selection. *Drosophila melanogaster* has a complex repertoire of mating behaviours including male courtship rituals to stimulate females (Bastock and Manning, 1955) and female choice behaviours within a polyandrous mating system, These features allow the potential for

increased 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 in the attention of many research groups, leading to numerous novel discoveries in this field, as summarised below.

Reproductive system

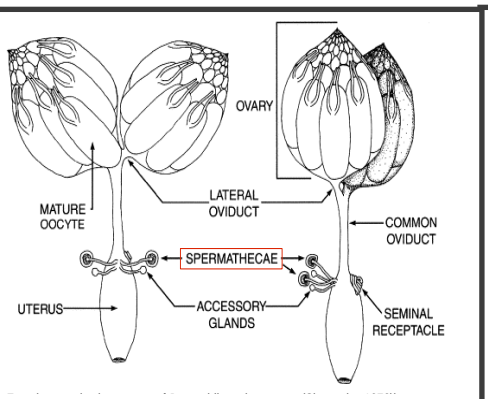


Figure 1: Female reproductive system of *Drosophila melanogaster* (Shorrocks, 1972)

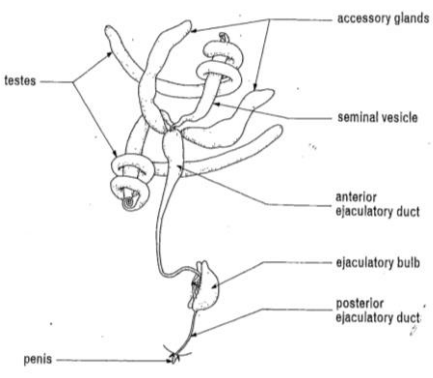


Figure 2: Male reproductive system of *Drosophila melanogaster* (Shorrocks, 1972)

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 storage: paired spermathecal glands (also called female

accessory glands) that produce a variety of substances for sperm maintenance and egg protection, two *spermathecae* (for long-term storage), as well as a *seminal receptacle* (the primary sperm storage organ) and ducts connecting these organs (Figure 1).

The reproductive system of male *D. melanogaster* consists of two testes each containing spermatozoa in various stages of development. At the anterior ends of the testes two *vasa deferentia* tubulars are swollen to 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. Following, females lay fertilized eggs often in clutches, on a soft substrate suitable for larval development. After embryogenesis, the larva hatches from the egg and starts immediately feeding on the soft substrate. The larvae transition through three larval instars, followed by pupation. The developmental time varies from species to species and 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. 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 one reproductive cycle. As a result, sperm of different males come in contact in the female reproductive system, where they have to compete for the fertilization of the ova (Imhof *et al.*, 1998). In general, female *Drosophila* reproductive success is mainly limited by egg production, whereas in males, reproductive success is limited by access to the females (Bateman, 1948). This divergence in interests of males and females leads to sexual conflict and sexual selection. In males, selection favours an increase in gamete productivity, at the cost of the contribution to zygote resources. In females, optimum ovum size is much larger, limiting the maximum number of gametes. These differing strategies, and the consequences that flow from them, 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 despite any cost to females, and females resist the mating costs inflicted by males (Rice, 1996). In addition, male harassment can be costly and harmful to females, also resulting in sexual conflict in *D. melanogaster*. Males have also evolved strategies based on the effects of seminal protein transfer, the side effects of which 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 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 is always some degree of choice about whether or not to mate. In *D. melanogaster,* selection is likely to act on the female to show discrimination in mate choice. This is because the female's contribution to ova exceeds male investment in sperm, hence the losses to females far exceed those of males for a given degree of progeny inviability. Therefore, selective mating seems likely to be performed principally by female *D. melanogaster*, accepting males with which they wish to mate and repelling males with which they do not. Males, on the other hand, do not seem to exert mating preferences and usually mate at random (Merrell, 1949). This "selective mating" within *Drosophila* females will not only act to minimise hybridization, but it will also affect the genetic structure of populations. This is an excellent example of the principle underpinning Darwin's theory of sexual selection (Darwin, 1871).

Male Behavior: Male *Drosophila* do not attempt to mate with a female without 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. 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* is much simpler. If receptive, female spread her wings and genitalia, and she permits copulation. If unreceptive, the female will refuse to mate and give several rejection signals. These rejection behaviours differ between virgin and fertilized females. Virgins usually escape from courting males by decamping and fending off, whereas the fertilized female typically extrudes her ovipositor, which physically prevents genital contact. Immature virgin also produces 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 days (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 Sex Peptide 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 sperm receptacles (Manning, 1967).

2) Intra-sexual Selection

Intra-sexual selection and harmful mating strategies are observed to be more intense in species with greater last mate advantage, such as 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 the sperm compete within the female for fertilization of the ova.

- **Coercion:** The occurrence of forced mating in *D. melanogaster* is more common than has been suspected and may account for at least some of the observed sperm competition in *Drosophila* (Manning, 1962). After their first mating, female *D. melanogaster* become unreceptive and tend to reject courting males for at least 24h (Manning, 1967). However, males can overcome female rejection behavior and force females into re-mating (Manning, 1962). In addition, male *D. melanogaster* may also force newly emerged females to copulate, as these females appear incapable of performing any rejection behaviours (Markow, 2000). In many cases forced copulation appears to occur in the absence of courtship.

- **Contest competition:** Intense aggressive behavior has been detected in male *D. melanogaster*, when 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 opponents, 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 rise 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 extensive sperm storage capacity of females and because of high rates of multiple inseminations. *D. melanogaster* males have adopted different strategies to increase their reproductive success under the threat of sperm competition:

1. **Sperm quantity:** male *D. melanogaster* increase the number of sperm in their ejaculate when the perceived risk of rivalry is high. When males copulate in the presence of rivals, the copulating males 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), and males can even apparently retain a visual memory of the rival (Kim *et al*., 2012). 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, controlled by female (Snook and Hosken, 2004). *D. melanogaster* sperm have a long lifespan and can live up to 3 months at 10°C in female sperm storage or up to 30 days at higher temperatures (Muller and Settles, 1927; Parker, 1970).

3. **Mating plug**: To reduce the probability that a male ejaculate will be displaced during a subsequent mating by the same female, male *D. melanogaster* deploy mating plugs akin to chastity belts. These are formed after sperm transfer at the distal part of the female's reproductive tract, to facilitate sperm movement into storage, prevent a second insemination or prevent sperm loss. In insects, such mating plugs could also have arisen partly through natural selection, since males who are 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 from the accessory glands (Lung and Wolfner, 2001; Chapman and Davies, 2004; Ram and Wolfner, 2005; Avila *et al.*, 2011). The plug is transferred just before sperm transfer, so that initially the sperm are more caudal than the plug, but later pass around it or through it; after separation of male and female the sperm lie above the plug in the upper uterus and sperm stores (Parker, 1970). The plug facilitates sperm movement into the female reproductive tract, preventing sperm from flowing back.

4. **Sperm displacement/ sperm precedence:** One strategy used in sperm competition is the displacement of previously stored sperm during mating (Lefrve and Jonssom, 1962). In *D. melanogaster*, when females re-mate, the last male sires a majority of the offspring, out-competing his predecessor (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 from storage (Price *et al*., 1999). The mechanism behind sperm displacement is physical, the incoming second-male sperm physically displace the resident sperm from the seminal receptacle and the spermathecae (Manier *et al.*, 2010). In *D. melanogaster* the proportion of the offspring's sired by the second male (P2) is high and 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 female attractiveness to other males, and therefore increasing male sexual success. **1)** The **Cuticular hydrocarbon (7-tricosene)** is a major pheromonal component of male *D. melanogaster* and it is usually absent from virgin females. During mating, male *D. melanogaster* transfer 7-tricosene externally to the female cuticle. This pheromone reduces female attractiveness to future males for 3-4 hours after mating (Tompkins and Hall, 1981; Scott, 1986). 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 she is able to 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 is transferred along with the sperm during mating (Brieger and Butterworth, 1970). Once it is transferred in the female tract, the fluid

enzyme esterase 6 metabolize 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 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 help to ensure reproductive success for both sexes and may include adaptations that have been shaped by both sperm competition and sexual selection. However, many seminal fluid proteins appear to function specifically to increase a male's paternity share. The changes in female behavior caused by seminal fluid proteins can favour male reproductive success in two main ways: Firstly, the longer the female remains unreceptive to mating with rival males, the longer the period the competition with other rival sperm is avoided. Secondly, the more offspring a female produces within her period of unreceptivity, the greater will be the male's share of his mate's lifetime reproductive success (Simmons, 2001). *Drosophila melanogaster* males release seminal fluids, known as Sfps (Seminal fluid proteins), from their accessory glands to reduce the likelihood of the female from participating in future copulations. These substances can act as an anti-aphrodisiac, causing a rejection of subsequent copulations, and they also stimulate ovulation and oogenesis (Wolfner, 1997). They may even be toxic to the female, making re-mating costly to her in terms of life span (Fowler and Partridge, 1989; Chapman *et al*., 1995).

Male Accessory Glands

An early transplantation attempt in *D. melanogaster* of male 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 *D. melanogaster* male accessory glands are a secretory tissue of the male reproductive system. They produce and secrete a complex mixture of accessory gland secretions (Chen, 1984). These secretions are transferred along with the sperms to female during copulation. Certain components of these accessory gland secretions 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 also 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 interdispersed "secondary" cells (Figure 3), each of which expresses a set 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 accessory gland matures with it, and 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). 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 they 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).

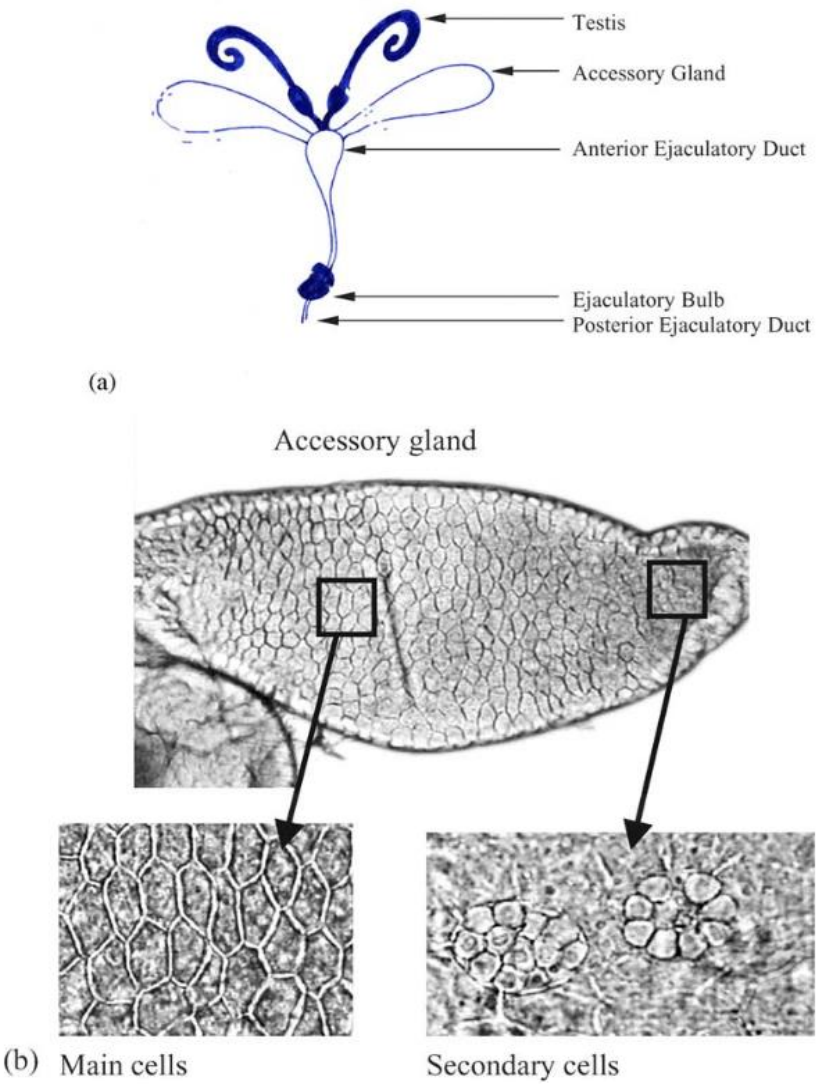


Figure 3: (a) Diagram of internal male *Drosophila melanogaster* reproductive system. (b) Male accessory gland, showing "main" cells and vacuolated "secondary" cells. Glands were dissected into phosphate buffered saline and images captured using a video camera at 100×magnification. After Chapman & Davies 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 bulb. Accessory gland secretions include proteins and peptides, free amino acids and amines, lipids and carbohydrates (Chen, 1984; Gillott, 2003). Male seminal products have been recognized as having significant impact on female reproductive physiology and behavior (Gillott, 2003; Avila *et al.*, 2011). These changes include decreasing receptivity in females to re-mating (Manning, 1962; Manning, 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 increased post-mating aggression in females (Bath *et al.*, 2017). The accessory proteins also contribute to mating plug formation (Lung *et al*., 2001). The well-known genetics of *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 fluid and sperm appear to be temporally separated events in *D. melanogaster,* where copulation 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 thus 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* have approximately 163 seminal fluid proteins (Sfps) that are transferred from males to females during copulation, and most of these Sfps have post-mating effect on females (Avila *et al.*, 2011; Sepil *et al.*, 2018). The functions of many of these substances are 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 the chromosome. The Sfps 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 and further details are given in the next 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
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
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
Sperm storage	Acp36DE	Bertram *et al.*, 1996; Neubaum and Wolfner, 1999; Chapman *et al.*, 2000
Longevity	Acp70A Acp62F CG8137 CG10433	Lung *et al,* 2001; Wigby and Chapman, 2005; Mueller *et al.,* 2007
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
Mating plug	Acp62F Acp76A CG6289 CG9334	Wolfner *et al.*, 1997; Ram and Wolfner, 2005
Immune response	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). Sex Peptide forms part of the seminal fluid that is released during copulation and binds to sperm tails via its N-terminal end (amino acids from 1 to 7). When the sperm are stored in the female reproductive tract, the C-terminal end of Sex Peptide is subsequently released from sperm by a cleavage that occurs between amino acids 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 storage and enters 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 internal sensory neurons that innervate the female uterus and project into the central nervous system, eliciting post-mating responses 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 transfer more Sex Peptide, increasing male reproductive fitness. It is possible that the 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 alters 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).

In *D. melanogaster*, 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. How Sex Peptide precisely regulates female post-mating behavior is still unknown, although its action requires activation of the Sex Peptide receptor. This G-coupled-protein receptor (*CG16752*), is required in neurons that express *fruitless*, *doublesex* and *pickpocket* (Haussmann *et al.*, 2013). Circulating Sex Peptide in the hemolymph reaches the central nervous system, activates the Sex Peptide receptor in a subset of specific neurons, which 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), increased feeding, altered food preferences (Carvalho *et al.*, 2006; Ribeiro and Dickson, 2010), loss of sleep (Isaac *et al.*, 2014), decreased receptivity (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. The behavioral modifications induced by Sex Peptide typically last for approximately a week, which matches the approximate time for which sperm are stored in females from the initial mating.

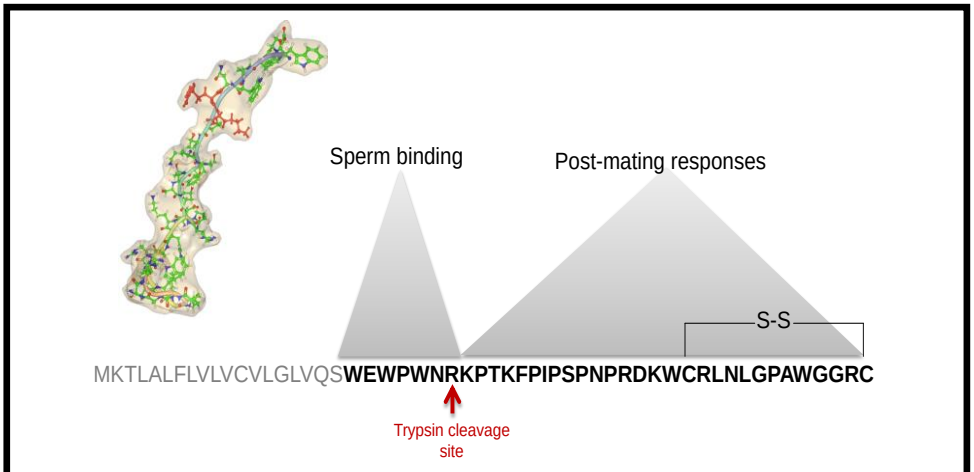


Figure 14: 3D representation and Amino Acid sequence of mature Sex-peptide. The amino acid sequence of mature Sex-peptide of 36 aa (Black), and the precursor of Sex-peptide of 19aa (Grey). After Domanitskaya *et al.*, 2007.

Functions of Sex Peptide

1. **Egg laying:** In *D. melanogaster*, 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, the decrease in receptivity to mating lasts 1-3 days. Alternatively, 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 this 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, that in these assays, females have usually been maintained under conditions that lack an oviposition substrate. This could imply that the wakefulness of females mated with SP^+ males reflects 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., 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 decrease 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 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, 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 to females. When female *D. melanogaster* are exposed to wild-type males, they show significantly lower fitness and lower reproductive success compared to females exposed to Sex Peptide deficient males. Thus, Sex Peptide decreases female fitness and female lifespan (Wigby and Chapman, 2005; Mueller *et al.*, 2007). However, the mechanism(s) by which Sex Peptide decreases female longevity are still unknown.

7. **Immune response:** Sex Peptide induces a post-mating immune response. It is suggested that Sex Peptide acts 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 also induces female aggression directed toward other females after mating. Sex Peptide influence the female social competitive environment, with potentially evolutionary consequences and could be an important fitness consequence for females and their offspring (Bath *et al.*, 2017).

Genetic Variation

Various biotic and abiotic forces throughout time 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 the **del**etions (INDELs) of DNA bases or single nucleotide polymorphisms (SNPs). Mutations are considered 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, 1997). 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, 1997).

The gap in our understanding for the relationship between genotype and phenotype results from the complex interactions between genes and environment. With the advent of next generation molecular sequencing methods, it is now possible to study both the genotype and phenotype, and characterize the genetic variation that occurs within a population. With the availability of full Genomic sequence 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, 1997). 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 referred to as **non-synonymous** substitutions. Those that do not alter the protein sequence; are known as **synonymous** (or silent) substitutions. The evolutionary rate 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 involved in reproductive processes can evolve rapidly. This could be the result of sexually antagonistic co-evolution between the sexes, but also because of ecological conditions. Interestingly, in *D. melanogaster*, the genes encoding male accessory gland proteins are among the most rapidly evolving genes (Hartl and Clark, 1997). The evolutionary rate of non-secreted and non-reproductive proteins is significantly slower in comparison to secreted accessory gland proteins and proteins involved in reproduction (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 suggests that genetic diversification is beneficial for reproduction. This could be due to within and between sex interactions within sexual selection. 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 protein (Sex Peptide) that affect female survival, and because mating alters female immune response (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, 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 the genomics approach to investigate the extent and nature of the genetic variation in Sex Peptide susceptibility traits in mated females as well as the 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 longevity 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 functional 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 a 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 longevity. 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 also 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.