
# The role of sexual selection in fertilisation within the *Drosophila obscura* species group

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## Abstract

Sexual selection, in which one sex typically males - competes for access to the other sex while females exert partner choice, along with sexual conflict which occurs when the reproductive interests of the sexes are not aligned, has the ability to drive the evolution of reproductive traits. The forces of sexual selection and sexual conflict can target traits across the whole organism, including behaviour, physiology, and anatomy. I assessed the effects of sexual selection and sexual conflict in driving reproductive trait evolution across the whole organism in the *Drosophila obscura* species group. In chapter 2, I examined remating behaviours in experimentally evolved populations of *Drosophila pseudoobscura* manipulated under different sexual selection and sexual conflict regimes. I found that sexual selection/conflict has targeted mating behaviour. When males and females were allowed to interact continuously, sexually selected males courted females more frequently than males that did not evolve in an environment with sexual selection. However, this did not affect the number of rematings by females that either had or had not evolved under sexual selection. I also found evidence of sexual conflict in that females who had not evolved under sexual selection (and therefore no sexual conflict) had significantly more progeny over their lifetime than females who had evolved under sexual conflict. In chapter 3, I investigated whether males from these experimental sexual selection populations had evolved differences in reproductive physiology by describing and quantifying proteins from the accessory glands of male *D. pseudoobscura*. Accessory gland proteins (Acps) transferred to the female during mating can alter her behaviour and physiology. Using mass spectrometry, I identified a subset of 395 proteins carrying a secretion signal, as it is these proteins that are candidates for being transferred to females during mating. Subsequent gene ontology analysis showed enrichment for predicted biological functions relating to reproduction, such as sperm competition and post-mating female receptivity, suggesting that I had identified relevant proteins. While there are some unique proteins arising in the different sexual selection populations, the majority of these had no known function or were not annotated as being associated with reproductive functions. In chapter 4, I asked whether variation in sperm morphology, a target of sexual selection due to

sperm competition and cryptic female choice, influences intracellular sperm-egg interactions (ISEI) that could then mediate successful fertilisation. To address this question, I developed a novel methodology by which various ISEI parameters could be measured. Data analysed demonstrates the effectiveness of such an approach, which can be used in future studies to quantify ISEIs and test the extent to which sexual selection can influence them both within and among species. Overall, in this thesis, I find evidence for substantial effects, across the whole organism, in which sexual selection can influence multiple episodes of selection, from pre-copulatory through to post-copulatory effects.

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# Chapter 1. Introduction

Sexual selection is responsible for driving the evolution of traits associated with reproduction and therefore has the ability to influence the reproductive success of individuals (Andersson, 1994). The differential investment of females and males in the size of their gametes means that the sexes maximise their reproductive fitness in different ways; females typically benefit from producing a greater number of progeny over their lifetime (Bateman, 1948; Schärer et al., 2012; Fritzsche & Arnqvist, 2013), whereas males benefit from acquiring a greater number of females (Arnqvist & Nilsson, 2000). Owing to these differences, males are usually in competition for females and, as such, males can seek to improve their reproductive fitness by evolving a variety of behavioural, morphological and/or physiological traits. For example, males have evolved elaborate courtship displays in response to competition with other males (precopulatory; Snook et al., 2005; Debelle et al., 2014). Males invest in precopulatory reproductive traits such as these elaborate courtship displays, to appear more attractive to females with the aim of securing access to a greater number of copulations. But, as females are naturally promiscuous in most organisms (Andersson, 1994), sexual selection can continue to act after copulation, until fertilisation occurs (Tregenza & Wedell, 2000). So-called postcopulatory sexual selection comprises both male-male competition (known as sperm competition) and cryptic female choice and can act on postcopulatory traits, for example, components of the male seminal fluid that can alter female physiology and behaviour (Seminal Fluid Proteins, hereafter, SFPs; Chen, 1984; Avila et al., 2011; Civetta & Reimer, 2014) and female control over paternity (Pitnick et al., 1999). Some traits, such as the production of giant sperm (Pitnick et al., 1995a) in some species of *Drosophila*, carry fitness costs that can only be explained by having been shaped by sexual selection. The cost of such traits can only be outweighed by the increase in reproductive fitness as a consequence of possessing them. In this thesis, I examine a variety of both pre- and post-copulatory traits that could be shaped by sexual selection and/or sexual conflict.

First, I will discuss pre- and post-copulatory sexual selection, sexual conflict and reproductive isolation. I will then summarise the reproductive traits that form the focus of this thesis. Finally, I will then outline the key techniques used to study reproductive trait evolution in a model species group.

**Precopulatory sexual selection**

Sexual selection has the ability to drive divergence in mating traits and their associated preferences (Fisher, 1930). Precopulatory traits that can be shaped by sexual selection include ornaments, such as the peacock's tail, and weaponry (Andersson, 1994), for example, antlers in male deer. These traits can be seen by females as honest signals of male quality because such traits are energetically expensive to produce and could cause the bearer to be conspicuous to potential predators.

An example of precopulatory sexual selection discussed in more detail through this thesis is courtship behaviours. Males can employ elaborate courtship displays to outcompete rival males when females mate preferentially. These can be in the form of resources provided to the female, such as nests or nutrients (Andersson, 1994) or displays such as courtship dances and/or courtship songs (Snook et al., 2005). For example, females from populations of *D. pseudoobscura* selected under experimentally biased sex ratios (which modifies the strength of sexual selection; Kokko et al., 2006) prefer the courtship song of their coevolved males (Debelle et al., 2014). Males can attempt to coerce females to mate with them by exhibiting these, and other, "attractive" traits before mating. Once females mate, interactions between the sexes continue within the female reproductive tract (postcopulatory interactions).

**Sperm competition (postcopulatory sexual selection)**

Sperm competition is a form of male-male competition that occurs when the ejaculates of two or more males reside in a common female reproductive tract and therefore are in competition with each other for fertilisation of that female's eggs (Parker, 1970). Sperm competition is widespread in insects because

females are typically promiscuous and they store sperm (Andersson, 1994). Since male reproductive fitness is linked to the number of partners they acquire (Arnqvist & Nilsson, 2000), males evolve a variety of traits that enhance their competitive ability in response to sperm competition. For example, males can alter testis size in response to selection (Pitnick et al., 2001a; Hosken & Ward, 2001), modify the number of sperm they transfer per ejaculate or alter sperm morphological traits, such as sperm length. Sperm length has been shown to evolve rapidly in response to selection (Pitnick et al., 2003). For example, in some organisms, longer sperm confer a selective advantage (Pitnick & Markow, 1994; Bennison et al., 2014), in others, short sperm are preferentially utilised by females (Morrow & Gage, 2001). Some species of *Drosophila* demonstrate a phenomenon known as sperm gigantism (Pitnick et al., 1995a). Such long sperm carry fitness costs, such as delayed sexual maturity (Pitnick et al., 1995b), so can only be explained by serving some adaptive function in response to sexual selection.

Males can also modify components of their seminal fluid, known as seminal fluid proteins (SFPs), which when transferred to females upon mating, can alter female physiology and behaviour (Chen, 1984; Avila et al., 2011; Civetta & Reimer, 2014). For example, males can utilise SFPs to delay female remating (Chen et al., 1988; Fricke et al., 2009), increase egg laying in mated females (Herndon & Wolfner, 1995; Chapman et al., 2001) and affect sperm storage and usage (Lung et al., 2002; Wigby et al., 2009). In *Drosophila* species, strong male mating order precedence exists, with 80% of progeny sired by the last male to inseminate the female in *D. melanogaster* (Gromko et al., 1984). Males that can manipulate females to delay remating, for example, may benefit from an increased opportunity to fertilise more of a female's eggs during this time. Through evolving these traits, males can gain a significant advantage over other males and some studies have shown that males increase their accessory gland size (the tissue where some SFPs are produced) in response to sperm competition (Bangham et al., 2002; Baker et al., 2003; Crudgington et al., 2009; Wigby et al., 2009). Since the ejaculates of different males evolve in response to

sperm competition, this can create the potential for females to discriminate between these different ejaculates (known as cryptic female choice).

**Cryptic female choice (postcopulatory sexual selection)**

Multiple mating by females allows them to bias sperm usage (Eberhard, 1996). Cryptic female choice is any aspect of female behaviour, morphology and/or physiology that can affect paternity in favour of individual males after mating. These postcopulatory events can include sperm storage and use, oviposition and control over rematings (Ravi Ram & Wolfner, 2007; Avila et al., 2011).

For example, females have been shown to release sperm from the reproductive tract after copulation with a second male (sperm dumping; Snook & Hosken, 2004) and this does not require incoming ejaculates, neither sperm nor seminal fluids. Females can bias sperm storage and use by rapidly evolving sperm storage organs that selectively store sperm. For example, *D. melanogaster* females evolved longer sperm storage organs to selectively bias paternity to males with longer sperm. Consequently, males responded to female selection by evolving longer sperm (Miller & Pitnick, 2002).

Females can also drive the evolution of SFPs through cryptic female choice (Eberhard, 1996; Wolfner, 2009). Females may evolve to increase their threshold to the action of certain SFPs (Chapman, 2001), to ensure only males with the highest quantity and/or quality activate her full post-mating response. This can provide females the opportunity to protect against fertilisation by low quality males. For example, the action of sex peptide (Acp70A) delays female remating (Fricke et al., 2009) and given that, in *Drosophila* species, 80% of progeny are sired by the last male to inseminate the female (Gromko et al., 1984), females may suffer costs of being fertilised by low quality males. If a female can increase her response threshold to the action of Acp70A, she may remate faster if she received ejaculates of lower quality/quantity, than if she received higher quality and/or quantity of this seminal fluid protein. As such, cryptic female choice can select for increased quality and/or quantity of various seminal fluid components.

**Intersexual coevolution (mutual or antagonistic)**

Reproductive traits evolve in response to a female preference for them (either via sexual selection or sensory bias; Fisher, 1930; West-Eberhard, 1984) and this can promote correlated evolution between the sexes which impacts both male and female reproductive fitness (Pitnick et al., 1999; Gavrilets et al., 2001). In mutual coevolution, female preference drives the evolution of a particular male trait, for example, females evolved larger sperm storage organs to selectively bias paternity towards males with longer sperm in *D. melanogaster* (Miller & Pitnick, 2002). As such, males evolved longer sperm in a correlated response to changes in female morphology. The costs associated with evolving longer sperm, such as an increase in development time (Pitnick et al., 1995a; 1995b), are presumably outweighed by the benefits the male receives in fertilisation opportunity.

However, the reproductive interests of the sexes are rarely matched, owing to their differential investment in gametes (Bateman, 1948). These differences can drive the evolution of reproductive traits through a process known as sexual conflict (Parker, 1979; Stockley, 1997; Rice, 2000; Chapman et al., 2003). Both pre- and post-copulatory sexual selection can create the potential for conflict between the sexes. For example, male harassment as a result of courtship (precopulatory; Crudgington et al., 2010) and the evolution of SFPs (postcopulatory; Wolfner et al., 1997) can both cause harm to females as a by-product of male-male competition (Rice, 1996). When the fitness of one sex is impaired, selection favours the evolution of traits that counteract the fitness loss (Parker, 1979; Chapman et al., 1995) and this can drive antagonistic coevolution that can promote increasingly harmful traits (Holland & Rice, 1998; Arnqvist & Rowe, 2005). In particular, both male harassment over courtship and the action of some SFPs can decrease female lifespan (Partridge & Fowler, 1990; Chapman et al., 1995) and it is thought these effects are driven by sexual conflict.

**Reproductive isolation**

Both mutual and antagonistic forms of intersexual coevolution can generate inter-sexual specialisation, as females and males that engage in reproductive interactions are able to influence the selection of each other's traits. Sexual selection and/or sexual conflict can drive the divergence of reproductive traits and ultimately influence the ability of individuals (from the same or different populations) to mate and reproduce with each other (Clark et al., 2006). If coevolution occurs in different directions among populations, this can lead to individuals from one population being unwilling or unable to mate and reproduce successfully with individuals from another and could lead to reproductive isolation (Gavrilets, 2000). Therefore, the impact of sexual selection on reproductive traits can become a source of incompatibility between or within populations. There is evidence that sexual selection both with and without natural selection (good genes and runaway selection respectively) can drive the formation of isolating barriers (Panhuis et al., 2001; Coyne & Orr, 2004).

As reproductive proteins are known to evolve rapidly (Swanson & Vacquier, 2002; Clark et al., 2006), traits associated with both pre- and post-copulatory interactions can quickly respond to selection. As such, reproductive isolating barriers are subdivided into pre- and post-copulatory, i.e. behavioural isolation and gametic isolation (Coyne & Orr, 2004). Behavioural isolation includes traits that reduce the attractiveness of potential mates and is created by selection on mating preferences and signals (Panhuis et al. 2001; Ritchie, 2007), such as a female preference for a particular courtship song or ritual. Gametic isolation involves barriers that act between mating and fertilisation, for example, fertilisation failure when gametes interact and the disadvantage of heterospecific sperm compared to conspecific sperm (Howard, 1999; Matute & Coyne, 2010).

**Patterns of remating**

Females are naturally promiscuous in a variety of organisms (Andersson, 1994) and often mate multiple times. The benefits of female remating are divided into two main categories; those that are direct (i.e. benefit the female personally) and those that are indirect (i.e. benefits a female by providing for her progeny). Direct benefits are those which provision the female in some way, such as ensuring an adequate supply of sperm (Ridley, 1988), resource acquisition (Ursprung et al., 2009), harassment avoidance (Watson et al., 1998) and/or providing the opportunity for the female to select the "best" partner. Indirect benefits are more controversial due to the difficultly in directly testing for the mechanisms by which they occur but include progeny attractiveness (females are able to select sperm that will make their progeny the most attractive), progeny viability (females select for sperm that will improve the survival of her progeny) and/or genetic compatibility between sperm and egg (see Birkhead & Pizzari, 2002). As a result of this selection of sperm, the remating rate of females can strongly influence the intensity of sperm competition (Arnqvist & Rowe, 2005) and be costly to males as sperm competition can reduce paternity. Females can also suffer costs of remating, such as reduced lifespan (Fowler & Partridge, 1989; Chapman et al., 1995) and exposure to disease and/or parasites (Kirkpatrick & Ryan, 1991).

The optimum remating rate from the perspective of the female, the mating male and any rival males are a source of sexual conflict and as such, males evolve traits that either stimulate or inhibit remating depending upon their selective environment (e.g. the order in which the males mate with the female). For example, males can manipulate females by coercing them to remate more than their reproductive optimum (Parker, 1979), if the costs associated with resisting remating, (i.e. courtship harassment; Magurran & Seghers, 1994; Watson et al., 1998; Gay et al., 2009), are greater than costs associated with multiple matings (e.g. premature female death; Chapman et al., 1995). Females may also remate when they are sperm depleted (Trevitt et al., 1998). This benefits the female as she can produce a greater number of progeny. Female remating interval can also

be manipulated by the action of SFPs, which can act to delay female remating as a result of competition between males (Chen et al., 1988; Wigby et al., 2009).

The advantage for males remating seems obvious, the more females he mates with, the more progeny he could potentially sire. Males evolving under greater intensity of sexual selection show an increase in their mating capacity when encountering multiple females sequentially, compared with males selected under enforced monogamy (Crudgington et al., 2009). Since sexual selection results in an interacting phenotype (Bacigalupe et al., 2008), the effects of selection on both males and females needs to be taken into account for the most comprehensive investigations to be achieved. Evidence for sexual conflict over remating rate came from one experiment in which females were prevented from coevolving with males of *D. melanogaster* (Rice, 1996). Males evolved to coerce previously mated females to remate. These males received a fitness benefit from remating at the expense of female fitness, whose survival was reduced as a result of toxic male SFPs. The toxic effect of these males was thought to be a by-product of male-male competition (Rice, 1996). Female remating behaviour can be costly to males as sperm competition can potentially reduce paternity. As such, female remating behaviour can strongly influence the intensity of sexual selection (Arnqvist & Rowe, 2005) and has been suggested as a source of sexual conflict (Parker et al., 1979; Holland & Rice, 1999; Pitnick et al., 2001a).

**Seminal Fluid Proteins (SFPs)**

Males of many organisms transfer a suite of seminal fluid proteins (SFPs) to females, alongside sperm, during mating (Chen, 1984; Chapman, 2001; Findlay et al., 2008; Baer et al., 2009). SFPs are produced by the secretory tissues of the male reproductive tract (Figure 1.1). Once transferred, SFPs are responsible for initiating a suite of changes to female behaviour and physiology (Wolfner, 1997; Gillott, 2003; Chapman & Davies, 2004), such as increased egg laying (Chen et al., 1988), decreased female receptivity to remating (Chen et al., 1988; Lung et al., 2002) and facilitating sperm storage (Tram & Wolfner, 1999). Consequently, SFPs can provide reproductive benefits to both males and females (Civetta & Singh 1995; Swanson et al. 2001; Swanson & Vacquier 2002; Clark et al. 2007).

Some of the most well studied examples of SFPs are produced by the accessory glands of the male reproductive system of insects (Figure 1.1; Wolfner et al., 1997; Chapman, 2001; Ravi Ram & Wolfner, 2007). These accessory gland proteins (Acps) constitute a key component of the seminal fluid (Wolfner, 1997). For example, Acp26Aa (ovulin) increases female egg laying (Herndon & Wolfner, 1995). The benefit to both sexes is clear, males increase their chances of siring more progeny and females increase their reproductive fitness through producing more progeny. As this protein is clearly beneficial to both sexes, males should increase their reproductive fitness by transferring higher quantities or of Acp26Aa than rival males and females may evolve to ensure only males with the highest quality of Acp26Aa activate her post-mating response, by increasing her threshold to the action of this Acp. Acp26Aa is processed into its active form after transfer to females (Park & Wolfner, 1995) and evolves very rapidly (Wong et al., 2006). Consequently, postcopulatory sexual selection (i.e. sperm competition and cryptic female choice) has been suggested as the driving force behind SFP evolution.

Such rapid evolution of SFPs could also be driven by sexual conflict. For example, Acp62F stimulates egg laying and sperm storage in mated females but can also shorten female lifespan (Lung et al., 2002). It is expected that this effect of SFPs on females is a by-product of male-male competition. In response,

females could resist the inflicted costs by increasing their response threshold to the action of these SFPs, potentially generating antagonistic coevolution between the sexes over SFP complements and their quantities. Since reproductive proteins evolve rapidly (Swanson & Vacquier, 2002) this can happen over relatively short timescales.

Another Acp, Acp70A (sex peptide) decreases female receptivity to remating (Chen et al., 1988), thereby delaying the onset of sperm competition. Males that transfer sex peptide to females have been shown to have higher reproductive success than males that did not transfer sex peptide (Fricke et al., 2009). In addition, males that had larger accessory glands transferred higher quantities of sex peptide and experienced higher competitive fitness than rival males with smaller accessory glands (Wigby et al., 2009). However, sex peptide has been shown to reduce overall female fitness (Wigby & Chapman, 2005), so is likely to play a role in sexual conflict.

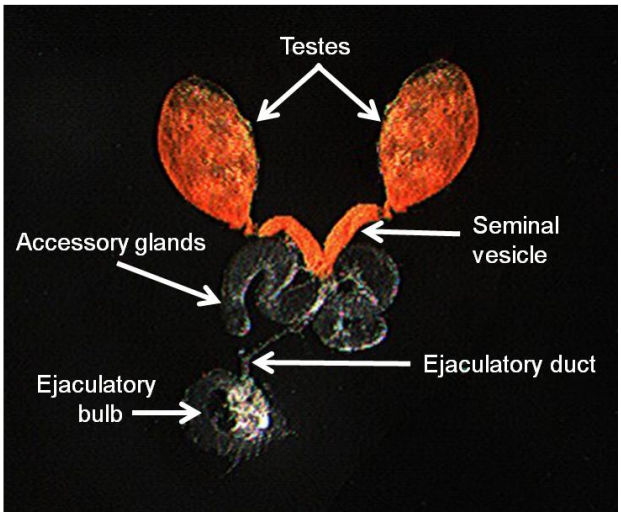



**Figure 1.1 *Drosophila pseudoobscura* male reproductive tract. The testes, seminal vesicles, accessory glands, ejaculatory duct and bulb are shown.**

Whatever the selection mechanism driving the evolution of these proteins, males with larger accessory glands appear to gain higher reproductive fitness, as shown in another study on *D. melanogaster* (Bangham et al., 2002) and in *D. pseudoobscura*, where males experiencing a greater intensity of sexual selection, also evolved larger accessory glands (Crudgington et al., 2009). However, this study did not test whether the Acp profiles or quantities were altered compared to males with smaller accessory glands. If sexual selection and/or sexual conflict drive the rapid evolution of Acps, then males with larger quantities or a greater number of unique Acps should have higher competitive success than rival males with lower quantities of Acps.

**Sperm-egg interactions**

Fertilisation is the union of male and female gametes to form a developing zygote and involves a suite of complex morphological, physiological and biochemical interactions. Traits associated with this union of gametes can become targets of incompatibility between the sexes as they directly influence the outcome of whether fertilisation is successful.

Evolutionary responses to sperm competition result in morphological diversity of sperm characteristics within and between species (Stockley, 1997; Swallow & Wilkinson, 2002). For example, sperm tail length between species of *Drosophila* show the greatest variation of any genus, ranging from 77 μm in *Drosophila persimilis* (Snook, 1997) to 58,290 μm in *D. bifurca* (Pitnick et al., 1995). Such changes must be accommodated by the egg for fertilisation to be successful and as such, coevolution between sperm and egg must occur. It has been shown in *D. melanogaster* that females increase the length of their sperm storage organ to accommodate longer sperm and therefore selectively bias paternity (Miller & Pitnick, 2002). Consequently, longer sperm are selected for as they provide males with a competitive advantage over rival males. The increased sperm length could be advantageous to females, as sperm contribute more than just their half of the genetic material required to propagate the next generation (Karr, 1991; Krawetz, 2005; Dorus et al., 2006). The paternal contribution to the developing

zygote has historically been underestimated. It has now been shown that males contribute almost their entire structure during fertilisation, including RNAs (Ostermeier et al., 2004) and a variety of proteins, such as tubulin (Green et al., 1979). Sperm also contribute to embryonic development by determining embryo polarity (Pedersen, 2001) and some component of the sperm tail must be present to elicit sperm aster formation and pronuclear fusion in insects and vertebrates (Moomjy et al., 1999; Sutovsky & Schatten, 2000).

After syngamy in most organisms, the entire sperm enters the egg and interacts with the egg cytoplasm (Shapiro et al., 1981; Karr, 1991; Simerly et al., 1995; Karr & Pitnick, 1996; Krawetz, 2005). These intracellular interactions between sperm and egg straddle the traditional divisions of pre- and post-zygotic reproductive isolation but are critical for fertilisation success. Once inside the egg, sperm must assume a specific structure to facilitate zygote formation and development (Ohsako et al., 2003; Lassy & Karr, 1996) and it has been suggested that this three-dimensional folding and coiling structure is species-specific (Karr, 1991; 1996; Snook & Karr, 1998). Any changes in sperm tail length must be incorporated into this intracellular choreography between sperm and egg. The precise mechanism by which sperm structure affects fertilisation success is unknown, but one suggestion is that the sperm functions to correctly align the sperm and egg pronuclei for fusion (Karr, 1991). Such intracellular sperm-egg interactions could represent a potential source of reproductive isolation, if the sperm of one species is unable to assume the appropriate structure or if the egg of one species is unable to accommodate the sperm of another.

### *Drosophila* as a model system

*Drosophila* species make good models for studying the interaction between the sexes. Their short generation time makes it possible to study the effects of sexual selection using experimental evolution in relatively short timescales. Such research is helped due to the relative ease with which both fertilised eggs and seminal fluids can be obtained for experiments. In addition to this, *Drosophila* species share many genes in common with mammals, including humans (Karr, 2007), making them an excellent study system, particularly for advancing our understanding of reproductive biology.

It has been established in the literature that *Drosophila* and mammals share a number of common reproductive traits. For example, the whole sperm enters the egg during fertilisation (Simerly et al., 1995; Alberts et al., 2002). However, the function of this process is currently unknown. As *Drosophila* are relatively simple to study, this means they make an excellent model system for improving our knowledge of fertilisation. It is possible, therefore, that any advances made in the study of *Drosophila* sperm and egg interactions could have wide ranging applications to the study of human reproduction and assisted reproduction techniques.

Within this thesis, both pre- and post-copulatory traits are studied within *Drosophila*. I collected data to assess the effects sexual selection has in driving reproductive trait evolution in the *D. pseudoobscura* subgroup of the *Drosophila obscura* species group (Figure 1.2). Species within this group demonstrate some interesting reproductive traits, for example, they produce two distinct sperm morphs (Snook et al., 1994; Snook, 1997; 1998). However, relatively little information is known about *D. pseudoobscura* seminal fluid (in particular their SFPs; Wagstaff & Begun, 2005; Richards et al., 2005). SFPs have, though, been extensively studied in *D. melanogaster* (e.g. Herndon & Wolfner, 1995; Park & Wolfner, 1995; Neubaum & Wolfner, 1999; Tram & Wolfner, 1999; Chapman et al., 2001; Lung et al., 2002; Findlay et al., 2008). As the *D. obscura* species group have been well studied in the literature due to their interesting sperm

morphs, it is important to further understand the role of other seminal fluid components in male-female interactions.

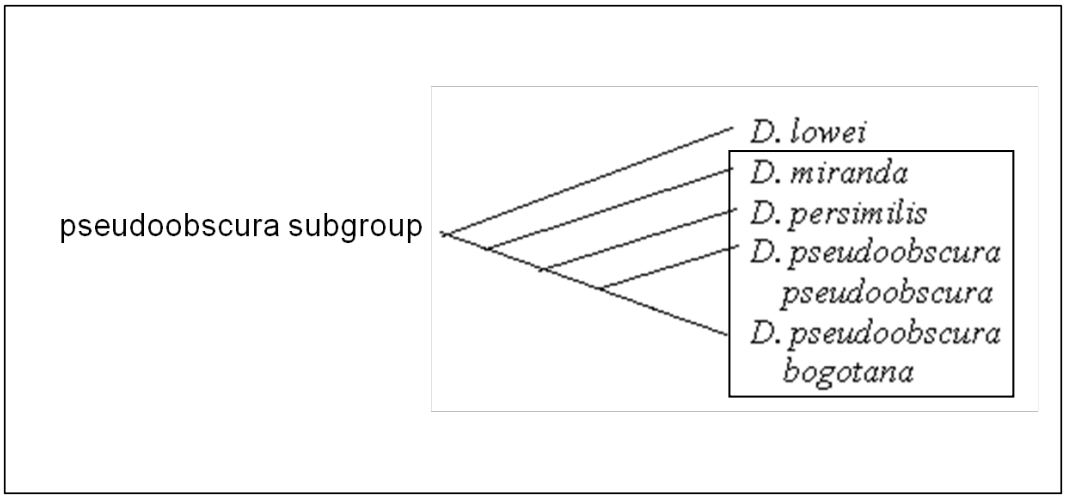



**Figure 1.2: Phylogeny of the *D. pseudoobscura* subgroup of the *Drosophila obscura* species group used in the chapters that follow. Phylogeny courtesy of Flybase. The species used in this thesis are highlighted.**

**Summary of the key techniques**

*Experimental evolution*

Experimental evolution studies provide a valuable opportunity to examine the effects of selection on reproductive trait evolution (Holland & Rice, 1999; Pitnick et al., 2001a,b; Crudgington et al., 2005). By manipulating the operational sex-ratio and therefore, the intensity of sexual selection, it is possible to experimentally examine if and how particular reproductive traits respond. Experimental evolution techniques have been used successfully to manipulate the intensity of both sexual selection and sexual conflict (Rice 1996; Holland & Rice, 1999; Pitnick et al., 2001a,b; Crudgington et al., 2005).

I used experimental evolution populations in chapters 2 and 3, to assess the effects of sexual selection intensity on various reproductive traits. These populations were created by manipulating the adult sex ratio, either to enforce monogamy (M; 1♀, 1♂) or to elevate polyandry (E; 1♀, 6♂). Each treatment is replicated four times. At the end of each generation (which lasts 28 days), progeny are collected and pooled together within each replicate line of M and E. The next generation is composed of a random sample of these pooled offspring, which takes account of the differential offspring production across families. For more information, the setting up and maintenance of the selection lines are described in more detail in Crudgington et al. (2005; 2009; but see Figure 1.3 for an updated version). Previous research has shown that sexual conflict is occurring in these treatments (Crudgington et al., 2005; Crudgington et al., 2010) and some traits had responded to selection. For example, males with greater opportunity for sexual selection had evolved to sing a faster courtship song to females (Snook et al., 2005; Debelle et al., 2014), larger accessory glands and a greater male mating capacity (Crudgington et al., 2009).

In chapter 2, I examine the effects of sexual selection on female fitness parameters in these experimentally evolved *D. pseudoobscura* populations. During interactions between the sexes, each individual provides the selective environment for the other and the result is an interacting phenotype (Bacigalupe

et al., 2008). As such, I aimed to determine whether pre- and post-copulatory traits had responded to sexual selection and/or sexual conflict by examining the interacting phenotypes between the sexes. To achieve this, I housed coevolved flies in their operational sex-ratios (i.e. within their selective environment) and observed them over the full course of the selection line set up (as shown in Figure 1.3).

In chapter 3, I used these same experimentally evolved populations of *D. pseudoobscura* to examine the suite of proteins present in the accessory gland tissue of the male reproductive tract (Figure 1.1). This was based on the findings that the accessory glands of E males are larger than those of M males (Crudgington et al., 2009). I used mass spectrometry to identify and analyse the quantities of proteins from the accessory glands from both M and E treatment males.

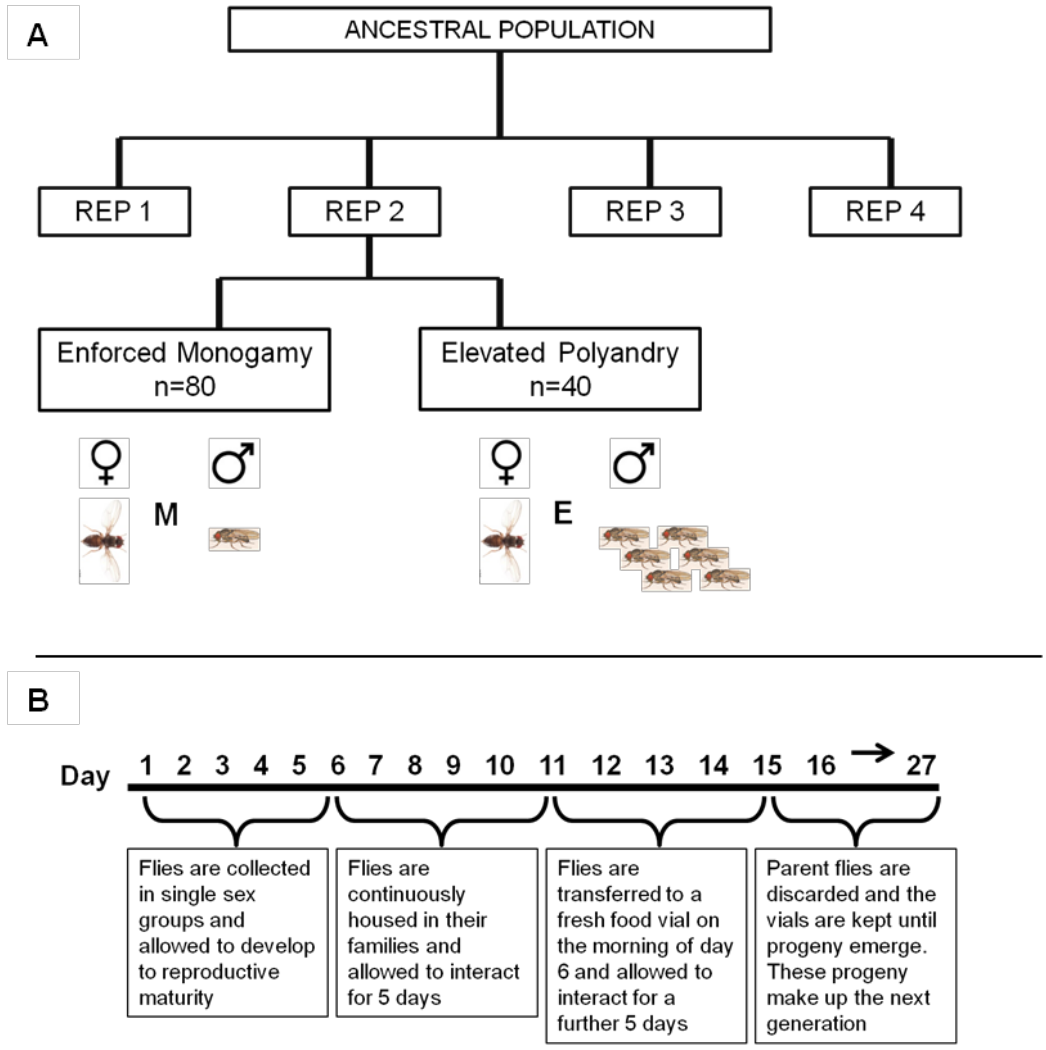



**Figure 1.3: Selection line set up (A) and maintenance (B). Flies are between 4-7 days old when housed in their families.**

*Mass spectrometry*

Mass spectrometry measures the mass-to-charge ratio of ions to identify and quantify molecules. Proteomic analyses can identify thousands of proteins in complex biological samples (Karr, 2008). Selection acts on the structures that proteins form and mass spectrometry provides qualitative and quantitative analyses of proteins. There are multiple advantages and disadvantages to different types of quantification methods in mass spectrometry (Bantscheff et al., 2007). I decided to use label-free quantification, because it enables a shotgun proteomics approach, whereas labelling methods require a much more targeted approach (Bantscheff et al., 2007). Since the aim of the study was to identify which proteins are present in the different samples, labelling the samples would have been very restrictive. Label-free quantification also takes longer to run on the mass spectrometer but this is advantageous because it is generally better at identifying proteins. Labelling approaches are faster but this means they are more likely to miss lower abundance proteins that may be of interest (for example, some SFPs may be relatively low abundance in the accessory gland tissues compared to more abundant, e.g. housekeeping, proteins).

I used an Orbitrap Elite Mass Spectrometer to perform tandem mass spectrometry (LC-MS/MS), which provided high mass accuracy, high resolution and high sensitivity data. The accessory gland proteome was then analysed and the proteins identified with a signal sequence were extracted, which constitutes the secretome. These secretome proteins form the majority of the analyses for chapter 3. Secretome proteins are those which are actively transported out of the cell (Hathout, 2007). Proteins with a signal sequence are candidates for being transferred to females during mating (Wolfner et al., 1997) and therefore provide a putative identification of many proteins that may be involved in interactions between the sexes and contain those which are identified as Acps.

*Modelling sperm-egg interactions (ScanIP)*

In chapter 4, I discuss in detail a methodology which I have developed to model the intracellular interactions between sperm and egg. Using a combination of confocal microscopy and computer-generated three-dimensional models, I demonstrate the possibilities for measuring a variety of intracellular sperm-egg interactions. Fertilised eggs were collected and stained from species within the *D. obscura* species group. Two-dimensional images of eggs were taken using confocal microscopy and then stacked to create a three-dimensional model using imaging modelling software called "ScanIP". Sperm tails and pronuclei, as well as other areas of interest, were highlighted and measured. These measures were exported to Mathematica and Python where analyses were performed on various gamete parameters and intracellular sperm-egg interactions. As *Drosophila* make good model organisms for studying human reproductive traits (Alberts et al., 2002), this technique could have wide ranging applications to the study of human reproduction and assisted reproduction techniques. This technique could also be applied to extract various parameters from biological systems when high resolution three-dimensional imaging is possible.

# Chapter 2. The effects of sexual selection on female fitness parameters in *Drosophila pseudoobscura*

## Introduction

Sexual selection has the ability to drive the rapid evolution of traits that affect the reproductive success of individuals (Andersson, 1994; Birkhead & Pizzari 2002). Given that it is usually females who are the choosier sex, males must compete for access to them (i.e. fertilisations) by evolving a variety of behavioural, morphological and/or physiological traits that can improve their competitive success. Selection can act on precopulatory traits, such as the evolution of elaborate courtship displays (Andersson, 1994; Snook et al., 2005; Debelle et al., 2014) and alterations in male mating capacity (Harcourt et al., 1981; Crudgington et al., 2009). As females are naturally promiscuous in most organisms (Andersson, 1994), selection can also act on postcopulatory traits, such as female control over paternity (Birkhead & Pizzari, 2002) and the evolution of seminal fluid proteins (SFPs) that alter female behaviour and physiology (Chen, 1984; Avila et al., 2011; Civetta & Reimer, 2014). Males evolve traits in response to a female preference for them (either via sexual selection or sensory bias; Fisher, 1930; West-Eberhard, 1984) and this can result in iterative bouts of correlated evolution that can impact the reproductive optima of the sexes (Pitnick et al., 1999; Gavrilets et al., 2001) However, the sexes are rarely congruent in what maximises their fitness. In general, males benefit through acquiring a greater number of mates (Arnqvist & Nilsson, 2000), whereas females benefit through a greater number of progeny over their lifetime (Bateman, 1948; Schärer et al., 2012; Fritzsche & Arnqvist, 2013).

Such sex specific selection can result in sexual conflict promoting the bearer's fitness, even when those traits are costly to the reproductive fitness of their mate (Parker, 1979; Clutton-Brock & Parker, 1995; Arnqvist & Rowe, 2005). When the fitness of one sex is impaired, selection favours the evolution of traits that resist the fitness loss that is inflicted (e.g. females evolve counteradaptations to reduce the effect of harmful male traits; Parker, 1979; Arnqvist & Rowe, 2005). Such

interlocus sexual conflict can drive antagonistic coevolution (Rice, 1996; Holland & Rice, 1998) that promotes male traits, such as harassment as a result of courtship (behavioural; Bateman et al., 2006; Crudgington et al., 2010), conflict over female remating (Rowe et al., 1994; Arnqvist & Nilsson, 2000; Alonzo & Pizzari, 2013) and the evolution of seminal fluid proteins (SFPs; physiological; Wolfner et al., 1997; Holland & Rice, 1999), which can cause harm to females as a by-product of male-male competition (Chapman et al., 1995; Wolfner et al., 1997).

Male traits can have negative impacts on female fitness. For example, harassment as a result of courtship can reduce progeny production by altering female oviposition behaviour (Sakurai & Kasuya, 2008; Gay et al., 2009), reducing female opportunity for feeding (Magurran & Seghers, 1994) and decreasing female lifespan (Partridge & Fowler, 1990; Clutton-Brock & Langley, 1997). Female preference for a particular trait, for example male courtship behaviour, can become elevated to potentially harmful levels in response to intrasexual selection (Orteiza et al., 2005). Harassment costs to females over courtship are the only manipulation males can employ up until females mate (precopulatory). Once females have mated, the postcopulatory arena also comes into play and this is where the evolution of SFPs can provide considerable benefits to males (Chen et al., 1988; Wolfner et al., 1997), for example, delaying female remating (Wigby & Chapman, 2005) and causing females to oviposit earlier in their lifetime (Crudgington et al., 2010), which can inflict severe costs to females (Chapman et al., 1995) as a result of postcopulatory sexual selection and/or sexual conflict.

Females can benefit from remating both directly and indirectly (Jennions & Petrie, 2000) but at the same time suffer fitness costs (Chapman et al., 1995; Holland & Rice, 1999; Rice, 2000). Female remating behaviour can also be costly to males as sperm competition can potentially reduce paternity. As such, female remating behaviour can strongly influence the intensity of sexual selection (Arnqvist & Rowe, 2005) and has been suggested as a source of sexual conflict (Parker, 1979; Holland & Rice, 1999; Pitnick et al., 2001a). The optimum remating rate

from the perspective of the female, the mating male and any rival males are a source of sexual conflict and as such, males evolve traits that either stimulate, for example via courtship, or inhibit, for example via SFPs (Chen et al., 1988) female remating depending upon the selective environment (e.g. the order in which the males mate with the female). For example, males can manipulate females by coercing them to remate more than their reproductive optimum (Parker, 1979; Rice, 1996). This can occur when costs associated with resisting remating (i.e. harassment; Watson et al., 1998) are greater than costs associated with multiple matings (e.g. premature female death; Chapman et al., 1995 and a reduction in progeny production; Sakurai & Kasuya, 2008).

One recent method to identify when sexual conflict is operating and what traits are targeted is the use of experimental evolution in which the operational sex ratio of populations are manipulated, thereby modifying the strength of sexual selection (Kokko et al., 2006). The opportunity for sexual selection in naturally promiscuous populations is typically manipulated by enforcing monogamy, elevating the potential for female polyandry, or both and has provided evidence that sexual conflict can shape some reproductive traits. Males that have been selected for enforced monogamy court females less than males from populations in which multiple female matings can occur (*D. melanogaster*; Holland & Rice, 1999; *D. pseudoobscura*; Crudgington et al., 2010). Males evolving under greater intensity of sexual selection have been shown to delay remating of monogamous females longer than remating in promiscuous females (*D. melanogaster*; Pitnick et al., 2001b but see Crudgington et al., 2005) for *D. pseudoobscura*). Males are able to delay female remating through the action of SFPs, which have been shown to be costly to females by shortening their lifespan (Chapman et al., 1995). Since the effects of mating with promiscuous males are more pronounced in monogamous females compared to promiscuous females, these results suggest that, in the absence of sexual conflict, males have evolved to decrease both pre- and post- copulatory harmful effects on females (Holland & Rice, 1999) and monogamous females are, therefore, likely to be more susceptible to the costs of manipulation by males.

Males evolving under greater intensity of sexual selection show an increase in their mating capacity compared with males selected under enforced monogamy (Crudgington et al., 2009). Males from polyandrous experimental populations sired a greater number of progeny compared with monogamous males but this was when males were presented with multiple coevolved females, and so the remating rates of females was not considered in this experiment. If monogamous pairings are coevolving to be less harmful to each other, as predicted by sexual conflict theory, then one might expect to see a higher reproductive output from monogamous pairings compared to polyandrous pairings. Monogamous females do produce more progeny when mated to their coevolved monogamous males, than when mated to control promiscuous males (in *D. melanogaster*; Pitnick et al., 2001b). However, when the interaction between males and females is taken into account, lower courtship rates (Holland & Rice, 1999) in monogamous compared with promiscuous males, does not result in a greater number of progeny in *D. melanogaster* (Holland & Rice, 1999). However, in *D. pseudoobscura* females produce more progeny when housed with only one male, compared to when they are housed with six males, regardless of their sexual selection history (Crudgington et al., 2005). Since sexual selection results in an interacting phenotype (Bacigalupe et al., 2008), the effects of selection on females are just as informative as the effects on males.

Experimental evolution studies have shown that males with a greater opportunity for sexual selection have higher fitness. These males have both higher courtship rates and a greater mating capacity, which can lead to them siring an overall greater number of progeny compared to monogamous males (Crudgington et al., 2009). However, previous studies on the outcome of experimental evolution have altered the selective environment in which males and females have evolved by either altering the operational sex ratios during experimentation (see, Holland & Rice, 1999) or by investigating interactions between non-coevolved individuals. This makes the experimental environment different to that of the selective environment and masks how males and females interact *during* selection. Also, as females may have higher fitness when mating with males from non-coevolved

populations (Rowe et al., 2003), the outcome of selection on specific traits may be masked if females are not paired with coevolved males.

In this study, I measured various fitness parameters (both pre- and post-copulatory) by examining them in the selective environment, during selection as I wanted to investigate whether traits thought to be evolving under selection showed the same trajectories when they were observed for the full interaction period between the sexes. I also wanted to determine whether postcopulatory sexual selection was occurring in these selection treatments. I used the experimental evolution *D. pseudoobscura* system (first outlined in Crudgington et al., 2005) to study a number of key reproductive traits. Previous work on these experimental evolution treatments have found that whilst some traits thought to be important in reproduction have evolved (e.g. courtship song [Snook et al., 2005; Crudgington et al., 2010; Debelle et al., 2014]; male mating capacity which has been linked to larger accessory glands [Crudgington et al., 2009]; CHCs [Hunt et al., 2012] and the number of eggs per ovariole [Immonen et al., 2014]), others have not (e.g. neither testes mass, sperm length or sperm number [Crudgington et al., 2009] nor sex combs [Snook et al., 2013]). Sexual conflict operates in this system as females from the elevated polyandry treatment had higher fecundity and higher progeny hatching success than females in the enforced monogamy conditions when they were both mated to an ancestral population male (Crudgington et al., 2010). However, monogamous and elevated polyandrous males did not differ in the number of progeny they sired per mating, so both monogamous and elevated polyandrous females produce the same amount of progeny with their coevolved male. Perhaps elevated polyandrous males were only able to sire more progeny because they sequentially mated with more females (Crudgington et al., 2010). During selection, single females were housed with either one (enforced monogamy; M) or six (elevated polyandry; E) males and were observed in their selective environment (i.e. coevolved individuals were housed in their operational sex ratios) for the full 10-day interaction period. Consequently, by housing individuals in their sex ratios and by not changing their mating partners, I did not change the sexual environment against which each male and female is interacting and therefore coevolving and

was able to capture traits during the entirety of the interaction between the sexes. However, it was not possible to distinguish fully between differences in traits as a result of treatment effects or differences that may have arose as a result of the differences in contemporary sex ratios. Specifically, I measured a number of pre- and post-copulatory reproductive traits: (1) the number of courtship attempts by males, (2) the number of female rematings, (3) the remating interval and (4) the total number of progeny each female produced. I predicted that E males would court females more but sire a lower total number of progeny than M males (Holland & Rice, 1999; Pitnick et al., 2001a). I also predicted that E females would suffer greater costs associated with remating compared to M females over the 10 days, through a reduction in their lifespan or progeny number compared with M females (Pitnick et al., 2001b; Chapman et al., 1995).

## Materials and Methods

### Selection line treatments

An ancestral population of *Drosophila pseudoobscura* was established in 2001, using wild-caught, mated females from Tucson (AZ). *D. pseudoobscura* is a naturally polyandrous species, with females frequently shown to remate and be inseminated by at least two males (Anderson, 1974). From the ancestral population, four replicate populations were established (Replicates 1, 2, 3 and 4) for two sexual selection treatments; enforced monogamy (M; 1♀, 1♂) and elevated polyandry (E; 1♀, 6♂). I refer to these groupings as “families”. Flies were maintained in vials containing cornmeal-agar-molasses food media with added live yeast and housed at 22°C with a 12 hour light, 12 hour dark cycle (from here referred to as 12L:12D). Effective population sizes were equalised between the treatments, with the M treatment comprising 80 vials per replicate and the E treatment comprising 40 vials per replicate (Crudgington et al., 2005; Snook et al., 2009a). At the end of each generation (which lasts 28 days) progeny are collected and pooled together within each replicate line of M and E.

The next generation is composed of a random sample of these pooled progeny, which takes account of the differential progeny production across families. For more information, the setting up and maintenance of the selection lines are described in more detail in Crudgington et al. (2005; 2009; but also see chapter 1, Figure 1.3 for an updated version).

**Experimental design mimics selection line maintenance**

Flies from the pooled offspring groups mentioned previously were separated on the day of eclosion into single-sex groups using $CO_2$ anaesthesia and allowed to sexually mature (between 4-7 days after eclosion). For each replicate, virgin flies from coevolved lines were housed together in ratios appropriate to their selection treatment (M or E) and allowed to interact in their vials for 5 days, after which they were transferred in their families to fresh food vials for a further 5 days. Adult flies experienced a total of 10 consecutive days of interaction, which has been shown to be sufficient for both pre- and post-copulatory sexual selection to act (Turner and Anderson, 1983). However, it was not possible to assess whether any trait differences are as a result of treatment or as a result of differences arising from contemporary sex ratios. In previous studies, selection is relaxed before starting experiments, to reduce any maternal effects on offspring. Importantly, I did not relax selection prior to commencing the observations because I aimed to quantify what happens during selection in these lines. In this study, the set up used mimics the way in which these treatment groups of the experimentally evolving populations have been selected for over 150 generations.

I took a random sample of 30 vials from each of the four replicates of M and E (240 vials in total out of the 480 for all treatment replicates combined; M1, M2, M3, M4 and E1, E2, E3 E4) and assigned each family a unique identifier (numbered 1-240). Each day, both mating and courtship behaviour were observed for the duration of the 12-hour light photoperiod (between the hours of 8am and 8pm). For practical reasons, flies could not be observed in the controlled temperature room, so the vials were moved to the laboratory benches

and observed at room temperature. Temperature was checked at various intervals throughout the day to ensure it was consistent within a few degrees Celsius. It was not necessary to observe these flies during the 12-hour dark photoperiod, as *D. pseudoobscura* have been shown to have a peak of mating activity just before darkness (Partridge et al., 1987) and during the preliminary observations there was no evidence that these selection lines of *D .pseudoobscura* exhibit mating behaviour in the dark. Both the number of times and the day(s) each female remated across the 10 days were recorded.

Every 15 minutes during the light photoperiod, all 240 vials were scanned and I recorded whether or not the male(s) were courting the female. Consequently, each vial was scanned 480 times across the 10 day period and in total there were 115,200 data points for courtship (480 scans x 240 vials) across 10 days. I counted behaviour as a courtship event if a male was orientating towards the female, vibrating their wings (courtship song) or attempting copulation (mounting). If a pair was already copulating during the courtship scan, then this was recorded as copulation and not as a courtship event. Copulation duration in *D. pseudoobscura* is relatively short and so mating behaviour was observed continuously during the 12-hour light photoperiod. Following the 10-day observation period, adult flies were discarded and all of the vials were kept at 22°C with a 12L:12D cycle. All emerging adult progeny from each family were counted and then either used to start the next generation or discarded.

**Statistical Analysis**

Females that did not produce progeny (n=11 for M and n=3 for E) and females that remated more than twice (n=1 for M and n=2 for E) were removed from the entire dataset (Table 2.1 shows the final sample sizes for each replicate). Low numbers of progeny were thought to be due to problems related to sperm transfer and/or storage, fertility or oviposition rather than as a result of treatment (Pitnick et al., 2001a; Crudgington et al., 2005). There were significantly more M females, than E females, that did not produce any progeny ($\chi^2$=4.57, df=1, p=0.033), but qualitatively, the results remain unchanged for analyses when these females

were included. Of those females that produced zero progeny (n=14), 29% (n=4) did not mate at all. When these females (n=4) were removed from the dataset, there was no longer a significant difference between treatments for the number of females with zero progeny (n=10; $\chi^2$=1.6, df=1, p=0.21).

The total number of matings recorded for each female is the minimum number of matings that females engaged in. Females that had progeny but were labelled as having not mated during the experiment (n=19; 7.9% error across the whole dataset) have been changed to be counted as mated females. Families were being observed during the set up but many matings happen at once and as copulation duration in this species is relatively short, matings that were missed were likely to have been during this period. There were more missed matings in the M treatment (n=15) than in the E treatment (n=4), which could indicate that the overall level of missed matings was likely to have been underestimated and that mating frequency may be higher than reported in the E treatment. However, since these matings were likely missed during the set up, it is plausible that these were the only missed matings during the observation period.

**Table 2.1: The final sample size for each replicate used in all analyses**

| Treatment | M | | | | E | | | |
|---|---|---|---|---|---|---|---|---|
| **Replicate** | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| **Sample (n)** | 28 | 24 | 28 | 28 | 30 | 28 | 30 | 27 |

I analysed the total number of courtship attempts, the total number of matings and rematings (where females that did not remate were removed from the analyses) for each female and the propensity of females to remate. I also analysed the remating interval for each female and the total number of

subsequent progeny for each family. The results for replicate 3 for female remating interval showed a different pattern to the other three replicates. If replicate 3 was removed from analyses of female remating interval, this qualitatively changed the results. Replicate 3 has shown different patterns to the other replicates in previous work on these selection lines, so could potentially be an anomaly in general. However, I did not remove replicate 3 from analyses in this study on the whole because the purpose of this study was to examine what happens in these selection line treatments, during selection, to show whether or not postcopulatory selection is occurring and what effects these treatments have on female fitness. There was not enough evidence that replicate 3 consistently showed different patterns in these observations to exclude it completely. Sample sizes for the number of females that died during the 10-day observation period were low (7.62%; n=2 for M and n=15 for E) but were analysed using a chi-squared test. Sample sizes for female propensity to mate (n=4 females did not mate) were too low to be statistically meaningful, and so could not be assessed. It was not possible to analyse the number of progeny produced before the first remating because progeny number was only counted for each set of vials (before transfer from the interaction vials; IV and after transfer from the oviposition vials; OV) and so this information was not captured.

All statistical tests were performed using the open source software package R 3.1.0 (R Development Core Team, 2014). Data were tested for homoscedascity using a Levene's Test before commencing analyses. Courtship, mating, remating and progeny data were analysed using generalised linear mixed models (GLMMs; Table 2.2). These models were analysed using the library *lme4* (Bates et al., 2014). In all models, replicate was treated as a random effect nested within sexual selection treatment. Propensity to remate was analysed using a GLMM with a binomial distribution and all the other GLMM analyses were analysed with a Poisson distribution. Remating interval was analysed using a Cox Proportional Hazards Regression in the library *survival* (Therneau, 2014), with the frailty function used to add a simple random effect of replicate.

**Table 2.2: Statistical model statements and standard deviations (SD) for total courtship, propensity to remate and total progeny.**

| Response variable | Generalised linear mixed effects model using lme4 in R | Replicate SD |
|---|---|---|
| Total courtship | response~treatment +(1\|replicate) | 0.3075 |
| Propensity to remate | response~treatment +(1\|replicate) | 0.173 |
| Total progeny | response~treatment +(1\|replicate) | 0.1702 |

**Results**

Sexual selection treatment had a significant effect on the frequency with which males courted coevolved females. E males courted E females significantly more frequently than M males courted M females ($z=-5.715$, $df=1$, $p<0.0001$; Figure 2.1; SD shown in Table 2.2). When the average total number of courtship events is divided by the six males in the E treatment, each individual E male courted the female less on average than M males. Sexual selection treatment had no significant effect on either female propensity to remate ($z=-1.621$, $df=1$, $p>0.1$) or in the total number of matings females engaged in ($z=-1.017$, $df=1$, $p>0.3$; Figure 2.2). Sexual selection history had no significant effect on the total number of rematings for females, either in datasets including females that did not remate ($z=-1.864$, $df=1$, $p>0.06$) or when these females were removed from the analyses ($z= -0.353$, $df=1$, $p=0.72$). The number of females that remated accounted for 35.4% of the dataset ($n=32$ for M and $n=47$ for E females across all four replicates).

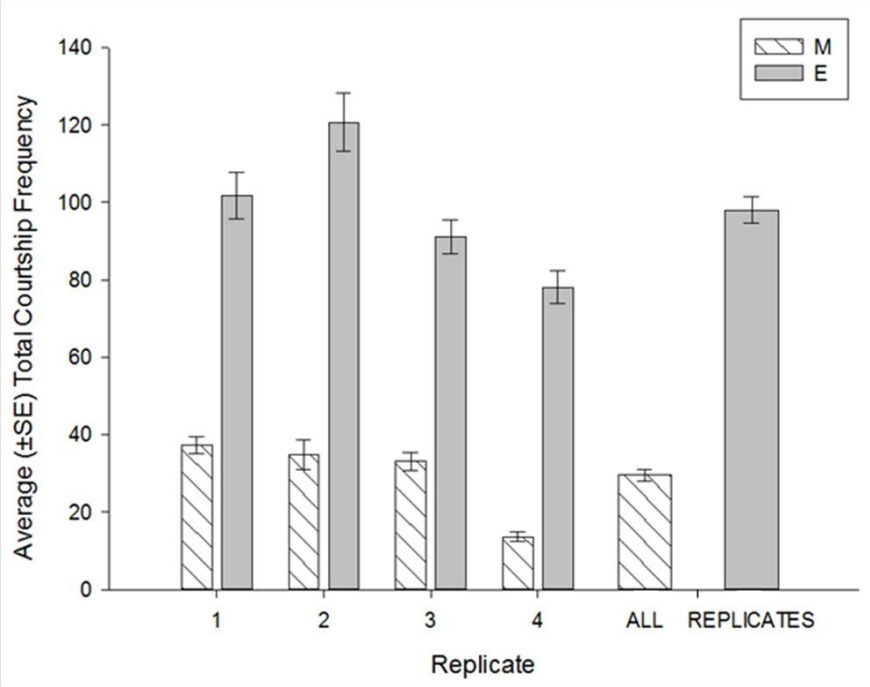



**Figure 2.1: Average (±SE) total number of courtship events per replicate across 10 days and the average (±SE) total number of courtship events for all replicates combined over 10 days ("ALL REPLICATES"). E courtship counted if any of the 6 males were courting the female during the observation scans.**

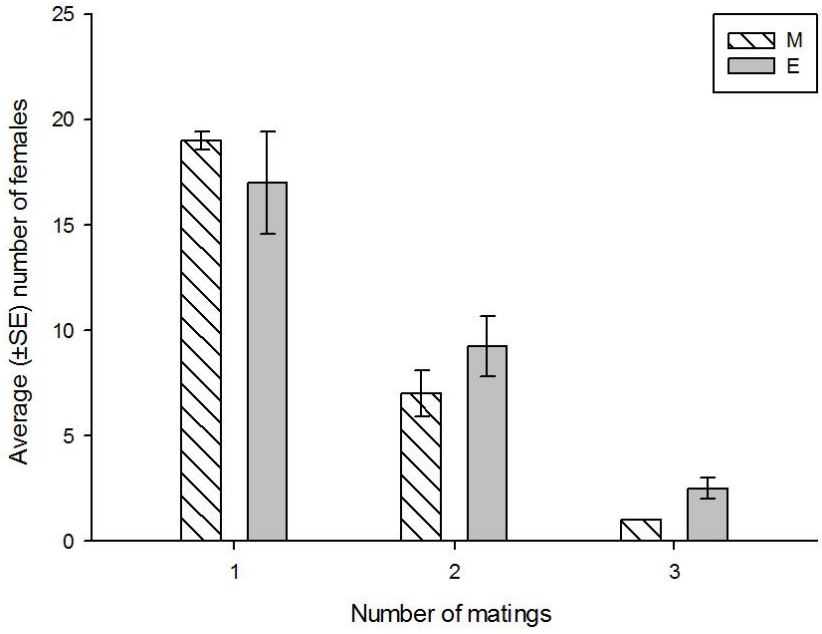



**Figure 2.2: Average (±SE) total number of females that either mated once (1), twice (2) or three times (3). The number of females (for each category of 'number of matings') was summed for each replicate within a treatment and then averaged. For M females that mated three times, there was no difference between replicates in the total number of females mating three times, and thus no error bar is shown.**

There was no significant difference in remating interval between enforced monogamy and elevated polyandry females ($\chi^2$=2.54, df=1, p=0.11; Figure 2.3). However, this was as a result of one replicate (Replicate 3). When replicate 3 is removed from the analysis, the interval to the first remating (in hours) is significantly shorter for M females compared to E females (z=-3.23, df=1, p=0.001).

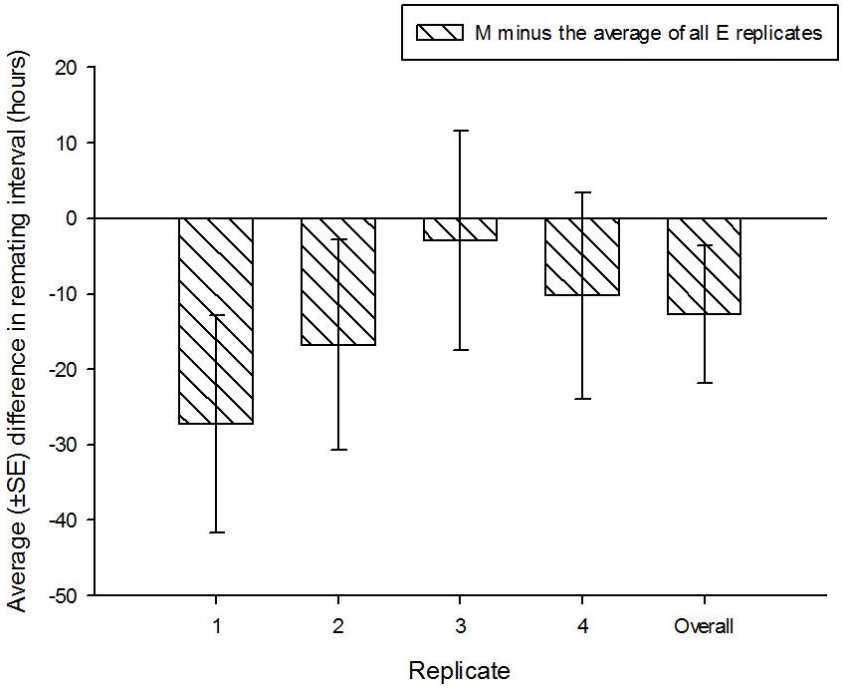



**Figure 2.3: Average (±SE) difference in remating interval (hours) compared using M replicates minus the average of all E replicates combined and overall M minus overall E, "Overall". M females remate faster than E females.**

Females in the elevated polyandry treatment had significantly lower survival (n=15/120 distributed roughly equally across replicates) than females in the enforced monogamy treatment (n=2/120 both for replicate M3; $\chi^2$=9.9412, df=1, p=0.0016). Females in the enforced monogamy treatment produced significantly more progeny than females in the elevated polyandry treatment (z=2.50, df=1, p<0.02; Figure 2.4). This was the case for both the first set of vials (the interaction vials; IV) before transfer (z=1.94, df=1, p=0.05) and the second set of vials (the oviposition vials; OV) after transfer (z=2.71, df=1, p=0.007).

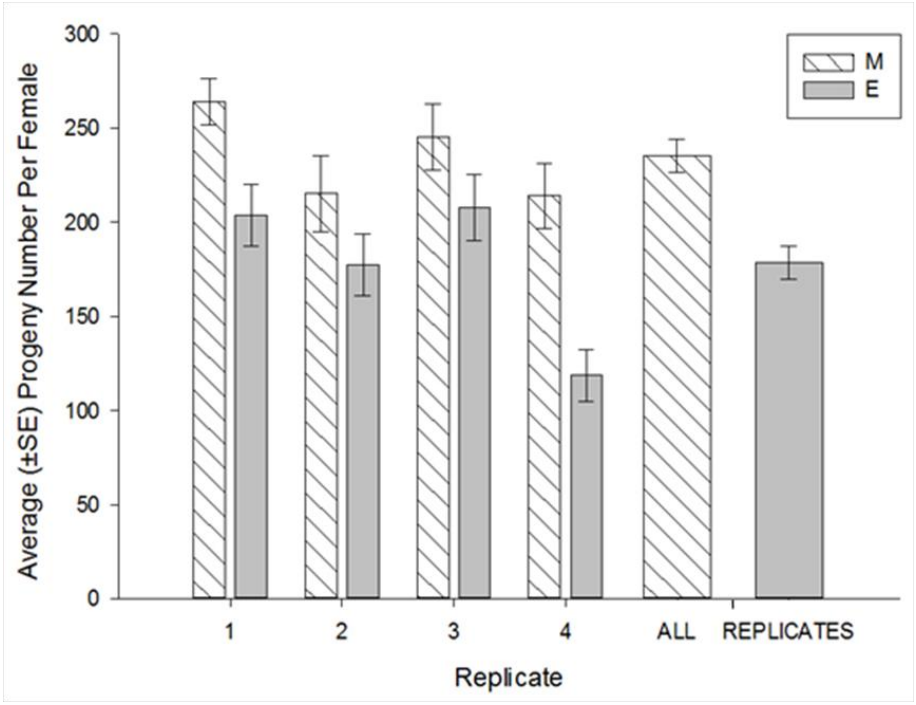



**Figure 2.4: Average (±SE) number of progeny per female by replicate within a treatment and the average (±SE) total number of courtship events for all replicates combined over 10 days ("ALL REPLICATES").**

## Discussion

To examine the effects of selection on a number of key reproductive traits during the interaction between the sexes, I used experimentally evolved populations of *Drosophila pseudoobscura* (E or M treatments). I predicted that E males would display elevated courtship frequencies compared to M males and this prediction was upheld consistently across all four replicates (Figure 2.1). Therefore, E females were likely to have been harassed as a result of persistent courtship displays. These results align with previous research on these populations that found E males courted ancestral females more frequently than M or ancestral (polyandrous) males (Crudgington et al., 2010), indicating that E males elevated courtship. However, an alternative or concurrent interpretation is that M males could be controlling the frequency of courtship, perhaps to allow females to oviposit more and not waste resources defending against unwanted matings. This idea corresponds with other experimental evolution studies in *D. melanogaster* that found monogamous populations had lower courtship frequency compared to control polyandrous populations (Holland & Rice, 1999). Coevolution under monogamy may have resulted in the sexes becoming more benign to each other, but it is unlikely to fully explain the difference in courtship frequency between treatments in this study because previously E males have been shown to elevate their courtship frequency more than a control promiscuous population (Crudgington et al., 2010). Therefore, it is likely that E males have evolved elevated courtship of females compared to more natural levels of courtship for this species.

Previous work has linked courtship harassment to reduced progeny production in a variety of organisms, including the dung fly, *Sepsis cynipsea* (Blanckenhorn et al., 2000; Martin & Hosken, 2003b) and two beetle species, *Callosobruchus maculatus* (Gay et al., 2009) and *C. chinensis* (Sakurai & Kasuya, 2008). As such, courtship behaviour is a possible source of sexual conflict that has the potential to negatively impact female fitness parameters. The results found in the current study are consistent with a precopulatory mechanism that impacts female reproductive fitness. This influence can be characterised in the following ways:

persistent courtship may reduce female productivity through altered oviposition behaviour (Clutton-Brock & Parker, 1995; Gay et al., 2009), reduced opportunity for feeding (Magurran & Seghers, 1994), and/or require increased energetic expenditure in resisting courtship (Watson et al., 1998) that reduces energy available for egg production. Such harassment could explain one of the other findings in the current study, that E females had fewer progeny than M females (Figure 2.4). However, it may not be exclusively the case that E males are more harmful *per se*, but rather the increased number of males in the E treatment contributes to the increased harassment. This is supported by the finding that individual E males court females less than each M male. However, E females are exposed to a higher average courtship frequency than M females, due to the cumulative courtship of the six males in the E treatment. It is also supported by a previous study on these populations, which showed that persistent housing with a greater number of males, regardless of selection history, had a negative impact on female lifetime progeny production (Crudgington et al., 2005).

Precopulatory harassment by males has also been linked to premature death of females, for example in *S. cynipsea* (Martin and Hosken, 2003a) and *D. melanogaster* (Partridge & Fowler, 1990), consistent with costs associated with sexually antagonistic coevolution via sexual conflict (Chapman et al., 2003; Rowe & Day, 2006). In the current study, significantly more E females died prematurely than M females, potentially as a result of harassment during courtship. However, it was not possible to separate the cost of harassment from other costs associated with mating, such as the action of SFPs and/or the timing of matings, as the mechanism for premature female death. When M females were housed with either monogamous or polyandrous males, there was no significant effect of elevated courtship frequency on female survival (Crudgington et al., 2010). There are a number of potential reasons for this variation in response to altering the selection environment. The previous study housed individual monogamous females with three selection line males and relaxed selection prior to the study. The current study housed individuals within their selective environment (i.e. with 1 or 6 males respectively), so perhaps reduced female survival shown in this

study is a consequence of the number of males a female is housed with, rather than the selection history of those males.

To assess differences in female remating patterns, I looked at both the number of rematings (Figure 2.2) and the remating interval (Figure 2.3) of each female. Previous work in *Drosophila* found that when males adapted to a static female phenotype, they evolved to increase female remating rate through coercion (Rice, 1996) and therefore, these males "win" in the sexual conflict between the sexes. As such, I predicted that E females would remate a greater number of times than M females. However, this prediction was not supported, as the number of female rematings did not differ between sexual selection treatments (Figure 2.2). This could be because the females in the previous study had a static, not interacting, phenotype and so there was no opportunity for coevolution between the sexes. The finding presented in this study aligns with previous research of *D. pseudoobscura* sexually selected populations, which showed no difference in male ability to coerce ancestral females to remate (Crudgington et al., 2005), despite E males having higher mating capacity than M males when allowed access to multiple females in sequential matings (Crudgington et al., 2009). However, the findings disagree with another study in *D. melanogaster*, which found that male biased lines coerced coevolved females to remate more (Wigby & Chapman, 2004). However, *D. pseudoobscura* might not be as promiscuous as *D. melanogaster* (Crudgington et al., 2005) and this could explain the differences in response to sexual conflict between these species.

Sexual conflict between the sexes can result in sexually antagonistic coevolution. At any given time, either males or females could be "winning" the conflict. At the evolutionary stage outlined in this study, perhaps females are winning against remating coercion because there were no significant differences in either female propensity to remate or in the number of rematings they engaged in. However, early research on these populations (Crudgington et al., 2005) showed similar results to those shown here (after 150 more generations of selection) in the number of female rematings. Earlier work has also shown a lack of divergence in sperm traits (Crudgington et al., 2009) between the populations, despite there

being enough heritability for some of these traits to evolve (Snook et al., 2010). This lack of trait divergence could be explained by the finding here that remating rate does not differ, suggesting that postcopulatory sexual selection is not a potent force in these populations. Still, it is unlikely that selection does not drive trait divergence at all in these populations, given that both pre- and post-copulatory reproductive traits have changed, for example, courtship song (Debelle et al., 2014) and accessory gland size (Crudgington et al., 2009), respectively.

Another possible explanation for there being no significant difference in the number of rematings between selection treatments here could be due to the high level of activity in each E treatment vial. When a male was attempting to copulate with a female, other E males were seen to be disrupting the pair and uncoupling them (previously shown in Punzalan et al., 2008). Without this factor, E females could have been receptive to more rematings than observed. However, previous research where male density was altered exclusive of the effect of selection history (Crudgington et al., 2005) also showed no significant difference in the number of female rematings, so this seems unlikely. Alternatively, perhaps males evolving under greater intensity of precopulatory sexual selection do not evolve greater manipulation of female remating behaviour, despite increasing their courtship frequencies. This makes sense if males have finite resources for mating, so that a trade-off between reproductive traits must be made. Males might evolve to invest more heavily in courtship either to be the male the female mates with when she is receptive to mating and/or to guard against other males mating. It is more likely to be the former of these suggestions for *D. pseudoobscura* because mate guarding has been shown not to occur (Crudgington et al., 2005).

While precopulatory ability to coerce females to remate does not appear to differ between treatments, I also examined whether there was a postcopulatory effect on remating interval. I predicted that E males would be able to delay remating of their mates' more than M males, as a result of postcopulatory sexual selection. Overall, there was no difference in the remating interval between treatments but

this was a consequence of one replicate; the other three replicates showed E males did delay remating of their mates longer than M males (Figure 2.3). This result suggests that postcopulatory sexual selection could be acting to manipulate female remating via the action of SFPs but further research would be needed to examine this in detail.

SFPs are known to alter female physiology, including delaying female remating (in *D. melanogaster*; Chen et al., 1988). As E males have larger accessory glands (where some of the SFPs are produced; Crudgington et al., 2009), they could be utilising either a greater number or a higher concentration of SFPs to manipulate females and delay female remating for longer. Alternatively or concurrently, M females could be getting direct benefits, such as sperm replenishment or another benefit from the male ejaculate (Trevitt et al., 1998; Gromko & Markow, 1993; Ravi Ram & Wolfner, 2007), from remating earlier compared to E females. This is supported by the finding of this study, that M females have higher productivity with their coevolved males compared to E females (Figure 2.4). Similarly, E females could have lower progeny production compared to M females because of their delay in remating. E females oviposited fewer eggs, so they are unlikely to become sperm depleted, whereas M females could be sperm depleted from ovipositing a much larger number of eggs. M females may remate faster to replenish their sperm in storage but these factors are unlikely to explain remating behaviour in *D. pseudoobscura*, since it is not affected by sperm load (Snook, 1998). However, sperm aging as a result of longer sperm storage in females may reduce fertility or zygote viability (Pizzari et al., 2003) and so M females may remate to improve their fertility, not simply because they are sperm depleted.

The finding that M females have higher productivity is similar to other studies in *D. melanogaster* which found that mating to monogamous males increased female productivity relative to mating with promiscuous males (Holland & Rice, 1999; Pitnick et al., 2001a) and is consistent with the prediction that monogamous pairings will evolve to be more benign to each other. When females remate relatively readily within a population, as in the M population in the current

study, selection favours traits that enhance fertilisation success, for example, investment in male ejaculates (Markow, 2002). However, when females do not remate readily, selection favours the evolution of traits that maximise mating success, for example, more intense courtship displays. If such a trade-off exists in these populations, then it appears E males are investing more in courtship displays but are not benefiting from an increased number of rematings. As a previous study has shown that sexual conflict is driving some reproductive traits in these populations (Crudgington et al., 2010), it is likely that E females are suffering costs in terms of reduced progeny production (as shown in the current study), which could be attributed to either courtship harassment or the action of SFPs, suggested to have negative fitness effects on females (Chapman et al., 1995; Crudgington et al., 2010).

I have demonstrated that by manipulating a species' natural mating system, the consequences of sexual selection on a range of both pre- and post-copulatory traits can be uncovered. The results presented here suggest that some traits have diverged in response to selection. Some of these responses are similar to those previously found in *D. pseudoobscura* (Crudgington et al., 2005; 2009; 2010) but differ from some of those in *D. melanogaster* (Rice, 1996; Wigby & Chapman, 2004). Such variation could be due to differences between species in the level of promiscuity. By observing the interaction between the sexes during selection, I found that courtship behaviour has responded to selection but does not translate into a difference in remating patterns in females, either for E males to coerce females to remate more or for M pairings to be more benign to each other as predicted by sexual conflict theory. I have also uncovered a potential postcopulatory mechanism, as I found that E females delay remating longer than M females, at least in three of the four replicates measured. This could be due to the decrease in productivity (consequently using fewer sperm) and/or the action of SFPs employed by males to manipulate female behaviour. The effects of SFPs in *D. pseudoobscura* have not been well studied, so future investigation would be needed to identify the role(s) of SFPs in this species.

# Chapter 3. Proteomic analysis of male accessory glands in *Drosophila pseudoobscura*

## Introduction

Males of many organisms transfer a suite of seminal fluid proteins (SFPs), alongside sperm, to females during copulation (Chen, 1984; Findlay et al., 2008; Baer et al., 2009). SFPs have received much attention in the literature because of their fundamental importance to the reproductive success of both sexes (Civetta & Singh 1995; Swanson et al. 2001; Swanson & Vacquier 2002; Clark et al. 2006). SFPs are produced by the secretory tissues of the male reproductive tract (Figure 3.1a) and can cause numerous behavioural and physiological effects in mated females (Wolfner, 1997; Gillott, 2003; Chapman & Davies, 2004). For example, SFPs have been shown to increase egg laying (Chen et al., 1988), decrease female receptivity to remating (Chen et al., 1988) and facilitate sperm storage (Tram & Wolfner, 1999; Neubaum & Wolfner, 1999). In *Drosophila*, primary amino acid sequences of some SFPs show rapid evolutionary change (Mueller et al., 2005). As the majority of such proteins associated with reproduction are known to evolve rapidly (Swanson & Vacquier, 2002; Clark et al., 2006; Panhuis et al., 2006), a more detailed analysis of how, or if, these reproductive traits respond to known selection pressures could provide an exciting avenue of research.

Given the importance of SFPs to reproductive fitness and their relatively rapid evolution, it is likely that sexual selection is the driving mechanism behind SFP evolution, either as a result of postcopulatory sexual selection (i.e. sperm competition and cryptic female choice) and/or sexual conflict. SFPs could have evolved in response to sperm competition to increase paternity assurance when females mate multiply (Clark et al., 1995). SFPs that are specifically produced by the accessory glands are known as accessory gland proteins (hereafter, Acps). In *Drosophila melanogaster*, males that do not transfer accessory gland protein Acp36DE suffer decreased fertilisation success because sperm are not stored efficiently by the female (Neubaum & Wolfner, 1999; Chapman et al., 2000).

Alternatively, or additionally, females may drive the evolution of SFPs through cryptic female choice (Eberhard, 1996; Wolfner, 2009). Females may evolve to increase their threshold to the action of certain SFPs, thereby ensuring only males with the highest SFP quantity and/or quality activate her full post-mating response (Chapman, 2001).

The rapid evolution of SFPs could also be in response to sexual conflict, potentially generating sexually antagonistic coevolution between the sexes. Certain SFPs, for example Acp62F, stimulate egg laying and sperm storage in mated females but can also shorten female lifespan (Lung et al., 2002). Such reproductive proteins allow males to benefit from increased fitness by expressing them, but females suffer costs as a result. In response, females will try to resist costs associated with SFPs by increasing their response threshold (Holland & Rice, 1997), potentially leading to the effects of these particular SFPs being exaggerated over evolutionary time. One well studied protein, sex peptide (Acp70A), has been shown to increase egg laying and decrease female receptivity to remating (Chen et al., 1988). To function within the female reproductive tract, sex peptide requires an interaction with a female G-protein coupled receptor called sex peptide receptor (SPR) and females that lack SPR fail to respond to sex peptide (Yapici et al., 2008). This example highlights how females could respond to the presence or quantity of SFPs and shows the importance of reproductive proteins in mediating reproductive success.

In *D. melanogaster* the transfer of Acp26Aa (ovulin) causes females to lay eggs earlier in their lifetime and increase the overall number of eggs laid (Herndon & Wolfner, 1995; Heifetz et al., 2000; Chapman et al., 2001). This could be an advantage to the male if a female lays a larger number of eggs before she remates, therefore increasing the chances of that particular male siring more progeny. *Acp26Aa* is one of the most rapidly evolving genes in the *Drosophila* genome (Wong et al., 2006) but has significant conservation of a structural backbone which may allow the protein to maintain its overall 3D configuration so that it can tolerate such high rates of evolution at other sites (Wong et al., 2006; 2010). Despite some SFPs showing rapid evolution of their primary amino-acid

sequences (Mueller et al., 2005), functional classes of SFPs are highly conserved (Mueller et al., 2004) within and between species supporting the idea that the functions of SFPs are important to reproductive success.

Males that are able to transfer a greater quantity of particular SFPs, and therefore gain greater control of female reproductive biology, could achieve a reproductive advantage over other males that cannot. Therefore, it is expected that males experiencing more intense sperm competition are expected to invest more heavily in SFPs to benefit from their effects on reproductive success (Wigby et al., 2009). Consistent with this prediction is the rapid evolution in the size of the accessory glands in response to more intense sexual selection, presumably to accommodate a greater number or higher quantities of SFPs. Larger accessory glands have been shown to increase male reproductive success in *D. melanogaster* (Bangham et al., 2002) and *Crytodiopsis dalmannni* (Baker et al., 2003), where males with larger accessory glands, but not testes, had a greater mating capacity. *D. melanogaster* males showed significantly higher levels of sex peptide and a greater reproductive fitness after only ~40 generations of artificial selection for larger accessory glands, compared to males with smaller accessory glands (Wigby et al., 2009). Experimentally evolved lines of *D. pseudoobscura* showed that males experiencing greater sperm competition risk had larger accessory glands and a greater sequential mating capacity (Crudgington et al., 2009). However, it is not known if these males have more SFPs, unique SFPs or a greater quantity of particular SFPs, than males experiencing relaxed or no selection. Conversely, males have been shown to decrease the transfer of particular SFPs in response to female mating status, potentially to exploit the action of SFPs transferred by previous males (Sirot et al., 2011).

Proteomic analyses can identify the form and function of thousands of proteins within complex samples. Such analyses have been used to identify SFPs in many insect species (Simmons et al., 2013; Wei et al., 2015), including *Drosophila* (Findlay et al., 2008; 2009; 2010). Advances in mass spectrometry have increased sensitivity of analyses and it is now possible to identify peptides with far greater accuracy than previously achievable.

In this study, I used experimentally evolved *D. pseudoobscura* populations (Crudgington et al., 2009) in which accessory gland size has increased under selection and performed liquid chromatography tandem mass spectrometry (LC-MS/MS) to analyse peptides for a whole tissue analysis of the accessory gland pairs (Figure 3.1b). The focus was not only to identify proteins present in the accessory gland tissue but also to identify those proteins which are secreted. By analysing the whole accessory gland tissue, I have identified 3770 proteins that comprise the accessory gland proteome. These proteins include house-keeping proteins as well as proteins associated with reproductive functions in the interaction between males and females (discussed previously). Acps must be secreted from the accessory glands to be transferred to females. Therefore, to be described as Acps, proteins must have predicted signal sequences (Wolfner et al., 1997) that allow extracellular secretion. By extracting those proteins with a signal sequence from the proteome dataset for a more detailed analysis, this chapter forms the first secretome analysis for *Drosophila* species, allowing this study to identify and quantify a suite of known and potential Acps. These findings demonstrate the power of combining approaches in evolutionary biology with proteomics to investigate fundamental aspects of reproductive biology.

## Materials and Methods

### Selection line treatments

An ancestral population of *Drosophila pseudoobscura* was established in 2001, using wild-caught, mated females from Tucson (AZ). *D. pseudoobscura* is a naturally polyandrous species, with females frequently shown to mate with and be inseminated by at least two males (Anderson, 1974). From the ancestral population, four replicate populations were established (Replicates 1, 2, 3 and 4) for two sexual selection treatments: enforced monogamy (M; 1♀, 1♂) and elevated polyandry (E; 1♀, 6♂). Flies are maintained in vials containing cornmeal-agar-molasses food media with added live yeast and housed at 22°C with a 12 hour light, 12 hour dark cycle. At the end of each generation, offspring are collected and pooled together within each replicate line of M and E. The next generation is composed of a random sample of these pooled progeny, which takes account of the differential progeny production across families. For more information, the setting up and maintenance of the selection lines are described in detail in Crudgington et al. (2005; 2009; but also see chapter 1, Figure 1.3 for an updated version).

### Experimental flies preparation

Flies from replicates 1-4 of the selection lines were collected from generations 157, 156, 155 and 153 respectively. In order to reduce any maternal effects, parental flies were collected and housed together on egg laying plates. Males and females were allowed to interact for 24hrs, after which a fresh egg plate was provided for females to lay their eggs on. Eggs were allowed to hatch and develop to the first instar larval stage, before being collected in groups of 100 and housed in standard molasses/agar food vials at 22°C. Males from these vials were collected on the day of eclosion and housed in vials of 10 individuals, until they were sexually mature. Males were dissected when they were 5 or 6 days old.

### Tissue preparation

Intact accessory glands were extracted from *D. pseudoobscura* males under a Leica dissecting microscope and using fine dissection needles. A total of 30 accessory gland pairs (AG) per replicate were dissected into a drop of Phosphate Buffered Saline (PBS) and transferred to a microcentrifuge tube containing a 30µl aliquot of Radio-Immunoprecipitation Assay (RIPA) buffer, phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors stored on ice (see Appendix 1.1 for details on the stock solutions). Once all AGs had been dissected, samples were freeze/thawed three times by first being frozen on dry ice (~5 mins), then thawed at 37°C for 30 seconds. After the freeze/thaw cycles, samples were vortexed for 30 seconds, centrifuged at 20 Kg for 5 minutes at 4°C and then stored at -80°C until processing. A Bradford assay was used to determine how much of each sample to run. Elevated promiscuity (E) males have larger accessory glands than enforced monogamy (M) males (Crudgington et al., 2009), so it would be expected that E males have a higher concentration of protein in their accessory glands. In light of this, the protein concentration being loaded onto the gels was standardised for E and M samples.

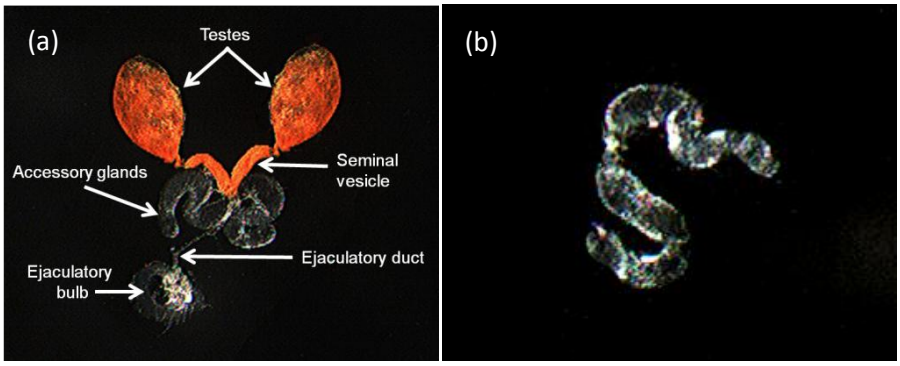



**Figure 3.1: (a) Reproductive tract of *Drosophila pseudoobscura* showing the testes, seminal vesicles, ejaculatory duct, ejaculatory bulb and accessory glands. (b) The intact accessory glands of male *D. pseudoobscura* dissected and separated from the other components of the male reproductive system.**

**1-Dimensional SDS-PAGE**

A 1 mm 4-12% NuPAGE Novex Bis-Tris Mini Gel was set up using the XCell II Mini-Cell system (Invitrogen) as per manufacturer's instructions. Fifty micrograms of AG protein (with DTT and $ddH_2O$) was loaded for each sample alongside a molecular weight marker. The gel was run all the way to the bottom at a 200 V constant. Following electrophoresis, the gel was stained using Brilliant Blue G Colloidal Concentrate (Sigma) electrophoresis reagent and images of the gel were taken (Figures 3.2-3.4). The gel was then cut so that each lane was separated from the others and cut into roughly equal pieces. Each gel piece was transferred to an empty siliconised microcentrifuge tube, labelled appropriately and destained by washing with 200 mM ammonium bicarbonate and 40% acetonitrile. Gel pieces were then dried down using a vacuum concentrator and stored at -20°C overnight.

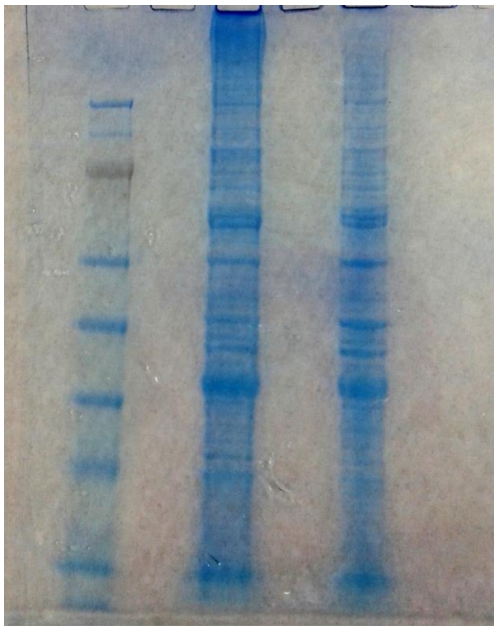


**Figure 3.2: Gel image showing the molecular weight marker and monogamy (M) and elevated polyandry (E) Replicate 1 (left to right).**

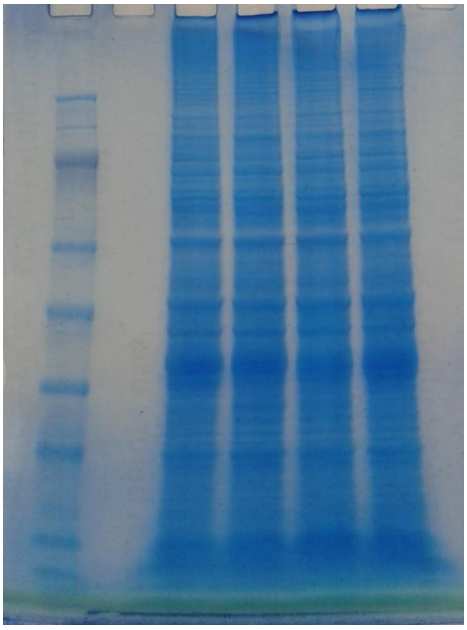


**Figure 3.3: Gel image showing the molecular weight marker, monogamy (M) treatment Replicates 2 and 3, the elevated polyandry (E) treatment Replicates 2 and 3 (left to right).**

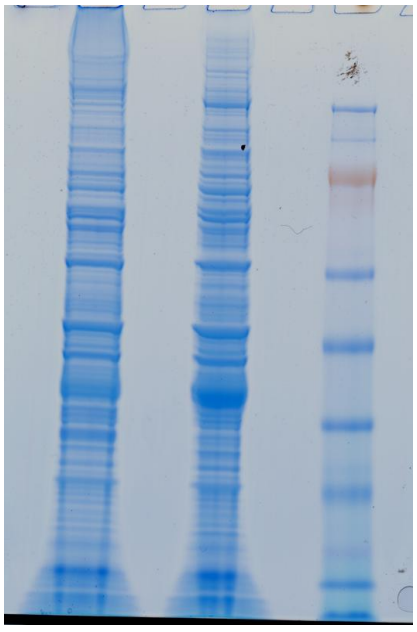


**Figure 3.4: Gel image showing the monogamy (M) and elevated polyandry (E) treatment for Replicate 4 and the molecular weight marker (left to right).**

**In-gel digestion of proteins**

A standard in-gel digestion protocol was performed on each gel piece. Gel pieces were reduced and alkylated using 200 µl of 50 mM ammonium bicarbonate (reduction buffer) and 55 mM iodoacetemide mixed with 50 mM ammonium bicarbonate (alkylation buffer) and centrifuged at 13 Kg for 10 seconds. Gel pieces were then dried down using a miVac Quattro vacuum concentrator (Genevac) for ~30 minutes (until all samples were dry). Once dried, 20 µl of trypsin (New England BioLabs) and 50 µl of acetonitrile was added to each gel piece and incubated overnight at 37°C. Peptides were extracted the following day using a standard method with 100% acetonitrile, 5% formic acid and a solution of 100% acetonitrile and 5% formic acid (Appendix 1.1) and dried down overnight in a vacuum concentrator at 30°C. Resulting peptides were resuspended in 7.5 µl of Switchos Solution (0.1% (v/v) formic acid, 3% (v/v) acetonitrile), sonicated in a water bath for 5 minutes and centrifuged at 13 Kg for 10 seconds, before being transferred to a sample vial and loaded into the autosampler tray of the Dionex Ultimate 3000 µHPLC system. Samples were set to run using the Xcalibur sequence system.

**LC-MS/MS analysis**

Extracted peptides were submitted to a µHPLC-MS/MS system comprising an Ultimate 3000 Nano LC System (Dionex) coupled to an LTQ Orbitrap Elite hybrid mass spectrometer (Thermo Scientific) equipped with an Easy-Spray (Thermo Scientific) ion source. Peptides were desalted on-line using a capillary trap column (Acclaim Pepmap100, 100 µm, 75 µm x 2 cm, C18, 5 µm; Thermo Scientific) and then separated using 60 min RP gradient (3-40% acetonitrile/0.1% formic acid) on an Acclaim PepMap100 RSLC C18 analytical column (2 µm, 75 µm id x 10 cm; Thermo Scientific) with a flow rate of 0.25 µl/min. The mass spectrometer was operated in standard data dependent acquisition mode controlled by Xcalibur 2.2. The instrument was operated with a cycle of one MS (in the Orbitrap) acquired at a resolution of 60,000 at m/z 400, with the top 20 most abundant multiply-charged (2+ and higher) ions in a given chromatographic window were subjected to CID fragmentation in the linear ion trap. An FTMS

target value of 1e6 and an ion trap MSn target value of 10000 were used. Dynamic exclusion was enabled with a repeat duration of 30 s with an exclusion list of 500 and exclusion duration of 30 s.

**Mass Spectrometry data analysis**

The raw mass spectra files were analysed using MaxQuant version 1.5.0.12 (Cox & Mann, 2008) searched against a combined UniProt (downloaded July 2014) *D. pseudoobscura* and *D. melanogaster* sequence database using the following search parameters: trypsin with a maximum of 2 missed cleavages, 7 ppm for MS mass tolerance, 0.5 Da for MS/MS mass tolerance, with acetyl (Protein N-term) and oxidation (M) as variable modifications and carbamidomethyl (C) as a fixed modification. A protein FDR of 0.01 and a peptide FDR of 0.01 were used for identification level cut offs (1% FDR using a decoy database). Match between runs with a 2 minute retention time window was enabled and label free quantitation (LFQ) was performed with a minimum ratio count of 2. Missing values were imputed using Perseus (1.4.1.3) and two sample t-testing was performed with a permutation based FDR calculation in Perseus.

The final dataset was filtered so that every protein must be identified by at least two unique peptides in any of the biological replicates. This was to ensure that the dataset was a robust one, containing only proteins that showed up consistently and to avoid potential misidentifications through using only one peptide to identify a protein. These datasets were used to identify gene ontology (GO) enrichment, to examine protein abundances and to make comparisons between the data presented in this chapter and known SFPs from the literature. To be included in the proteome and secretome datasets for comparison between the treatments of M and E, proteins had to be identified by at least two unique peptides in 3 out of the 4 replicates (see Appendix 1.2 for the number of proteins identified in 2 out of the 4 replicates; Figures 1.2.1; 1.2.3 and in all 4 replicates; Figures 1.2.2; 1.2.4). By ensuring each protein was identified in 3 out of the 4 replicates, the chance of misidentifying a protein was reduced without being too strict (for example, by only including proteins that were in 4 out of the 4

replicates) and potentially missing proteins that were consistently present in the dataset.

**Secretome analysis**

SignalP version 4.1 (Petersen et al., 2011) and Phobius version 1.01 were used to identify the proteins with predicted signal peptide sequences. Default settings were used for both SignalP and Phobius, i.e., default D-cutoff values to optimise for correlation and "Eukaryotes" as the organism group. Proteins that were identified in both SignalP and Phobius were collated to form the secretome list for further analysis. The secretome list contains 396 proteins which comprises 10.5% of the entire dataset and closely correlates with the predicted secretome in humans (~10% of the total proteins encoded by the genome; Pavlou & Diamandis, 2010).

**Gene ontology and protein abundance**

Gene ontology (GO) was established using the ClueGO plugin version 2.2.4 (Bindea et al., 2009) of Cytoscape version 3.2.1. *D. melanogaster* orthologues were assigned to each secretome protein (where available) using FlyMine. These orthologues were compared to a reference population for statistical analyses and to generate figures of enrichment for cellular components and molecular function. The reference population is all of the genes (for *D. melanogaster*) that have a GO annotation. GO Cellular Component Annotations were compared using statistical tests for gene enrichment (two-sided hypergeometric test) with Holm-Bonferroni multiple test correction implemented. Network parameters were set as follows: GO Tree Levels (min = 2, max = 5), GO term restriction (min#genes = 4, min% = 4), and GO Term Connection Restriction (Kappa score threshold = 0.53). Only terms with a p-value ≤ to 0.05 were shown and groups sharing >50% of terms were merged.

For protein quantification, the secretome dataset was filtered to only include proteins that had been identified by at least two unique peptides and the top 20 (5.1% of the secretome identified) most abundant proteins were selected for analysis (as suggested by Skerget et al., 2015). In this study, LFQ intensities

were used to quantify abundance because they are normalised to reflect the relative amounts of proteins (MaxLFQ; Cox et al., MCP, 2014).

## Results

The accessory glands of male *Drosophila pseudoobscura* were analysed using mass spectrometry. In total, 3770 proteins were identified by 44182 unique peptides (Table 3.1). Of those proteins identified in the proteome, 395 proteins had a signal sequence (Table 1) and were grouped as the secretome. These secretome proteins were identified by 5938 unique peptides, which is an average of 15.03 peptides per protein for the secretome (Table 3.1).

**Table 3.1: Summary of Mass Spectrometry Results. Each protein had to be identified by at least two unique peptides in any replicate/treatment.**

| | Proteome | Secretome |
|---|---|---|
| Total proteins identified | 3770 | 395 |
| Total unique peptides identified | 44182 | 5938 |
| Average peptide hits per protein | 11.72 | 15.03 |

**Proteome.** The proteome dataset was analysed for any differences between the treatments of M and E. 1949 out of the 3770 proteins (Table 3.1) were found to be in 3 out of the 4 replicates within a treatment. This strict filtering allowed for a robust but conservative number of proteins to be analysed. These 1949 proteins that comprised the filtered proteome were used for the remainder of the proteome analyses.

1651 of the proteins identified were found to be in both M and E (Figure 3.5). 97 proteins were found only in the M treatment and 201 proteins were found only in the E treatment (Figure 3.5). However, there was no significant enrichment of any GO terms within a treatment for the proteome. For the proteins only identified in a

single treatment, there is no evidence that these proteins are completely absent from the other treatment, only that they were not identified by enough peptides to be included when using strict filtering. There was a large amount of overlap between the replicates of the proteome; 1390 proteins were present in all 4 replicates of M (Figure 3.6) and 1528 proteins were present in all four replicates of E (Figure 3.7).

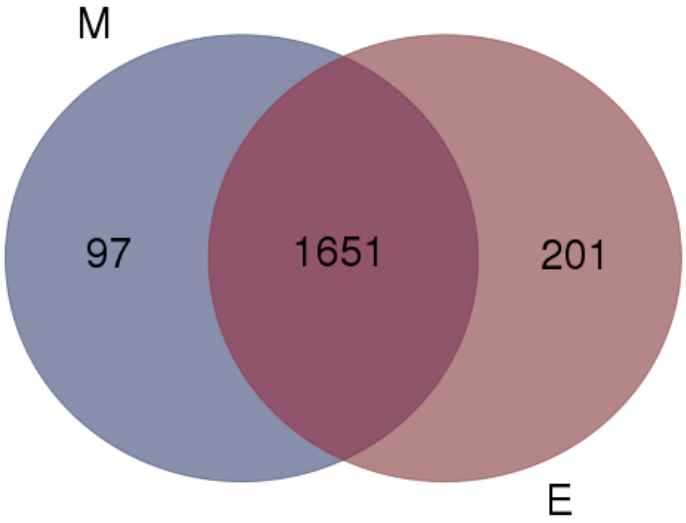



**Figure 3.5: Venn diagram illustrating the overlap of protein identification between the two treatments (M and E) of the proteome. 1651 proteins were found to be common to both treatments. The proteome dataset was filtered so that each protein identified had to be present in 3 out of the 4 replicates (with at least two unique peptides to identify it in each replicate) within a treatment. 84.7% of those proteins found in 3 of the 4 replicates were found in both M and E.**

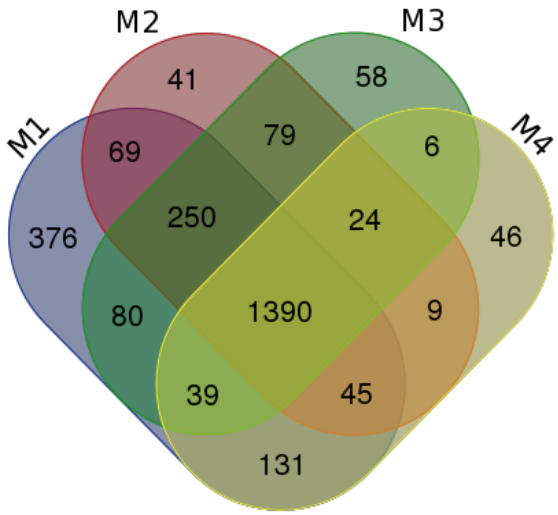



**Figure 3.6: Venn diagram illustrating the overlap of protein identification between the monogamous treatment replicates of the proteome. 1390 proteins were found to be common to all four replicates. The proteome dataset was filtered to only include proteins that were identified by at least two unique peptides within each replicate.**

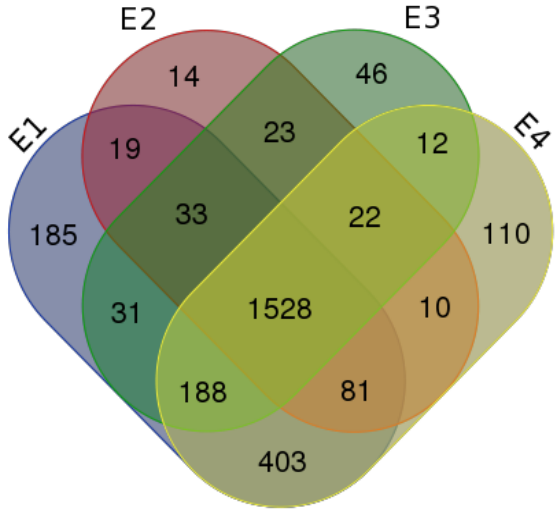



**Figure 3.7: Venn diagram illustrating the overlap of protein identification between the elevated polyandry treatment replicates of the full proteome. 1528 proteins were found to be common to all four replicates. The proteome dataset was filtered to only include proteins that were identified by at least two unique peptides within each replicate.**

**Secretome.** The 395 proteins identified as the secretome (Table 3.1) were filtered so that each protein must be identified by at least 2 unique peptides in at least 3 of the 4 replicates within a treatment. With this stricter filtering, the secretome was comprised of 274 proteins. Of these, 247 proteins were found to be present in both M and E (Figure 3.8). 11 proteins were found only in the M treatment and 16 proteins were found only in the E treatment (Appendix Table 1.2.1) but 91% of those for M and 100% of those for E were found to be in both treatments at some level when examining the unfiltered dataset. There was considerable overlap between the replicates of the secretome. The M treatment had 224 proteins common to all four replicates (Figure 3.9) and the E treatment had 235 proteins common to all four replicates (Figure 3.10).

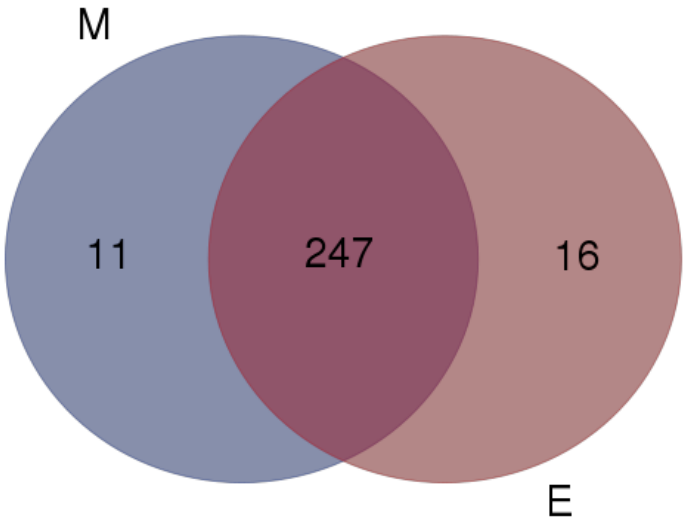



**Figure 3.8: Venn diagram illustrating the overlap of protein identification between the two treatments, M and E of the secretome. 247 proteins were found to be common to both treatments. The secretome dataset was filtered so that each protein identified had to be present in 3 out of the 4 replicates (with at least two unique peptides to identify it) within a treatment. 90% of the proteins found in 3 of the 4 replicates were found in both M and E.**

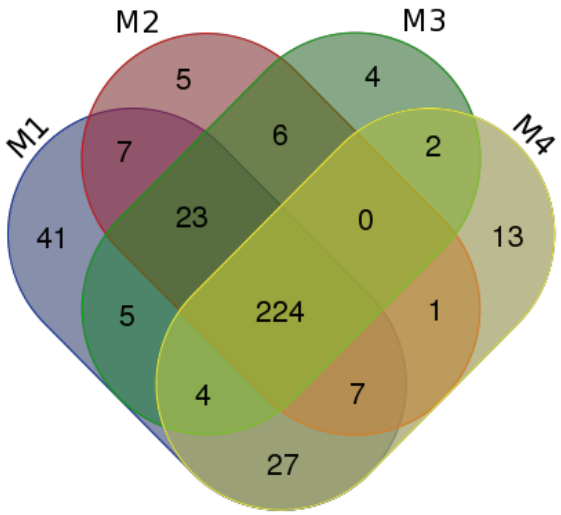



**Figure 3.9: Venn diagram illustrating the overlap of protein identification between the monogamous treatment replicates of the secretome. 224 proteins were found to be common to all four replicates. Each protein had to be identified by at least two unique peptides within the replicate.**

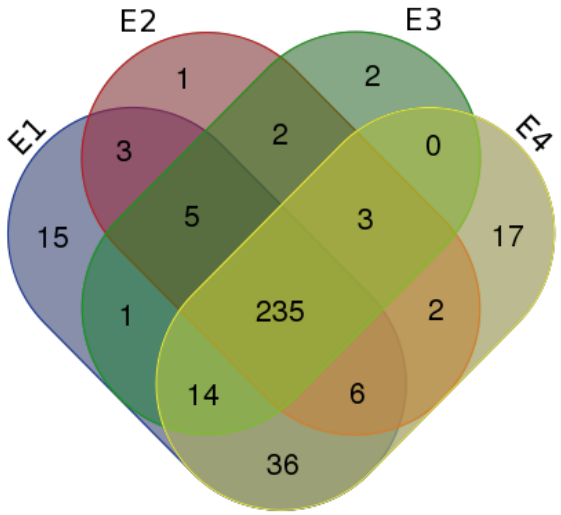



**Figure 3.10: Venn diagram illustrating the overlap of protein identification between the elevated polyandry treatment replicates of the secretome. 235 proteins were found to be common to all four replicates. Each protein had to be identified by at least two unique peptides within the replicate.**

**Gene ontology (GO).** Of the 395 total secretome proteins, 365 of those have *D. melanogaster* orthologues. These orthologues show gene ontology (GO) enrichment of multicellular organism reproduction (n=86, p=4.71e-11), reproduction (n=92, p=5.11 e-10) and insemination (n=10, p=4.78 e-6) biological processes. 22 of the secretome proteins are enriched in multi-organism behaviour (p=0.001) and 8 of the secretome proteins show enrichment in the regulation of female receptivity, post mating (Appendix Table 1.2.2; p=9.09 e-4). Both sets of proteins (22 for multi organism behaviour and 8 for female receptivity) are present in both treatments, M and E. A large proportion of the proteins identified in the secretome are annotated as extracellular and plasma membrane proteins (Figure 3.11; Figure 3.12). The secretome is enriched in proteins that have hydrolase activity (n=23, p=1.46 e-12) and protein disulfide isomerase (n=25, p=3.81 e-12) molecular functions.

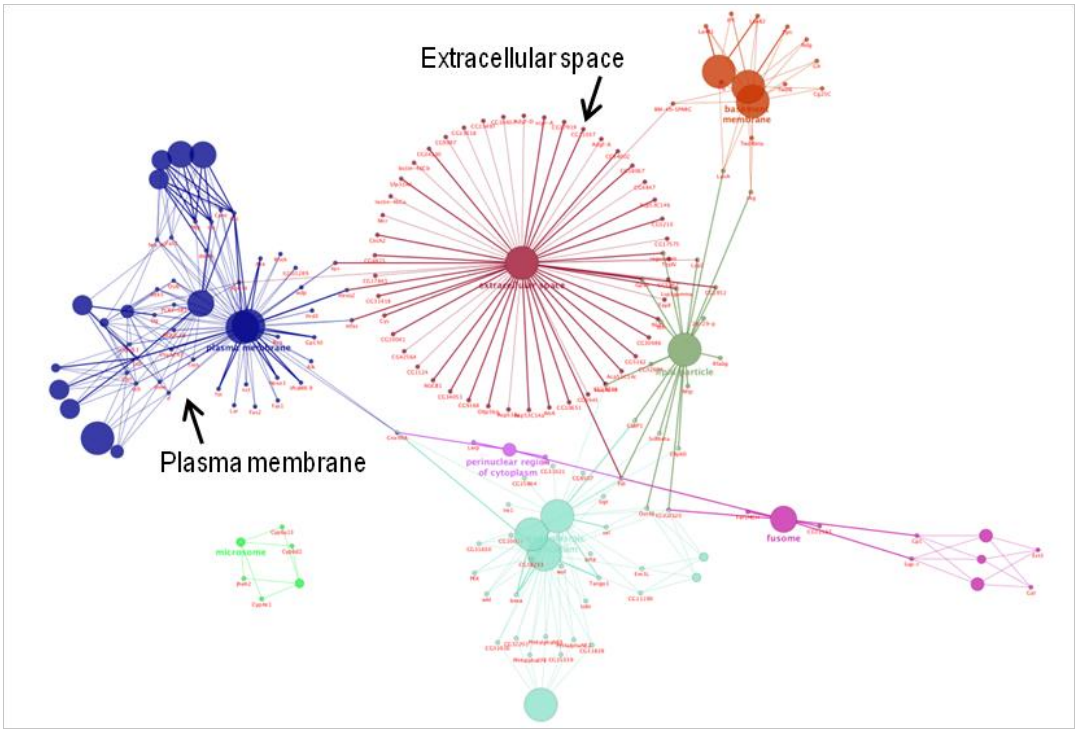



**Figure 3.11: Gene ontology enrichment for the cellular component of the secretome proteins (identified by the *D. melanogaster* orthologues). Both treatments, M and E, were included. Each of the genes identified as being part of the secretome are plotted around the cellular nodes.**

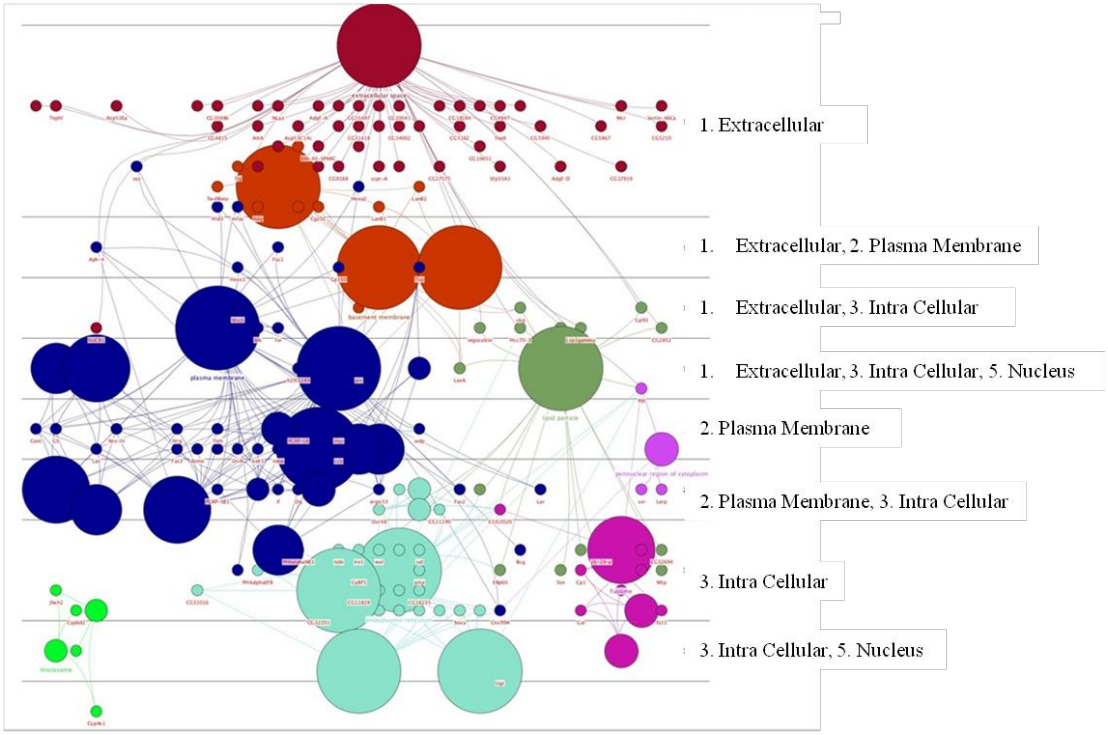



**Figure 3.12: Gene ontology enrichment for the cellular component of the secretome proteins (identified by the *D. melanogaster* orthologues). Both treatments, M and E, were included. The same data as Figure 3.11 is presented but also showing the cellular locations of the proteins.**

**Overlap with known SFPs.** There are 64 secretome proteins that overlap with known SFPs (Figure 3.13), and these correlate with 68 *D. melanogaster* orthologues. These *D. melanogaster* orthologues show GO enrichment of biological processes such as reproduction (n=58, p=2.07 e-37), sperm competition (n=9, p=4.79 e-11), copulation (n=10, p=1.62 e-11) and regulation of female receptivity, post mating (n=8, p=5.19 e-9). The majority of these overlapping proteins are annotated as extracellular proteins (n=63, p=2.62 e-52). The named Acps found in the secretome presented in this chapter are (Dpse\) Acp26Aa, Acp53Ea, Acp53C14a, Acp53C14b, Acp53C14c and Acp32CD.

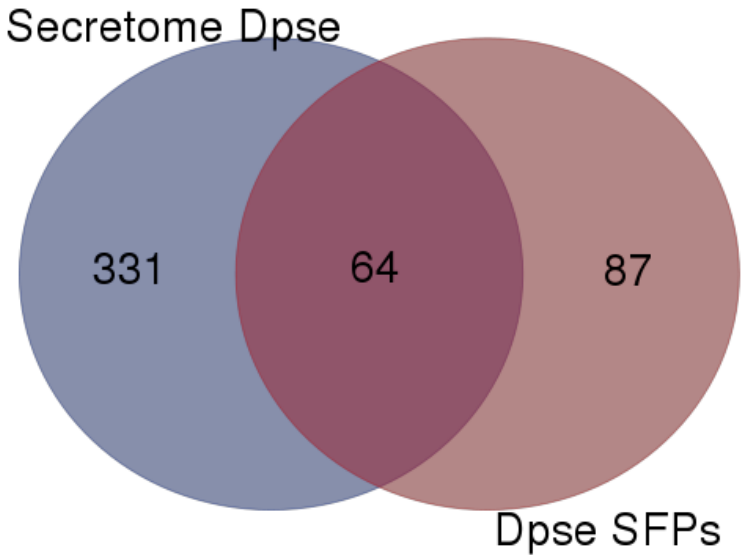



**Figure 3.13: Venn diagram illustrating the overlap of protein identification between the secretome (Secretome Dpse) and the known seminal fluid proteins (Dpse SFPs) converted to *D. pseudoobscura* identifiers from *D. melanogaster* identifiers. 64 proteins were found to be common to both datasets. The secretome dataset was filtered so that each protein had to be identified by at least two unique peptides in any of the biological replicates.**

**Protein abundance.** Secretome proteins were organised by average LFQ intensity across both treatments (M and E) and the top 20 most abundant proteins (5.1% of the total secretome dataset) are shown in Table 3.2. These intensity values provide a relative abundance of the proteins of the secretome. Some of the most abundant proteins in the secretome are named Acps: Acp53Ea, Acp26Aa and Acp53C14b. These known Acps were also found in the top 20 most abundant proteins for both M and E treatments when considered separately, which provides further indication that there are no significant differences between the treatments. The most abundant proteins are annotated as extracellular proteins (n=14, p=3.49 e-13) which are involved in a variety of biological processes relating to reproduction; sperm competition (n=4, p=3.06 e-4), insemination (n=4, p=4.44 e-4), copulation (n=4, p=0.001) and regulation of female receptivity, post mating (n=3, p=0.004). There was no molecular function enrichment for the top 20 most abundant secretome proteins.

**Table 3.2: The top 20 most abundant secretome proteins by average LFQ intensity for both treatments, M and E, combined.**

| | Flybase Id | Protein Name | *D. melanogaster* Orthologue | Average LFQ intensity (±SE) |
|---|---|---|---|---|
| **1** | FBgn0080005 | Dpse/GA20009 | Pdi | 3.84E+10 (2.70E+09) |
| **2** | FBgn0077997 | Dpse/GA17988 | Hsc70-3 | 2.27E+10 (1.11E+09) |
| **3** | FBgn0248215 | Dpse/GA26842 | SP1029 | 1.21E+10 (1.31E+09) |
| **4** | FBgn0078945 | Dpse/GA18946 | Gp93 | 1.17E+10 (1.22E+09) |
| **5** | FBgn0249994 | Dpse/GA28634 | CG17843 | 6.31E+09 (4.92E+08) |
| **6** | FBgn0070792 | Dpse/GA10736 | Rfabg | 4.69E+09 (3.10E+09) |
| **7** | FBgn0245536 | Dpse/GA24137 | ERp60 | 4.59E+09 (3.14E+08) |
| **8** | FBgn0245588 | Dpse/GA24189 | - | 4.54E+09 (2.22E+08) |
| **9** | FBgn0075503 | Dpse/GA15486 | CG2852 | 4.5E+09 (5.17E+08) |
| **10** | FBgn0243575 | Dpse/Acp53Ea | Acp53Ea | 4.08E+09 (6.03E+08) |
| **11** | FBgn0082796 | Dpse/Acp26Aa | CG8982 | 4.03E+09 (4.35E+08) |
| **12** | FBgn0074591 | Dpse/GA14563, A | CG17575 | 3.94E+09 (1.26E+08) |
| **13** | FBgn0077417 | Dpse/GA17404, B | mfas | 3.78E+09 (2.26E+08) |
| **14** | FBgn0079900 | Dpse/GA19904 | Ugt | 3.77E+09 (2.59E+08) |
| **15** | FBgn0074106 | Dpse/GA14075 | lectin-46Cb | 3.33E+09 (4.35E+08) |
| **16** | FBgn0245732 | Dpse/GA24335 | lectin-46Ca | 3.14E+09 (3.50E+08) |
| **17** | FBgn0249580 | Dpse/GA28217 | Glg1 | 3.01E+09 (2.69E+08) |
| **18** | FBgn0248690 | Dpse/GA27323 | Calr | 2.93E+09 (1.55E+08) |
| **19** | FBgn0073882 | Dpse/Acp53C14b | Acp53C14b | 1.98E+09 (6.54E+08) |
| **20** | FBgn0247756 | Dpse/GA26382 | CG42564 | 1.92E+09 (6.22E+08) |

## Discussion

Shotgun proteomics has been used to identify the proteome of *Drosophila pseudoobscura* male accessory glands. The accessory glands of *D. pseudoobscura* males from two selection line treatments, enforced monogamy (M) and elevated polyandry (E) were analysed using LC-MS/MS. Each treatment consisted of four biological replicates, but due to equipment access constraints, technical replicates of the same biological material were not performed. However, there was a large amount of overlap of the proteins identified between the biological replicates for both the proteome (Figures 3.6 and 3.7) and the secretome (Figures 3.9 and 3.10). The relatively large overlap between replicates suggests that both the dissections (Figure 3.1) and the subsequent sample processing were of a consistently high standard. Additionally, strict filtering criteria for peptide and protein annotation were applied, which provided a conservative but robust number of proteins identified. The average number of peptides to identify each protein, 11.72 and 15.03 for the proteome and secretome respectively (Table 3.1), also demonstrated the quality of the dataset. Consequently, the biological replicates within a treatment were combined for the majority of the analyses into a single treatment group. I identified a total of 3770 proteins as the accessory gland proteome (Table 3.1). However, for the remainder of the analyses, I only included proteins which were identified by at least two unique peptides in 3 out of the 4 biological replicates within a treatment. This was so that I only presented protein identifications which consistently appeared and for which I could be confident were present, particularly when combining replicates into a single treatment for further analyses. However, I did not restrict the list to such an extent as to only include proteins that were present in all of the replicates, because I did not want to exclude proteins which were likely to be present in the proteome. The proteome contained 1949 proteins that were present in 3 out of the 4 replicates (Figure 3.5), of which 1651 were identified in both treatments, M and E. This represents 84.7% of the proteome. There were no significant GO enrichment terms within a treatment and overall I found no significant differences in the quantification of any proteins between treatments.

The proteome of *D. pseudoobscura* accessory glands was analysed for proteins that had a signal sequence. These proteins are the ones which are secreted from cells and are the candidates for being most likely to be transferred to females during mating (Wolfner et al., 1997). As such, the secretome dataset is expected to contain the accessory gland proteins for *D. pseudoobscura*, both known and currently unannotated. Proteomics has proved successful in analysing seminal fluid proteins in a variety of organisms (Findlay et al., 2008; 2009; 2010; Baer et al., 2009; Simmons et al., 2013; Wei et al., 2015) and has been utilised here to identify and quantify the secretome. To my knowledge, this is the first secretome analysis for *Drosophila* species, but it has been performed in other organisms, such as the oriental fruit fly (*Bactrocera dorsalis*; Wei et al., 2015). This technique could be applied to future analyses of SFPs in *Drosophila*.

I identified a total of 395 secretome proteins, which comprises 10.5% of the total proteome dataset and closely correlates with the predicted secretome for humans (~10% of the total proteins encoded by the genome; Pavlou & Diamandis, 2010). In addition, a large proportion of the proteins identified as the secretome were annotated as extracellular (Figure 3.11) and the majority of them were located in the plasma membrane or in the extracellular space (Figure 3.12). The secretome was enriched in proteins that are associated with reproduction and female receptivity (Appendix Table 1.2.2). These are good indicators that the secretome in this chapter was predicted with reasonable accuracy, that the methodology and the programmes used to identify these proteins were robust and that the data produced is good quality. It also supports the idea that by identifying the secretome proteins, these analyses include putative Acps. Additionally, I compared the secretome identified in this chapter to known SFPs from the literature (Wagstaff & Begun, 2005; Ravi Ram & Wolfner, 2007; Findlay et al., 2008; 2009; 2010) and found that 64 proteins were present in both lists (Figure 3.13; *D. melanogaster* orthologues converted to *D. pseudoobscura* identifiers). Some seminal fluid proteins are located in the ejaculatory duct and the ejaculatory bulb, but I did not include these in the samples because it was not possible to consistently dissect them in tact within the given timescale. In addition, sex peptide was not found in the secretome dataset, possibly because it

is “unannotated” in Flybase for *D. pseudoobscura* or because it is easily missed in mass spectrometry analyses due to its small size (36 amino acids in *D. melanogaster*; Chen et al., 1988; Chapman & Davies, 2004). Sex peptide is predicted to be present in *D. pseudoobscura* (Wagstaff & Begun, 2005) but is unlikely to respond to sexual selection (Wong et al., 2012), so most likely would not have contributed to the analyses for the effects of sexual selection on the secretome proteins.

There were six named Acps present in the secretome; Dpse/Acp26Aa, Acp53Ea, Acp53C14a, Acp53C14b, Acp53C14c, Acp32CD and the majority of the overlapping proteins were annotated as extracellular proteins (n=63, p=2.62 e-52). Three of the named Acps were present in the top 20 most abundant proteins for both M and E (Table 3.2). One of these proteins, Acp26Aa, increases egg laying in mated females (Herndon & Wolfner, 1995; Chapman et al., 2001). An increase in egg laying can be beneficial to both sexes to a point, but if males could manipulate females beyond her optimum level, this could have negative fitness consequences for females (Chapman, 2001). Previous research has shown that *D. melanogaster* males can increase the transfer of at least two key reproductive proteins (Acp26Aa and Acp70A) to females after just ~40 generations of artificial selection (Wigby et al., 2009). The experimental evolution treatments used in this chapter have been selected for over 150 generations. Therefore, M males were predicted to have lower quantities and/or fewer Acps than E males because M pairings were expected to have reproductive interests which are more aligned than E pairings. E males were expected to have evolved an Acp complement which enabled them to manipulate females to a greater extent and therefore gain a competitive advantage over rival males in response to sexual selection and/or sexual conflict. Rapid evolution of Acps and SFPs in general has been reported elsewhere (Swanson et al., 2001; Mueller et al., 2005) and has been suggested to be a response to sexual conflict (Holland & Rice, 1999). However, there were no significant differences between M and E in the quantity of Acps in this study. Similarly, the top 20 most abundant secretome proteins, including the named Acps, were the same for both treatments (Table 3.2). Therefore, it is unlikely that postcopulatory sexual selection is a potent force

driving Acp evolution in these populations. The lack of differences between M and E Acp profiles could be explained by the finding of chapter 2, that there was no significant difference in remating between treatments (chapter 2; Figure 2.2).

The secretome contained proteins that were apparently unique to M or E (11 for M and 16 for E; Figure 3.8; Appendix Table 1.2.1). However, these proteins are not found exclusively in that treatment. Each “unique” protein was identified by at least one peptide in at least one of the replicates of the other treatment. As such, there was no evidence of absence. These proteins (or rather, peptides) may have been missed by the mass spectrometer or were not in high enough concentrations to be detected in some of the replicates. This meant they were filtered out by the relatively strict filtering applied to this dataset. Equally, there could be evidence that these proteins are unique to a treatment and were identified as a false-positive with only one peptide in an opposite treatment. The reason strict filtering was applied to the dataset was to decrease the chances of proteins being incorrectly identified by only one peptide. Further study could examine these “unique” proteins more closely using labelling techniques, such as Isobaric Tags for relative and Absolute Quantitation (iTRAQ) or Tandem Mass Tag (TMT) to track individual proteins within the samples and identify whether these proteins are truly unique to one treatment and at what abundance they are found in each sample.

The secretome proteins are characterised by enrichment in reproductive functions and this is reflected in the top 20 most abundant proteins as well. Since *D. pseudoobscura* proteins are not currently well annotated, *D. melanogaster* orthologues were used to identify their potential functions. Out of the top 20 most abundant proteins (Table 3.2), 19 of them have *D. melanogaster* orthologues. FBgn0245588 (GA24189) does not have a *D. melanogaster* orthologue but has been identified in the literature as a *D. pseudoobscura* protein (Richards et al., 2005; Clark et al., 2007). As yet, there is no gene ontology for this protein other than it is a signal peptide located in the extracellular space (Flybase, 2004). In total, 30 identified *D. pseudoobscura* secretome proteins (n=395) do not have *D. melanogaster* orthologues. These proteins were not significantly different in

quantity between M and E but functional annotation was not available, so it is possible there were undetected differences in functional enrichment. Annotating these (currently unannotated) *D. pseudoobscura* secretome proteins would be an interesting avenue for future study.

Finally, as it seems unlikely that postcopulatory sexual selection is shaping the evolution of secretome proteins in *D. pseudoobscura* accessory glands, the potential cause of the increase in accessory gland size cannot be uncovered by these analyses. Males have been shown to adjust their transfer of specific SFPs in response to environmental cues, such as female mating status (Sirot et al., 2011). This has been suggested as a response to potentially exploit the action of SFPs transferred by previous males. This plasticity of SFP composition is consistent with SFPs responding to selection and their role in mediating male reproductive success (Sirot et al., 2015). An area of future research could be to examine the experimentally evolved populations of *D. pseudoobscura* from within their selective environment. For example, it would be of interest to examine the proteins dissected from within the female reproductive tract after transfer. The majority of SFPs have target sites within the female reproductive tract and are modified in some way by the female (Park & Wolfner, 1995; Wolfner, 2002; Ravi Ram et al., 2006; Wong et al., 2010). SFPs could be taken from previously unmated females and from previously mated females to determine if males allocate different SFPs or different quantities of these SFPs to females with different mating status. A future study could also aim to provide information on the function(s) of currently unannotated *D. pseudoobscura* SFPs. This is especially interesting for those 30 secretome proteins in these selection line treatments which do not have *D. melanogaster* orthologues and for which relatively little is currently known.

# Chapter 4. A three-dimensional model of sperm inside the egg cytoplasm: A sperm's tail.

## Introduction

For fertilisation to occur, complex morphological, physiological and biochemical interactions must successfully take place between the sexes. Any changes in reproductive traits could become a source of incompatibility, if the sexes have not coevolved, since sexual selection has the ability to drive rapid evolution (Simmons, 2001; Birkhead & Pizzari, 2002). As such, sexual selection can strongly influence the probability that individuals from the same or different populations will successfully produce progeny, thereby potentially generating reproductive isolation (RI). Postmating, RI has been broadly divided into two categories: mechanisms that occur before the formation of the zygote (postmating, prezygotic); and those that occur after (postmating, postzygotic). Postmating prezygotic mechanisms include the failure of sperm and egg to interact properly, known as gametic isolation (Coyne & Orr, 2004). One example of this occurs in the lysin/VERL system of *Haliotis* abalone species (Panhuis et al., 2006). However, definitions of gametic isolation can be extended to include both problems arising during fusion at the gamete surface (syngamy) or before and those occurring during fusion of gamete pronuclei (karyogamy) to form the diploid zygote (Snook et al., 2009b). This broader definition than previously recognised allows the extensive intracellular interactions between the gametes to be taken into account.

Research shows that after syngamy in most organisms, the entire sperm enters the egg and interacts with the egg cytoplasm (in mammals, Krawetz, 2005; marine invertebrates, Shapiro et al., 1981; and insects, Karr, 1991). The entrance and subsequent persistence of the sperm tail within the egg suggests that sperm contribute non-genetic components to the egg and/or the developing zygote. These intracellular interactions are critical for fertilisation and early embryogenesis (Krawetz, 2005) and could represent a further source of RI. For example, some component of the sperm tail must be present to elicit sperm aster

formation and pronuclear fusion in insects and vertebrates (Moomjy et al., 1999; Sutovsky & Schatten, 2000). In *Drosophila*, mutations such as *misfire* (mfr) inhibit aster formation and karyogamy by failing to breakdown the sperm plasma membrane (Ohsako et al., 2003). The egg cytoplasm also plays a role in these intracellular interactions, as a maternal mutation *wispy* (wsy) prevents the proper configuration of pronuclei for karyogamy and early embryogenesis by stopping the female pronucleus migrating towards the male (Brent et al., 2000).

Sperm also contribute to embryonic development by determining embryo polarity (in *Xenopus laevis*, Danilchik & Black, 1988; and in rodents, Pedersen, 2001; Piotrowska & Zernicka-Goetz 2001; Gray et al., 2004). It has been shown that some aspect of the tail remains attached to the zygotic nucleus through the 2-cell stage in rodents (Sutovsky & Schatten, 2000) and throughout early embryogenesis in *Drosophila*, with the tail assuming different positions and morphologies throughout (Karr, 1996; Karr & Pitnick, 1996; Pitnick & Karr, 1998; Snook & Karr, 1998). Sperm tail structures are later sequestered to the developing midgut, stripped of proteins and then excreted as waste by larvae (Pitnick & Karr, 1998). In addition to this, research has shown that sperm contribute various components to provision the egg (Dorus et al., 2006), such as tubulin (Karr, 1991) and an RNA-binding protein (Ostermeier et al., 2005), which are critical to fertilisation success (Krawetz, 2005).

Evolutionary responses to sperm competition (postcopulatory sexual selection) can result in sperm morphological diversity both between (Stockley, 1997; Simmons, 2001; Snook, 2005) and within a species (Swallow & Wilkinson, 2002; Gage & Morrow, 2003; Till-Bottraud et al., 2005). Such changes must evolve alongside counteradaptations in the female for fertilisation to be successful. One example of this is the correlated evolution of sperm tail length with the female sperm storage organs which has been demonstrated in *Drosophila* (Miller & Pitnick, 2002). Species within this genus exhibit the greatest variation in sperm tail length, ranging from 77 µm in *D. persimilis* (Snook, 1997) to 58,290 µm in *D. bifurca* (Pitnick et al., 1995b) and sperm length evolves rapidly (Pitnick et al., 2003). *D. melanogaster* females can accommodate sperm that are far longer

than the length of the egg, suggesting some coevolution of sperm length and specific mechanisms for sperm transport and compaction within the egg (Karr & Pitnick, 1996). However, females only have the opportunity to coevolve with conspecific males, so if the egg of one species is unable to accommodate the sperm of another, then this could result in embryonic failure and generate an isolating barrier (RI) between species.

Sexually selected changes in sperm morphology must be incorporated into the complex choreography of sperm-egg intracellular interactions. In *Drosophila*, sperm tails that enter the egg are confined to the anterior end and exhibit a putatively stereotypical three-dimensional folding and coiling structure that could be species-specific (Karr, 1991; 1996; Snook & Karr, 1998). Karr (1991) suggests that whole-sperm entry into the egg and the conformation it adopts functions to correctly align male and female pronuclei for karyogamy. Research has shown the importance of adopting the appropriate sperm configuration within the egg for zygote development (Ohsako et al., 2003; Lassy & Karr, 1996). For example, *Drosophila* sperm with the *mfr* mutation are typically not located in the anterior portion of the egg and adopt a different coiling structure to sperm that are commonly seen during normal development (Ohsako et al., 2003). As such, sperm of one species may be able to enter the egg of another but may not adopt the appropriate configuration for fertilisation. Even within a species, crosses between two geographical variants of *D. melanogaster,* M (cosmopolitan) and Z (Zimbabwe), have different fertilisation outcomes; some Z x M crosses have a higher percentage of eggs unsuccessfully fertilised than the reciprocal cross due to a) the entire sperm not entering the egg, b) sperm adopting an alternative 3D structure, or c) not being restricted to the anterior portion of the egg (Alipaz et al., 2001). However, whether these incompatibilities are due to sexually selected changes on sperm tails is unknown. The function of whole-sperm entry into the egg is not fully understood and the function of the three-dimensional sperm structure has not yet been explored.

Using a systems biology approach, I conducted a cross-discipline research project to develop a pipeline for studying intracellular sperm-egg interactions.

Specifically, the approach presented here allows for quantification of a variety of sperm and egg parameters (e.g. arc length and curvature) and the distance between the male and female pronuclei, which has not been performed previously. To assess the effectiveness of such an approach, I measured the sperm tail structures of various species within the *Drosophila obscura* group. *Drosophila* species provide an excellent study system in which to investigate this because they have huge variety in sperm tail length (Pitnick et al., 1995b; Snook, 1997) and they can form hybrids. However, hybrid crosses are not completely "successful", as usually the male progeny resulting from the crosses are sterile. Since it is not possible to model the sperm shape of completely incompatible crosses, these hybrids allow identification of how sperm structure might be disrupted when the sperm and egg of two species interact. The *obscura* group specifically are valuable to perform this experiment because they have relatively short sperm compared with other *Drosophila* species, making identification of the whole sperm shape achievable. I examined whether there was a species-specific 3D structure of sperm within the egg cytoplasm and to what extent this structure was disrupted in hybrid crosses. As hybrid crosses do fertilise and produce progeny, I predicted that if sperm formed a species-specific confirmation within their conspecific species eggs, then the sperm structure of hybrid crosses would align either to that of the conspecific male cross or to that of the female conspecific species cross, depending upon which one of the sexes is responsible for controlling the structure of sperm within the egg. I also examined the sperm tail structure during early embryogenesis to examine any positional or morphological changes the sperm tail may undergo and to investigate whether it was possible to track and model sperm during early embryogenesis using this approach. Future study could feasibly adopt this approach to understanding human reproduction and to the development of assisted reproductive techniques, as *Drosophila* fertilisation is similar to that of vertebrates in that the sperm tail also enters the egg (Simerly et al., 1995; Alberts et al., 2002). More generally, the development of this protocol to extract various parameters from images could have applications to other biological systems that use high resolution 3D imaging to quantify the properties of biological materials.

## Materials and Methods

### Strains of flies used and their maintenance

Four species from the *obscura* group were used for this experiment: *Drosophila pseudoobscura* (Tucson, AZ, 2001), *Drosophila persimilis* (TDSC, 2004), *Drosophila bogotana* (1990) and *Drosophila miranda*. Flies were maintained in vials containing cornmeal-agar-molasses food media with added live yeast and housed at 22°C with a 12L:12D cycle.

*Drosophila pseudoobscura* were crossed with *D. bogotana*, *D. persimilis* or *D. miranda* (Table 4.1). All species were crossed with their conspecifics as a control. Sperm shape was studied at the pronuclei stage (hereafter, PN) for all crosses and at 2n, 4n and 8n stages of development for the *D. persimilis* crosses with *D. pseudoobscura* and for all the conspecific crosses. The *D. miranda* crosses with *D. pseudoobscura* and *D. bogotana* crosses with *D. pseudoobscura* were only studied at the pronuclear stage.

### Egg collection and staining

Males and females from stock populations were transferred to egg collection chambers in groups of 30. Each egg collection chamber was fitted with an egg laying (molasses/agar) plate, with added live yeast, to allow females to start ovipositing (Snook et al., 1994). After 24 hours, the first egg collection plate was discarded and replaced with a fresh one. Eggs were harvested every hour and a fresh plate was placed on the chamber each time. Immediately following harvesting, eggs were dechorionated in 50% bleach and 50% $dH_2O$, rinsed in detergent (Triton-X) and then fixed in a 50% methanol and 50% heptane mix (to remove the vitelline membranes of eggs so that staining was possible). The sperm inside eggs were stained using a sperm-specific polyclonal antibody (Snook & Karr, 1998) and shortly before observation, eggs were counter-stained using a DNA-specific fluorescence, DAPI (4',6-diamidino-2-phenylindole) to label nuclei. Eggs were mounted onto standard microscope slides in 80% glycerol/PBS and sealed.

**Table 4.1: Reciprocal and conspecific species crosses from which images were obtained**

| Species A (female) | Species B (male) | Stages of development | Number of images (PN; 2n; 4n; 8n) |
|---|---|---|---|
| ***D. pseudoobscura*** | *D. bogotana* | PN | 8 |
| ***D. bogotana*** | *D. pseudoobscura* | PN | 8 |
| ***D. bogotana*** | *D. bogotana* | PN | 10 |
| ***D. persimilis*** | *D. pseudoobscura* | PN, 2n, 4n, 8n | 8; 4; 10; 9 |
| ***D. pseudoobscura*** | *D. persimilis* | PN, 2n, 4n, 8n | 7; 10; 10; 11 |
| ***D. pseudoobscura*** | *D. pseudoobscura* | PN, 2n, 4n, 8n | 8; 5; 6; 10 |
| ***D. persimilis*** | *D. persimilis* | PN, 2n, 4n, 8n | 10; 1; 10; 10 |
| ***D. miranda*** | *D. miranda* | PN, 2n, 4n, 8n | 10; 12; 13; 12 |
| ***D. miranda*** | *D. pseudoobscura* | PN | 5 |
| ***D. pseudoobscura*** | *D. miranda* | PN | 1 |

**Image processing and analysis**

Confocal laser scanning microscopy was used to acquire images of antibody-labelled eggs (Snook et al., 1994; Snook and Karr, 1998). Images were standardised by capturing a 2D image every 2 µm through the z-stack of the egg. The developmental stage of each egg was determined by staining the egg nuclei with DAPI and counting the number of nuclei present.

For reconstructing a three-dimensional image, the confocal images were imported into ScanIP, a 3D image visualisation and processing software created by Simpleware Ltd. (Figure 4.1). Spacing values for each stack of images were

set to 0.002 mm to represent the images being taken at 2 μm through the z-stack. The entire sperm tail and all pronuclei/nuclei and polar bodies (where present) were "masked" using the paint with threshold function (Appendix 2.1) and reconstructed into a 3D model by the software. Finally, a curve was fitted to the sperm tail and the coordinates of each point along the tail, as well as the coordinates for all other masks (pronuclei/nuclei positions and polar body positions), were recorded using a purpose built plug-in. It was important to keep the spacing between points on the sperm tail curve consistent between sperm of different species and across images, so that measurements were consistent across species and samples. For example, if one sample used more points than another, it is expected that more detail could be taken in the sample with more points compared to the one with fewer points, so this was automated as part of the plug-in. The mean position of all the voxels in each mask was taken as the centre of the nucleus or polar body and marked using the plug-in accordingly.

**Measurements of parameters**

Analyses were performed using Mathematica (Wolfram Research, Inc., Mathematica, Champaign, IL 2014). Using the coordinates of sperm tail position (x,y,z), measurements of arc length, aspect ratio, curvature, torsion and writhe were calculated (Appendix 2.1). Arc length (mm) is defined as the total length along the tail; aspect ratio gives the arc length divided by the net length (and provides a measurement of how much the sperm tail deviates from a straight line). Curvature (1/mm) measures the average curvature across the sperm tail length using the radius of a circle and indicates the amount to which the sperm tail deviates from being flat. The radius of curvature equals the radius of a circle ($r$) and curvature $k$ is equal to $1/r$, so a larger $r$ equates to a more curved sperm tail. Torsion (1/mm) measures how tightly wound the helix (curve) is and whether the helix is right or left handed. Writhe measures the helical structure (the same as torsion) and the tendency of the helix to self-knot (overlap itself). However, measurements of torsion and writhe are very sensitive to small fluctuations and measurement error, so I do not use these measurements in this analysis.

For the pronuclei stage, I used the distance from the polar bodies to various points along the sperm tail as a landmark for where the sperm tail was positioned within the egg. Polar bodies are haploid cells that are formed during oogenesis (Tremblay & Caltagirone, 1973). One of the haploid nuclei becomes the female pronucleus and the other three haploid genomes, the polar bodies, fail to develop and migrate to putatively the same position within the egg. As such, they are potentially useful landmarks for the orientation of the egg. I also calculated the distance between the two pronuclei. These analyses were performed using Python (2.7.10, 2015).

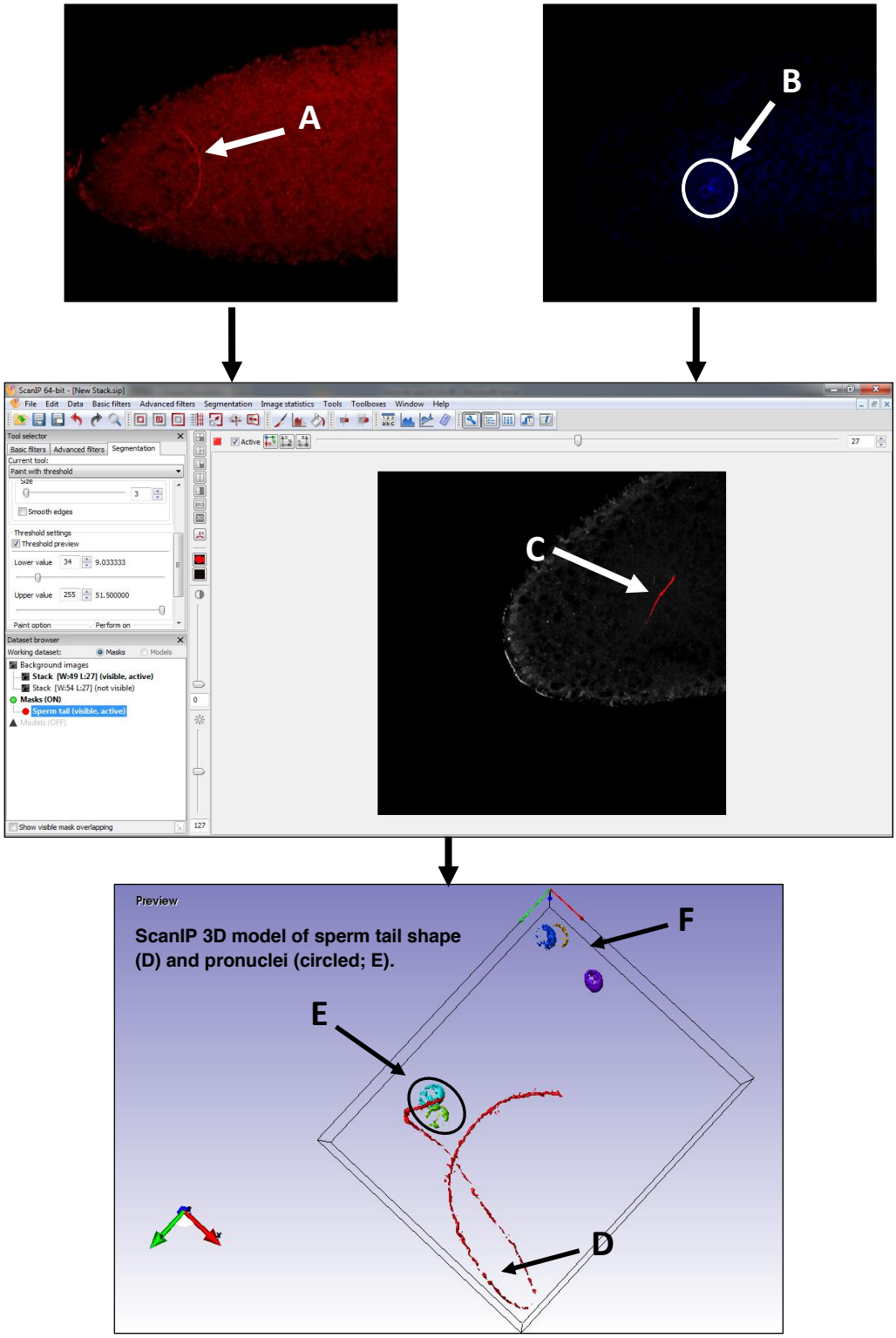



**Figure 4.1: A visual representation of the methodological pipeline. A) An example sperm tail stained red. B) Male and female pronuclei stained with DAPI (faint blue circles within the larger white circle). C) The red line is a partial sperm tail represented as a mask in ScanIP. D) The same sperm tail in full as the final three-dimensional model. E) The pronuclei from (B) represented in 3D as part of the ScanIP model. F) Polar bodies (not previously shown) segmented in ScanIP into the 3D model. The red, blue and grey regions (respectively) in the top three boxes of this figure correspond to the egg structure. The egg structure is not marked in the 3D model (fourth/final panel).**

## Results

Using the approach outlined here, a variety of sperm and egg parameters can be measured and analysed. This technique is methodologically robust and presents an interesting new avenue for research in the future. The majority of data discussed in this chapter demonstrate the technique; however they do not show statistically significant results on the whole due to the relatively low sample sizes. This protocol was developed to examine any differences in sperm and/or egg parameters for both between species and hybrid crosses. The following sections outline some putative differences between the sperm tail shapes of the various crosses, but the main purpose of these results is to demonstrate which measurements can be taken using this protocol using a test dataset as examples and to highlight potential differences which could be studied in future research.

### Is there a species-specific 3D sperm shape during the pronuclear stage?

I was able to quantify five key measures of sperm properties within the egg at the pronuclear stage. These measures were used to quantify and examine whether there was a species-specific sperm tail shape and were as follows:

(1) Arc length (Figure 4.2);

(2) Curvature (Figure 4.3);

(3) Distance between sperm and egg pronuclei (Figure 4.4);

(4) Distance between polar bodies and the sperm tail (Figure 4.5);

(5) Aspect ratio (Figure 4.6).

None of these measures are significantly different between species at the pronuclear stage ($p>0.05$) and so suggest there is not a species-specific sperm tail shape at this stage of fertilisation. However, Figures 4.2-4.6 demonstrate how the protocol developed in this chapter can be used to quantify various intracellular sperm-egg interactions. I briefly discuss each measure in this section.

Average arc lengths for all four species are very similar at the pronuclear stage (Figure 4.2) as has been previously demonstrated in the literature (Pitnick et al., 1995b; Snook, 1997; Snook, 1998). Further, there were no significant differences in average curvature (Figure 4.3) or positioning within the egg (Figure 4.4). Similarly, there does not appear to be differences in the distance between pronuclei (Figure 4.5), but this is expected in conspecific crosses as the pronuclei must fuse at this point. Of the parameters measured, it would be most interesting to collect more data for the aspect ratio of the different species during the pronuclear stage. There were no significant differences in average aspect ratio of sperm between crosses in these data, but this parameter appears to be the most likely to differ if a larger sample size were to be considered (Figure 4.6). It is possible, for example, that differences between *D. miranda* (which have straight sperm tails compared to the other species) and *D. pseudoobscura* (which have sperm tails that appear to deviate the most from a straight line) could be uncovered, however, this is currently just conjecture.

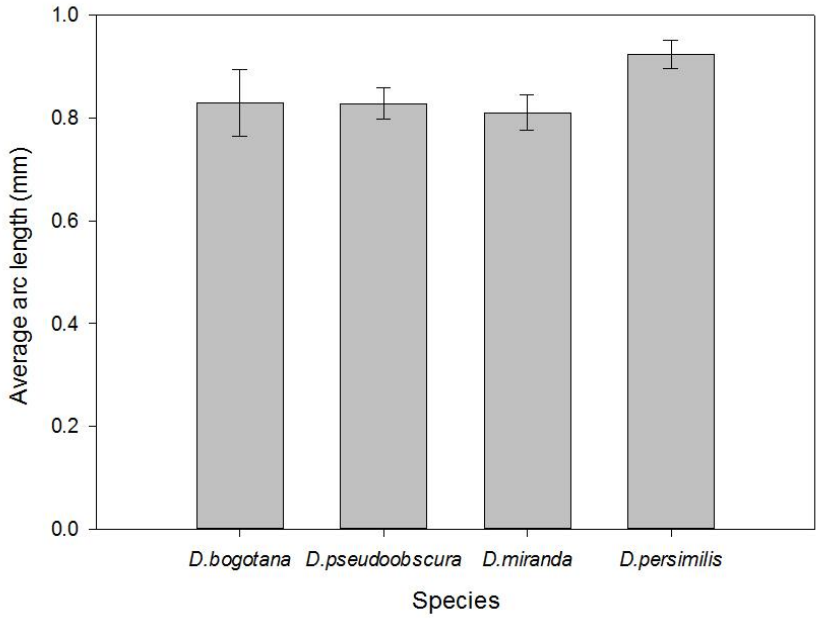



**Figure 4.2: Average (±SE) arc length for the conspecific crosses for the PN stage to identify whether or not there is a species specific-sperm tail shape. NS differences between species.**

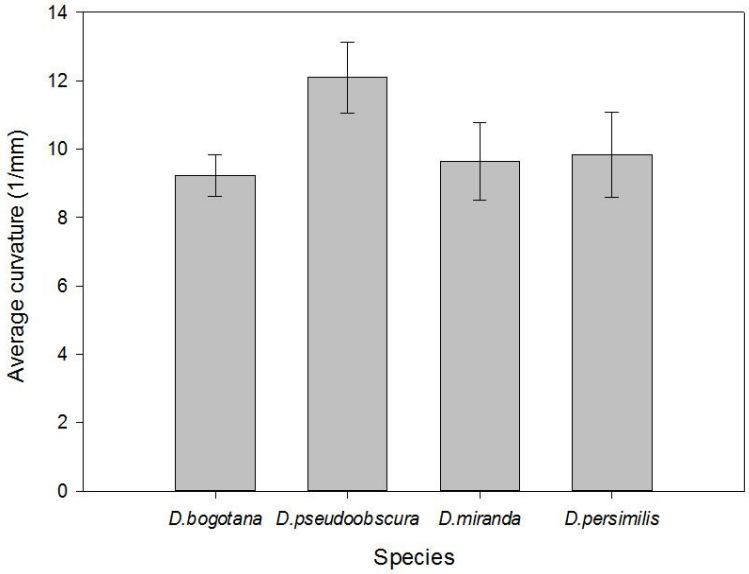



**Figure 4.3: Average (±SE) curvature for the conspecific crosses during the PN stage to identify whether there is a species-specific sperm tail shape. NS differences between species.**

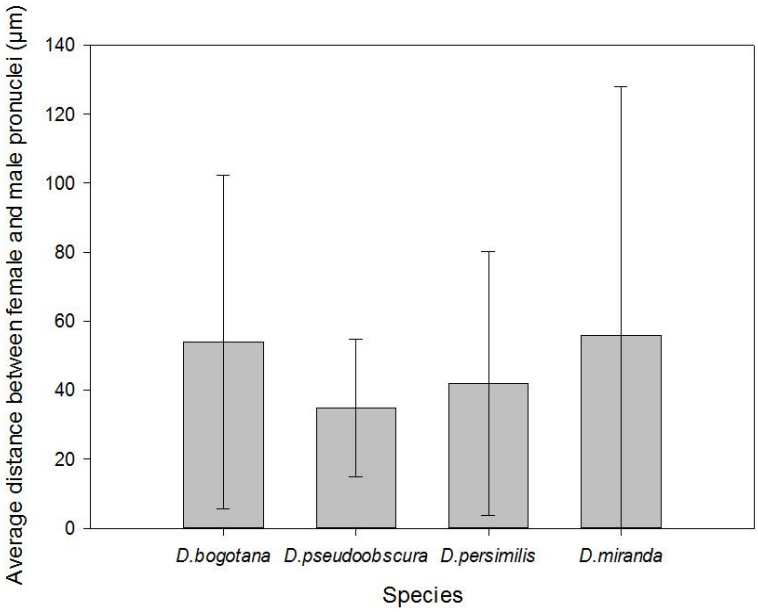



**Figure 4.4: Average (±SE) distance between sperm and egg pronuclei for the conspecific crosses during the PN stage. NS differences between species.**

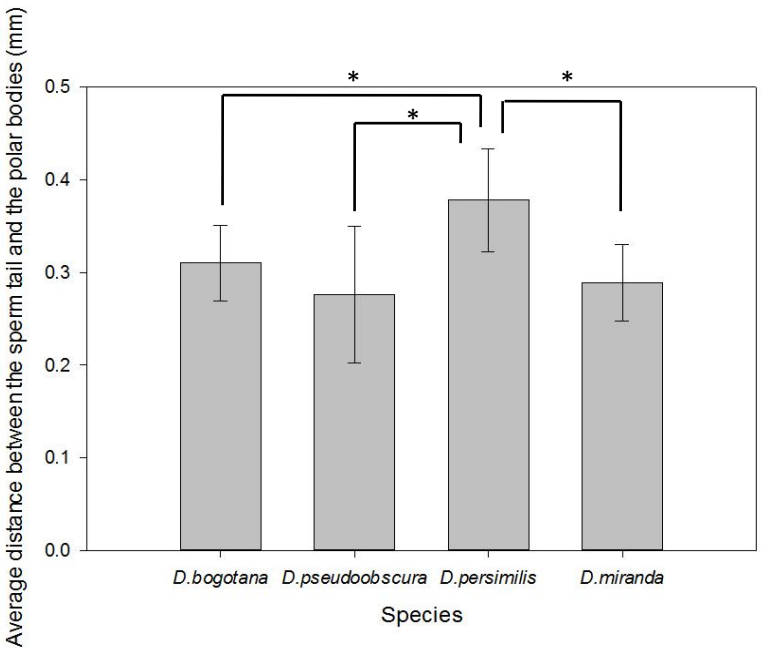



**Figure 4.5: Average (±SE) distance between points along the sperm tail and the polar bodies for conspecific crosses during the PN stage, * P<0.05.**

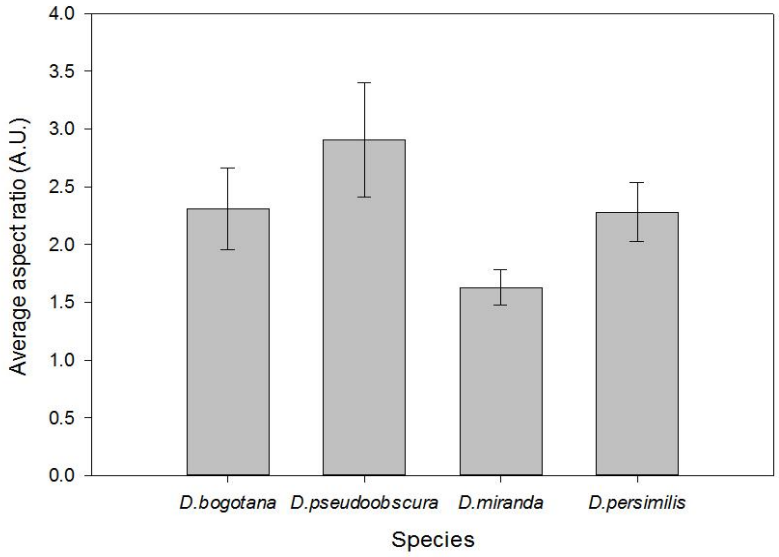



**Figure 4.6: Average (±SE) aspect ratio for the conspecific crosses during the PN stage to identify whether there is a species-specific sperm tail shape. NS differences between species.**

**Is there a species-specific 3D sperm shape during the developmental stages (2n, 4n, 8n)?**

I also applied the protocol demonstrated in the pronuclear section to studying the developmental stages of intracellular sperm-egg interactions. However, the pronuclei were not present during these stages so measures of the distance between pronuclei were not taken. Equally, measures of the distance between the sperm tail and polar bodies were not included. Therefore, only measures of (1) arc length, (2) curvature and (5) aspect ratio were examined for these stages. In addition to this, data for *D. bogotana* were not collected at these stages (see Table 4.1). In this section, I present the aspect ratio as an example of how this protocol can be used to track sperm tails at these stages of development. Figures for arc length and curvature at these developmental stages can be found in Appendix 2.2.

The aspect ratio of *D. miranda* sperm tails was significantly smaller than *D. pseudoobscura* at the 2n stage (Figure 4.7; F=5.17, df=2, p=0.05). Unfortunately only one data point was collected for *D. persimilis* for the 2n stage so no assertions can be made about this species. At the 4n stage, both *D. pseudoobscura* and *D. persimilis* were significantly larger than *D. miranda* (Figure 4.8; F=5.37, df=2, p=0.01). During the 8n stage of development, *D. miranda* had sperm tails with higher aspect ratio than *D. pseudoobscura* but this was not significant (Figure 4.9; p>0.05). Overall, this method has highlighted the dynamic changes occurring through time during sperm-egg interactions.

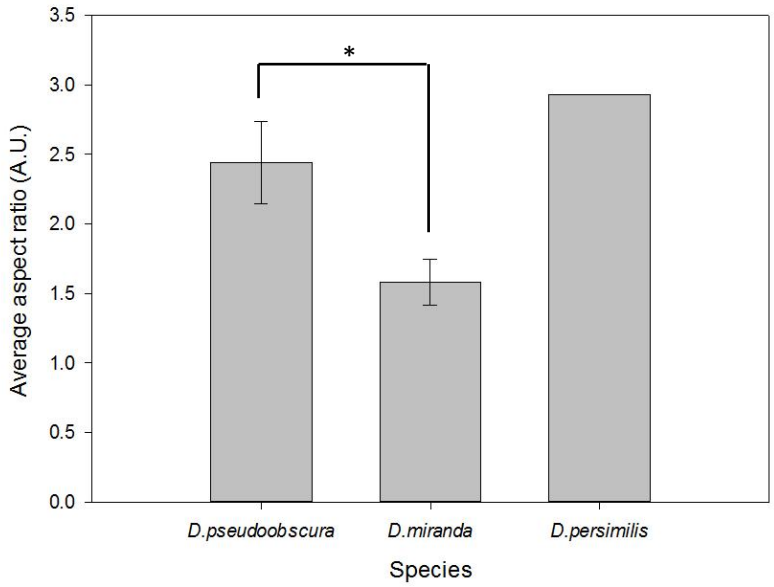



**Figure 4.7: Average (±SE) aspect ratio for the conspecific crosses during the 2n stage to identify whether there is a species-specific sperm tail shape, * P<0.05.**

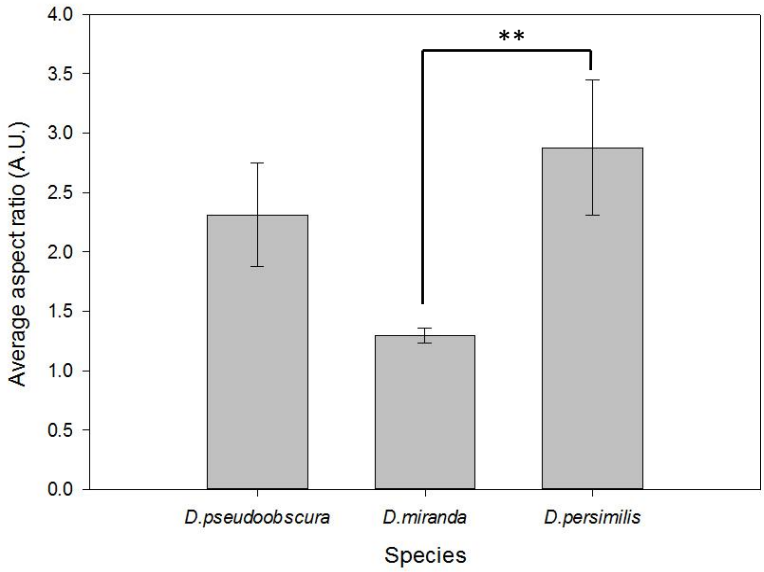



**Figure 4.8: Average (±SE) aspect ratio for the conspecific crosses during the 4n stage to identify whether there is a species-specific sperm tail shape, ** P<0.01.**

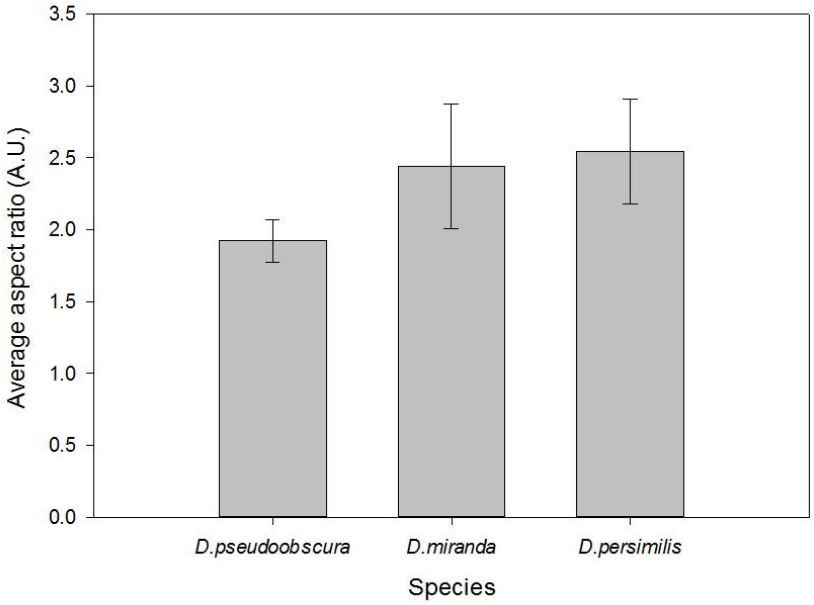



**Figure 4.9: Average (±SE) aspect ratio for the conspecific crosses during the 8n stage to identify whether there is a species-specific sperm tail shape. NS differences between species.**

**Comparisons *within* a species – are there changes in sperm tail parameters through development within each conspecific cross?**

The changes uncovered in the previous section indicate the need for study of the temporal properties of sperm tails within the egg. Therefore changes within a species at different stages of development were measured. Indeed, average aspect ratio for *D. miranda* is significantly larger at the 8n stage of development compared to the 4n stage (Figure 4.8 and Figure 4.9; F=4.13, d=3, p=0.012). In addition, the sperm tail for *D. pseudoobscura* was significantly longer at the 8n stage of development compared to the other stages (Figure 4.10; F=14.25, df=3, p=0.001). These within species changes in average arc length were also evident in *D. miranda* (F=8.05, df=3, p=0.002) and *D. persimilis* (F=15, df=3, p=0.001), both of which are presented as figures in Appendix 2.2.

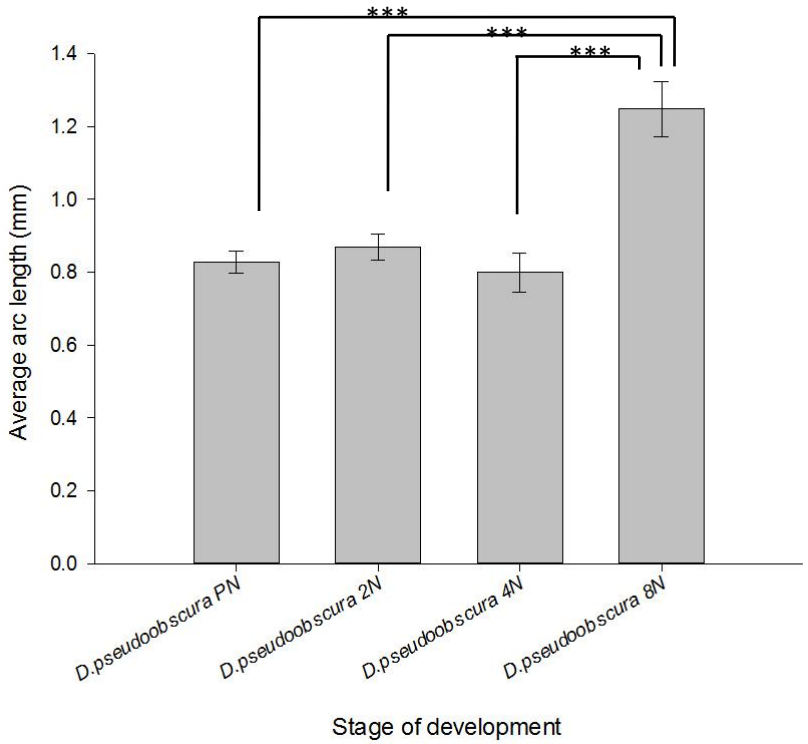



**Figure 4.10: Average (±SE) arc length for *D. pseudoobscura* from the PN stage to the 8n stage of development to identify changes in sperm tail shape within a species during development, *** P<0.001.**

**Are sperm tail shapes of heterospecific parental crosses disrupted in comparison to conspecific parental crosses during the PN stage?**

In addition to conspecific crosses, I used the protocol outlined in this chapter to identify whether sperm tail shapes were disrupted in hybrid crosses. For the pronuclear stage presented here, I was able to quantify five sperm traits within the egg (1) arc length (Figure 4.11); (2) curvature (Figure 4.12); (3) distance between sperm and egg pronuclei (Figure 4.13); (4) distance between polar bodies and sperm tail (Figure 4.14) and (5) aspect ratio (Figure 4.15). Once again, I present one example, in this case the *D. bogotana* and *D. pseudoobscura* conspecific crosses compared to hybrids of these species in both directions. I use these examples to demonstrate how comparisons between conspecific and hybrid crosses can be made using this protocol. Figures for the other comparisons between conspecific *D. persimilis* and *D. pseudoobscura* and their hybrid crosses and also comparisons between *D. miranda* and *D. pseudoobscura* with their hybrids are presented in Appendix 2.2.

The majority of the measurements for the pronuclear stage showed no significant differences between *D. bogotana* and *D. pseudoobscura* comparisons with their hybrid crosses. This is the case for arc length, the distance between pronuclei, the positioning of the sperm tail relative to the polar bodies and aspect ratio. In contrast with the prediction made about sperm positioning the pronuclei, the distance between the female and male pronuclei did not differ significantly between the conspecific and hybrid crosses (Figure 4.13); however, the small sample size studied here may explain this result.

Of the sperm tail measures within the egg, it would be the most interesting to collect more data for the average curvature of hybrid crosses and compare them to the conspecific sperm tail curvature. Average curvature was significantly lower for sperm tails from the *D. bogotana* x *D. pseudoobscura* hybrid compared to the *D. pseudoobscura* conspecific cross (Figure 4.12; $F=3.64$, $df=3$, $p=0.023$) and sperm tails possibly appear to assume the curvature structure of the female's conspecific cross. Further research with a larger sample size should be conducted to test this.

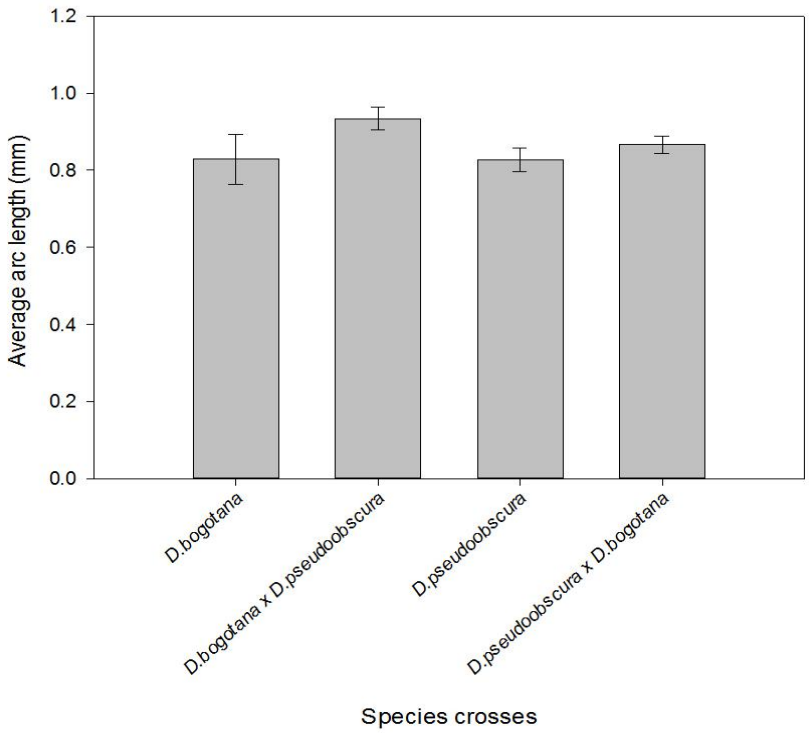



**Figure 4.11: Average (±SE) arc length of *D. pseudoobscura* sperm tail shapes compared with *D. bogotana* and their respective hybrid crosses during the PN stage. NS differences between crosses.**

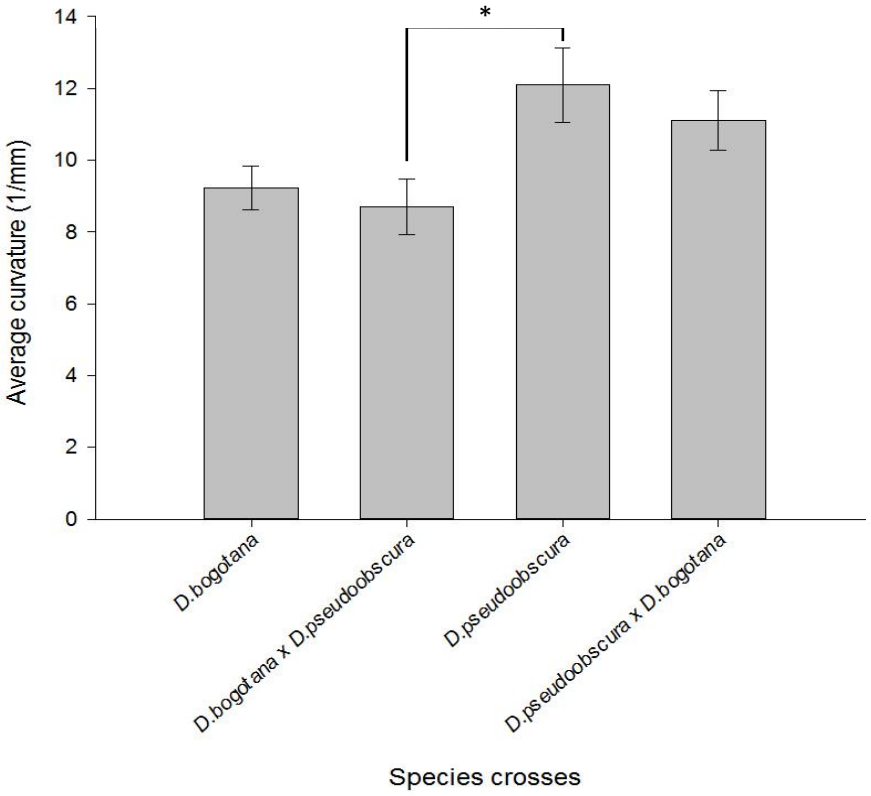



**Figure 4.12: Average (±SE) curvature of *D. pseudoobscura* sperm tail shapes compared with *D. bogotana* and their respective hybrid crosses during the PN stage, * P<0.05.**

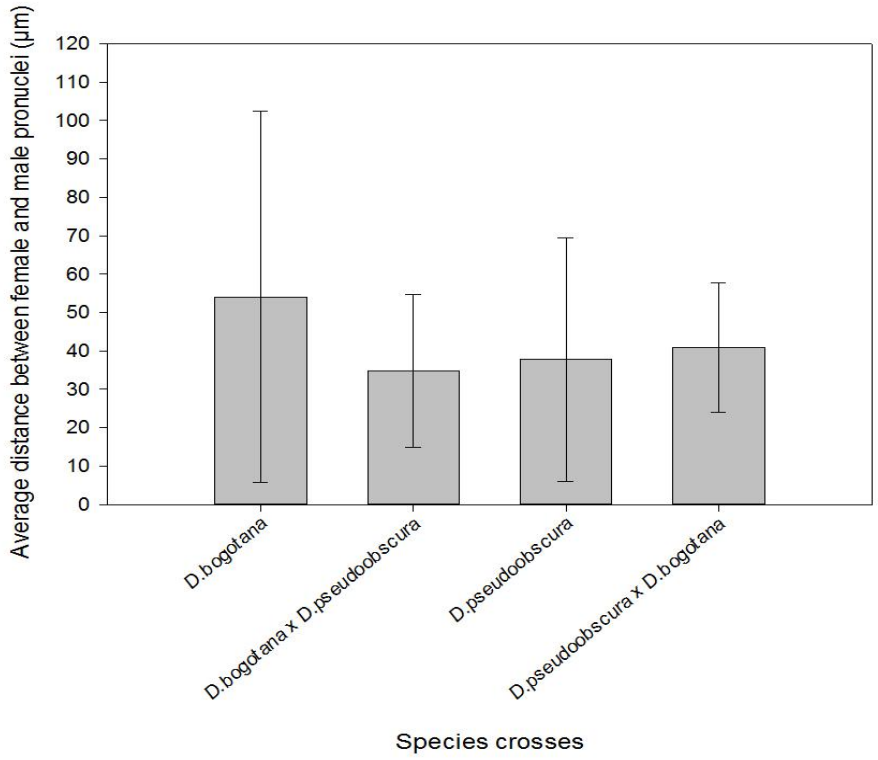



**Figure 4.13: Average (±SE) distance between the sperm and egg pronuclei of *D. pseudoobscura* and *D. bogotana* and their hybrid crosses during the PN stage. NS differences between crosses.**

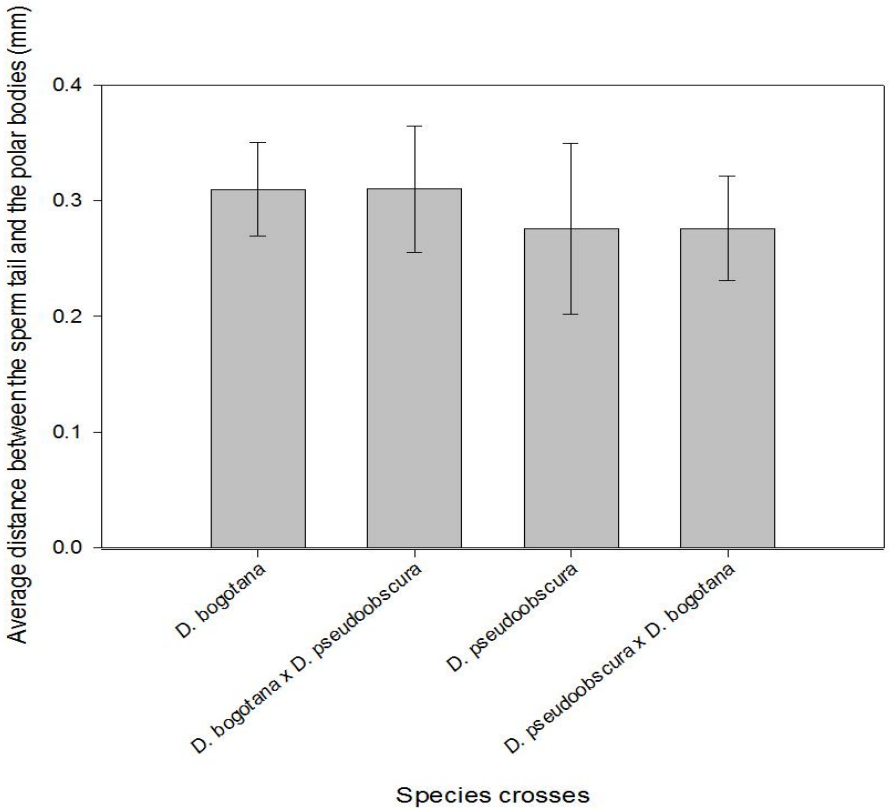



**Figure 4.14: Average (±SE) distance between points along the sperm tail and the polar bodies of *D. pseudoobscura* and *D. bogotana* and their hybrid crosses during the PN stage. NS differences between crosses.**

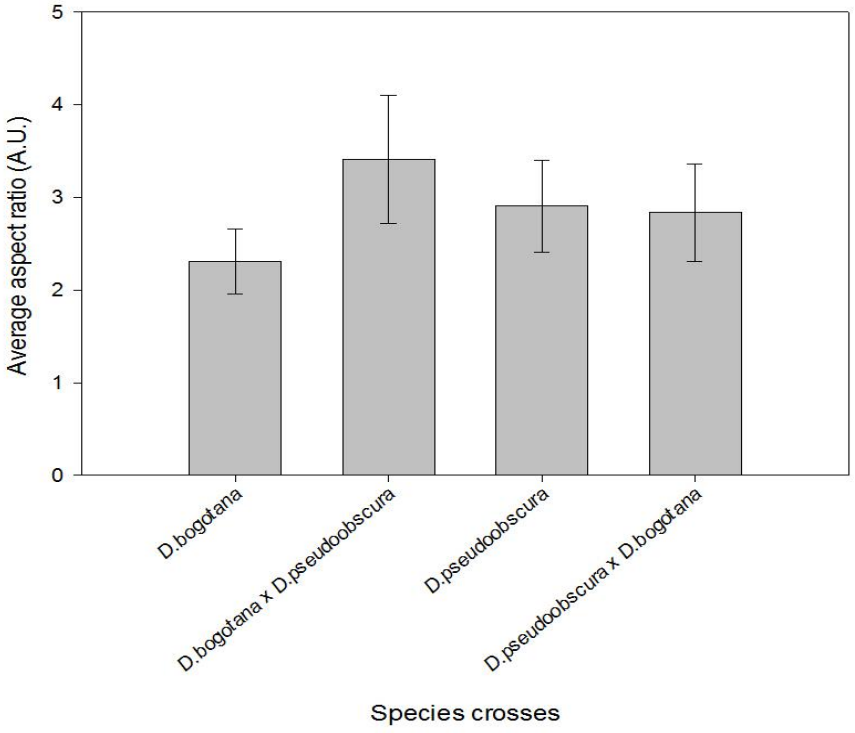



**Figure 4.15: Average (±SE) aspect ratio of *D. pseudoobscura* and *D. bogotana* and their hybrid crosses during the PN stage. NS differences between crosses.**

**Are the 3D sperm tail shapes of heterospecific parental crosses disrupted in comparison to conspecific parental crosses during the developmental stages?**

Measures of sperm tail properties in both conspecific and hybrid crosses were examined through early development. As the pronuclei were not present during these stages, measures of the distance between pronuclei were not taken. Equally, measures of the distance between the sperm tail and polar bodies were also not included. As in the between conspecific species developmental section previously, measures of (1) arc length, (2) curvature and (5) aspect ratio were examined. Only data for *D. persimilis* and *D. pseudoobscura* hybrids were collected past the pronuclear stage so I present this as the example of how sperm tail measures from hybrids during early development can be compared to conspecific crosses. In this section, I present the average aspect ratio as an example of how this protocol can be used to track sperm tails at these stages of development. Figures for arc length and curvature at these developmental stages can be found in Appendix 2.2.

During the 2n stage of development there were no significant differences in any measures taken (Figure 4.16; $p>0.05$). During the 4n stage of development, the average aspect ratio was significantly larger in *D. persimilis* compared to the *D. pseudoobscura* x *D. persimilis* hybrid (Figure 4.17; $F=3.36$, $df=3$, $p=0.03$). By the 8n stage of development, the *D. persimilis* x *D. pseudoobscura* hybrid's aspect ratio was significantly larger than the *D. pseudoobscura* conspecific cross (Figure 4.18; $F=4.46$, $df=2$, $p=0.02$). These rapidly changing results once again highlight the dynamic interaction between the sperm within the egg.

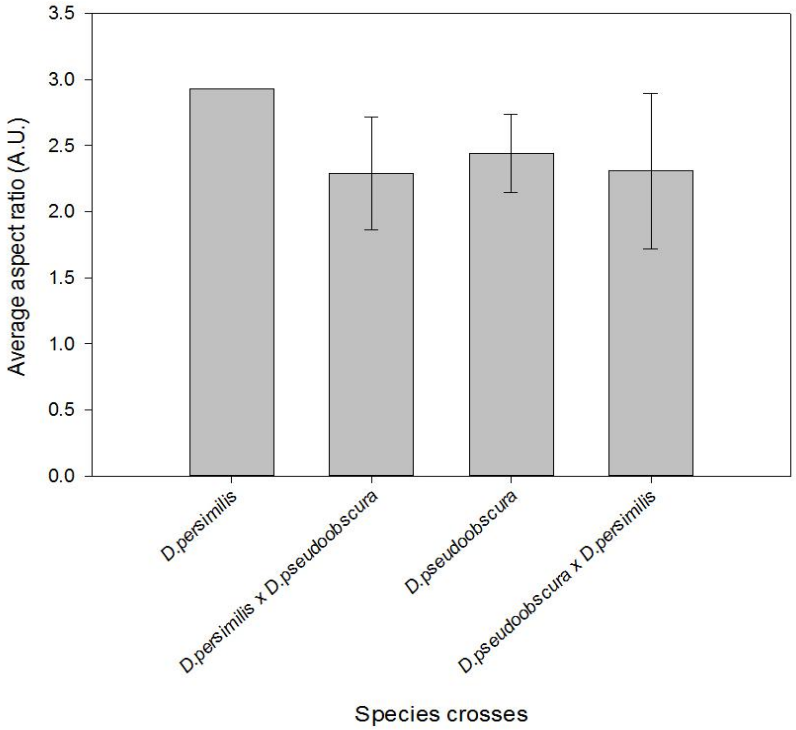



**Figure 4.16: Average (±SE) aspect ratio of *D. pseudoobscura* sperm tail shapes compared with *D. persimilis* and their respective hybrid crosses during the 2n stage of development. NS differences between crosses.**

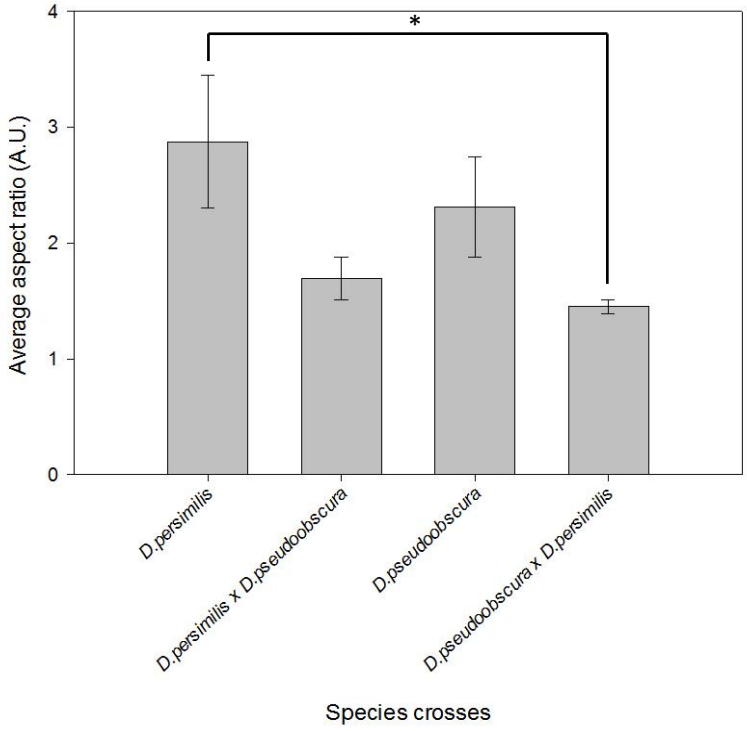



**Figure 4.17: Average (±SE) aspect ratio of *D. pseudoobscura* sperm tail shapes compared with *D. persimilis* and their respective hybrid crosses during the 4n stage of development, * P<0.05.**

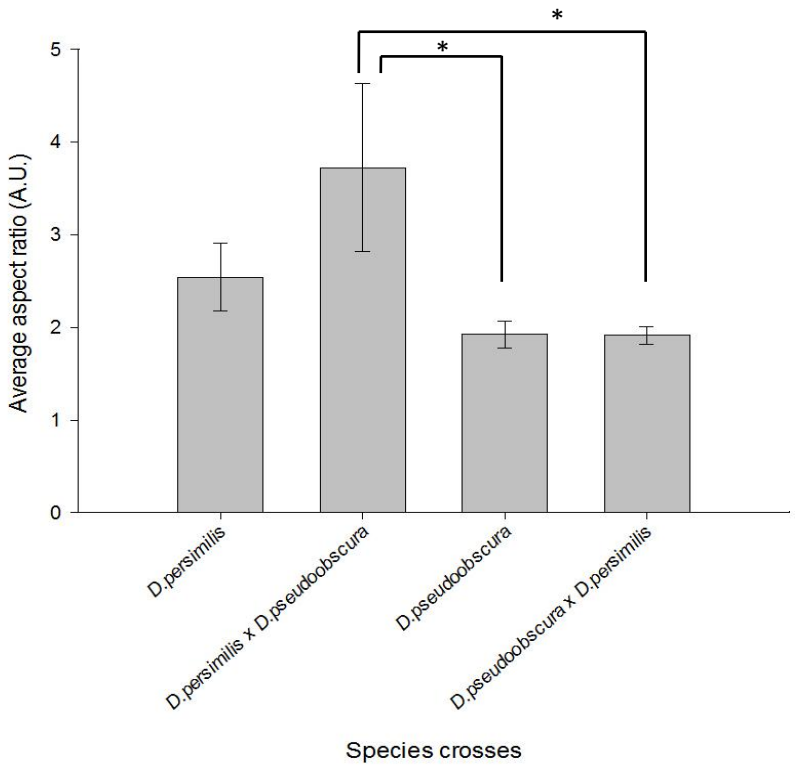



**Figure 4.18: Average (±SE) aspect ratio of *D. pseudoobscura* sperm tail shapes compared with *D. persimilis* and their respective hybrid crosses during the 8n stage of development, * P<0.05.**

## Discussion

The approach demonstrated here has proved to be successful in collecting data for studying intracellular sperm-egg interactions. Quantification of a variety of both sperm and egg parameters has been made possible using this pipeline (Figure 4.1) and therefore demonstrates proof of principle. In this chapter I have presented results for arc length, aspect ratio and curvature of sperm tails as well as showing the distances between female and male pronuclei during the PN stage. I have also shown that it is possible to track changes in sperm tail morphology throughout development to compare both within and between species.

Evolutionary responses to sperm competition have resulted in a diversity of sperm traits both within (Swallow & Wilkinson, 2002; Gage & Morrow, 2003; Till-Bottraud et al., 2005) and between species (Stockley, 1997; Simmons, 2001; Snook, 2005). Such sexually selected sperm morphology must be incorporated into the complex choreography of sperm-egg intracellular interactions for fertilisation to be successful. I aimed to examine the effects of sexual selection on intracellular sperm-egg interactions in *D. pseudoobscura*. However, within experimentally evolved populations with different sexual selection intensity, sperm traits have not evolved along different evolutionary trajectories (Crudgington et al., 2009). Hence, I have demonstrated the application of the developed protocol in this chapter using examples from a comparison of sperm-egg interactions between *D. pseudoobscura* and three other species from within the same species group, *D. obscura* (see chapter 1, Figure 1.2). Differences in sperm length between these species were used as a proxy to show that sexual selection may have shaped sperm length evolution. I chose *Drosophila* for analysis because they demonstrate huge disparity in sperm tail length, from 77 μm (Snook, 1997) to 58,290 μm (Pitnick et al., 1995b) and *Drosophila* eggs have been shown to accommodate sperm that are twice their length (in *D. melanogaster*; Karr & Pitnick, 1996). This suggests that there must be some coevolution of sperm length and specific mechanisms for sperm transport and compaction within the egg (Karr & Pitnick, 1996). The effect of sexual selection

on sperm traits and the evidence for the interaction between sperm and egg make *Drosophila* an ideal organism for developing the protocol presented in this chapter (Figure 4.1). However, this protocol could be applied to a variety of other organisms for which the sperm enter the egg and interact with the maternal cytoplasm (e.g. mammals, Krawetz, 2005; Simerly et al., 1995; marine invertebrates, Shapiro et al., 1981).

These intracellular interactions are critical for successful fertilisation and early embryogenesis (Krawetz, 2005), with some component of the sperm tail needed for pronuclear fusion in insects and vertebrates (Moomjy et al., 1999; Sutovsky & Schatten, 2000). The importance of adopting the correct sperm configuration has been shown (Ohsako et al., 2003), with some suggestion that the three-dimensional sperm structure may be species-specific in *Drosophila* (Karr, 1991; 1996; Snook & Karr, 1998). I found some putative differences in sperm tail shapes within the egg cytoplasm. For example, *D. obscura* group species could potentially have different average aspect ratios (Figure 4.6) during the PN stage. However, this study shows no significant differences between these species during the PN stage. To examine the positioning of the sperm within the egg, I measured the average distance between the sperm tail and the polar bodies, which I used as landmarks for the egg position (Figure 4.5). There were no significant differences between the species in their relative positioning within the egg, but further study could analyse this in more detail.

The data presented in this chapter confirm the previous observations that sperm tails persist through early embryogenesis and assume different morphologies throughout (Karr, 1996; Karr & Pitnick, 1996; Pitnick & Karr, 1998; Snook & Karr, 1998). For example, *D. miranda* aspect ratio was significantly lower than *D. pseudoobscura* in the 2n stage and both *D. pseudoobscura* and *D. persimilis* in the 4n stage of development (Figure 4.7 and Figure 4.8; respectively). These results show that *D. miranda* sperm are consistently straighter than the other species through these stages of development but during the 8n stage of development, *D. miranda* sperm tails have larger average aspect ratio than *D. pseudoobscura* (Figure 4.9). These observations indicate that there may be

species-specific differences in sperm tail shapes during the developmental stages. Perhaps sperm have to form particular shapes within the egg to contribute to development, or alternatively, eggs control sperm shape through development. Further study would be needed to identify whether sperm form a species-specific 3D shape consistently during development.

Why the sperm tail enters the egg during fertilisation is unclear, but one suggestion is that the sperm functions to align the male and female pronuclei for successful fertilisation (Karr, 1991). If this were the case, the expectation is that hybrid crosses may experience an incorrect alignment of male and female pronuclei. To test this idea, I measured the distance between pronuclei for both conspecific and hybrid sperm-egg interactions. I found no significant differences in the distance between pronuclei for conspecific compared to hybrid crosses (Figure 4.13). This could be a consequence of the fact that sperm and egg interactions were measured only in crosses that fertilise so therefore the pronuclei would need to interact at some point and this could be the point captured for these data. However, these measurements had larger standard errors and as such, collecting a greater number of fertilised eggs in future study could show clearer and potentially very interesting results.

Finally, I compared the sperm tail shapes of hybrid crosses with their parental conspecific crosses, to determine if sperm shapes were disrupted. Sperm tails of hybrid crosses putatively show average curvature more similar to that of their female conspecific. For example, hybrid *D. bogotana* x *D. pseudoobscura* (Figure 4.12) appear to have similar average curvature to the *D. bogotana* conspecific cross. This pattern of the hybrid being more similar to the female conspecific cross is also shown at the 4n stage of development, with sperm of the *D. pseudoobscura* x *D. persimilis* hybrids showing similar average curvature to the *D. pseudoobscura* conspecific cross (Appendix 2.2; Figure 2.2.17). This provides some indication that sperm tail shape within the egg could be under female control. However, through development in general hybrid crosses do not show any clear patterns of similarity to either conspecific cross. Hybrids of *D. persimilis* x *D. pseudoobscura* show more similarity to each other in aspect ratio during the

4n stage (Figure 4.17), with the *D. pseudoobscura* x *D. persimilis* hybrid being significantly straighter than the *D. persimilis* conspecific cross (Figure 4.17).

Future work could also examine potential quantification methods that are possible using the protocol outlined in this chapter, but were not presented. For example, it is possible to map the position of the micropyle, so that the point of sperm entry can be incorporated into future models of sperm-egg interactions. Torsion and writhe (as outlined in the methods) could also be incorporated into future analysis if large sample sizes are taken. These measurements could provide further insight into how much a sperm tail is coiled relative to sperm tails of other species. Sperm tail length has been shown to evolve rapidly in response to sexual selection (Pitnick et al., 2003) and so subtle differences between conspecific and hybrid sperm shapes could lead to the formation of an isolating barrier between species if the egg of one species became unable to accommodate divergence in sperm traits of another.

Overall, I used species of the *D. obscura* species group to demonstrate that this protocol is applicable to a variety of species, but also because these species have relatively short sperm compared to other *Drosophila* species (Snook, 1997; Pitnick et al., 1995b). This made quantification of a variety of sperm parameters clearer, but a future challenge would be to model the sperm of species with much longer sperm tails. Arc length for the *D. obscura* species tested showed an increase in length during development (e.g. *D. pseudoobscura*; Figure 4.10). This demonstrates that sperm of different lengths can be measured using the protocol outlined in this chapter. Arc length (Figure 4.10) and aspect ratio (*D. miranda*; Figure 4.8 and Figure 4.9) both changed between stages of development.

The primary focus of this chapter was to develop the protocol that future research could build upon to develop our understanding of the function of whole sperm entry into the egg and investigate further the sperm tail structure of different species and hybrid crosses. Here, I demonstrate the first application of this approach and show there are some differences in sperm morphology but more data is needed to fully address whether sperm form a species-specific 3D structure and to what extent this structure is disrupted in hybrid crosses. The

application of the pipeline presented here could have far reaching applications for biological systems that use high resolution 3D imaging to investigate the properties of biological materials.

# Chapter 5. General Discussion

Sexual selection has the ability to drive the rapid evolution of reproductive traits, both pre- and post-copulatory. In this thesis, I assessed the effects of sexual selection and sexual conflict in driving reproductive trait evolution in the *Drosophila obscura* species group. Specifically, I have found that some precopulatory traits have responded to sexual selection and/or sexual conflict, such as courtship frequency and progeny production in the experimentally evolved populations of *D. pseudoobscura*. However, other reproductive traits do not appear to have diverged in response to sexual selection, for example, the accessory gland secretome of *D. pseudoobscura*.

Previous work on these experimentally evolved populations showed that sperm traits have also not responded to selection (Crudgington et al., 2009). Consequently, to examine the effects of sexual selection on sperm traits, shown in other studies (Miller & Pitnick, 2002; Gage & Morrow, 2003), I have made some interspecific comparisons between the *Drosophila obscura* species group. Specifically, the purpose of collecting data on sperm-egg interactions was to develop a novel technique by which to assess the structure of sperm inside the egg cytoplasm and to determine whether sperm form a species-specific shape. I assessed what effects sexually selected sperm traits had in the interactions between sperm and egg. In the following sections, I present the key findings of each chapter and discuss how they are interrelated.

### Chapter 2

In chapter 2, I used experimentally evolved populations of *Drosophila pseudoobscura* (Crudgington et al., 2005; Figure 1.3 of chapter 1) to examine various fitness parameters. Specifically, I studied mating behaviours, remating interval and progeny production within these populations to encompass both pre- and post-copulatory reproductive trait evolution. I demonstrated the importance of studying the effects of sexual selection within the selective environment. Since sexual selection results in an interacting phenotype (Bacigalupe et al., 2008), the effects of selection on females are just as informative as the effects on males.

Previous work has shown that sexual conflict operates in this system (Crudgington et al., 2010) and that some traits thought to be important in reproduction had evolved in response to selection (e.g. courtship song [Snook et al., 2005], male mating capacity [Crudgington et al., 2009] and accessory gland size [Crudgington et al., 2009]), while others had not (e.g. testes mass and sperm traits [Crudgington et al., 2009]).

I found that courtship behaviour had evolved to be more frequent in the elevated polyandry (E) treatment compared to the enforced monogamy (M) treatment. This finding aligns with previous research on these same populations which demonstrated that E males have elevated their courtship more than a control promiscuous population (Crudgington et al., 2010). Harassment as a result of increased courtship has been linked to reduced progeny production in a variety of organisms (Blanckenhorn et al., 2000; Sakurai & Kasuya, 2008; Gay et al., 2009). In chapter 2, I found that there was a significant difference in progeny production between the M and E populations. E females produced significantly fewer progeny than M females and this could demonstrate a role for sexual conflict to negatively impact female fitness. E females may be prevented from ovipositing as a result of increased harassment by males (Sakurai & Kasuya, 2008) and/or they could have less energy to invest in egg production because they are using more energy to avoid remating (Watson et al., 1998). Alternatively, or concurrently, males and females in the M treatment could have evolved to be more benign to each other and this interaction resulted in an increase in progeny production (Pitnick et al., 2001b). Finally, the differences in progeny production may not be as a result of male selection history but could be a factor of the different number of males involved in each interaction, i.e. females housed with six males could produce fewer progeny regardless of their selection history (Crudgington et al., 2005).

Both the propensity of females to remate and the number of female rematings were not significantly different between treatments, despite E males courting females more frequently than M males. E males had previously been shown to have a higher mating capacity than M males when allowed access to sequential

matings with different females (Crudgington et al., 2009). Here I showed that when females and males have interacting phenotypes, males do not experience this increase in their reproductive success through a greater number of matings. I discussed that perhaps at this point in their selective history, females are "winning" in the conflict over remating rates by resisting coercion from males (i.e. increased male courtship frequency). Since remating rate does not differ between treatments, it is possible that postcopulatory sexual selection is not a driving force in these populations. This idea is supported by the lack of sperm trait and testes size divergence in these selection treatments shown previously (Crudgington et al., 2009). Other postcopulatory reproductive traits have evolved, for example, accessory gland size (Crudgington et al., 2009), potentially remating interval (chapter 2; Figure 2.4) and progeny production (chapter 2; Figure 2.5). Remating interval was significantly different between treatments in 3 out of the 4 replicates. Overall, E females took longer to remate than M females. This result suggests that E males could be delaying E female remating through the action of their SFPs. Alternatively M females could be remating faster than E females, potentially to replenish sperm numbers and/or to gain some other direct benefits through remating (Gromko & Markow, 1993; Ravi Ram & Wolfner, 2007). This idea is supported by the finding that M females have more progeny than E females (chapter 2; Figure 2.5). However, it seems unlikely the M females remate faster to replenish sperm numbers, as previous work has shown that remating behaviour in *D. pseudoobscura* is not linked to sperm load (Snook, 1998).

Finally, I observed that significantly more E females died than M females during the 10-day interaction period. This finding is consistent with costs to female fitness associated with sexually antagonistic coevolution via sexual conflict. However, it was not possible to separate the effects of harassment by courtship from other costs associated with matings, such as the action of SFPs and/or the timing of matings, as the mechanism of premature female death within this study. It is also possible that the number of males a female is housed with, rather than their selection history, is responsible for premature death of females. Similarly, population density in general could have resulted in premature death of both sexes, but this would need further examination.

**Chapter 3**

In chapter 3, I studied the experimentally evolved populations used in chapter 2 to (1) identify proteins from the accessory glands that may be involved in manipulating female behaviour and physiology and (2) assess any relative differences in the quantity and presence/absence of particular proteins. Chapter 3 was based on the previous observations that (1) E females showed a greater delay in remating than M females (chapter 2), (2) significantly more E females than M females prematurely died during the interaction between the sexes (chapter 2) and (3) E males had larger accessory glands than M males (Crudgington et al., 2009). Both a delay in remating and premature female death could be caused by the action of SFPs (Chen et al., 1988; Chapman et al., 1995). Also, there was no difference in the number of female rematings between the treatments, and so I wanted to investigate whether postcopulatory sexual selection could have acted on the evolution of SFPs or whether the differences in remating interval and premature female death in the E treatment were due to other potential factors (as discussed previously).

SFPs are transferred to females in the ejaculate, along with sperm, during copulation (Chen, 1984) and cause numerous behavioural and physiological effects in mated females. The effects of SFPs include reduced female receptivity to remating, increased egg laying and premature death of females (Chen et al., 1988; Chapman et al., 1995; Fricke et al., 2009). Reproductive proteins have the potential to evolve faster than non-reproductive proteins (Swanson & Vacquier, 2002; Clark et al., 2006). Such rapid evolution of SFPs could be in response to sexual selection and/or sexual conflict, potentially generating sexually antagonistic coevolution between the sexes. SFPs are known to factor in sexual conflict in *D. melanogaster* (Chapman et al., 1995). For example, Acp62F stimulates egg laying and sperm storage in mated females but can also shorten female lifespan (Lung et al., 2002). Such proteins are expected to be major components in sexual conflict, because males benefit from manipulating female behaviour, e.g. females storing their sperm over rival males' sperm, but inflict costs to female fitness, in this case reducing her lifespan.

In chapter 3, I analysed the whole tissue of the accessory glands dissected from experimentally evolved populations of *Drosophila pseudoobscura*. A high quality dataset was produced using a high sensitivity, ultra-high resolution mass spectrometer and by applying strict filtering criteria to the identified peptides/proteins. Using LC-MS/MS, I identified a total of 3770 proteins that are present in the accessory glands of *D. pseudoobscura*, identified by an average of 11.72 peptides per protein. There were no significant differences between treatments of the proteome and neither selection treatment had enrichment for any GO terms. By identifying which proteins had a signal sequence, it was found that 395 of these proteome identifications comprise the secretome. These secretome proteins are the most likely to be involved in the interactions between the sexes and this is the first time the secretome has been characterised for *Drosophila* species. To be defined as Acps, proteins must be secreted from the accessory glands to be transferred to females and therefore, must have signal sequences (Wolfner et al., 1997).

I found that a large number of proteins of the secretome overlapped between treatments (n=247 out of a total of 274 after filtering). These proteins showed enrichment for reproduction, insemination and regulation of female receptivity and this supported the idea that the secretome contains proteins involved in the molecular interaction between the sexes. The secretome was enriched in proteins identified as extracellular, which supports the criteria of the Acps having predicted signal sequences that permit extracellular secretion (Wolfner et al., 1997; Ravi Ram & Wolfner, 2007). I showed there was overlap of the secretome identified in chapter 3 with known seminal fluid proteins (n=64). This list of proteins found in both the literature and the dataset in chapter 3 contained named Acps; Acp26Aa, Acp52Ea, Acp53C14a, Acp53C14b, Acp53C14c and Acp32CD. These are all good indicators that the secretome was identified accurately.

I identified a list of the top 20 most abundant proteins of the secretome. Of these 20, 19 of them had *D. melanogaster* orthologues which were enriched for various functions associated with reproduction, as expected. This list contained three

named Acps: Acp26Aa, Acp53Ea and Acp53C14b, which were in the top 20 for both treatments, M and E (when considered separately). Acp26Aa is known to function to increase egg laying in females (Herndon & Wolfner, 1995). Increased egg laying can be beneficial to both sexes and so males from the M treatment could increase the quantity of Acp26Aa transferred to females. However, if males were able to manipulate females beyond an optimum level, this influence could have negative fitness consequences for females (for example, as demonstrated by Acp62F; Lung et al., 2002 and sex peptide; Wigby et al., 2009). This was the prediction made for E pairings and as such, it was expected that E females would be manipulated beyond their optimum to produce more eggs earlier in their life history. This could potentially result in E females producing more progeny than M females overall, if these eggs developed. If sexual selection and/or sexual conflict were acting on the evolution of Acps in the experimental evolution treatments, the assumption was that E treatment males would have more unique Acps and/or a greater abundance of particular Acps. However, there was no evidence to support either of these suggestions as there was no significant difference in quantities of any proteins of the secretome and there were no significant GO terms for either treatment. I found that M females had more progeny than E females (chapter 2), but this was most likely as a result of the reduced harassment during courtship compared to E females.

The secretome presented in chapter 3 showed no significant difference in the quantities of any secretome proteins and there was no evidence of absence for proteins from either treatment. In *D. melanogaster*, SFPs can respond to selection after relatively few generations, such as sex peptide and Acp26Aa (Wigby et al., 2009). Therefore, it is unlikely that selection has acted to shape SFP evolution in the treatments in this study because they had been selected for over 150 generations, but it is possible that selection on SFP evolution may just take longer in these populations.

**Chapter 4**

Both chapter 2 and 3 examined differences in reproductive traits within a species (*D. pseudoobscura*) under different selection conditions. Following on from chapters 2 and 3, chapter 4 examined the intracellular sperm-egg interactions of *D. pseudoobscura* in comparison to a variety of other closely related species within the *D. obscura* species group (see chapter 1, Figure 1.2 for phylogeny). It was not possible to examine the effects of sexual selection on sperm trait evolution using the experimentally evolved populations of *D. pseudoobscura* (used in chapters 2 and 3) because previous study has shown that sexual selection has not acted on sperm traits within *D. pseudoobscura* (Crudgington et al., 2009). Divergence in sperm traits between the *Drosophila obscura* species (Snook et al., 1994; Pitnick et al., 1995; Snook & Karr, 1998) was likely a response to sexual selection (Snook et al., 1994; Snook, 1997) and so, in chapter 4 I used measurable sperm properties as an identifier for the effects of sexual selection. Any effects of selection on sperm must be integrated into the choreography between sperm and egg for fertilisation to be successful.

I also examined intracellular sperm-egg interactions between species and in hybrid crosses of these species. The purpose of this chapter was to develop a methodology by which data on various sperm and egg parameters could be quantified. Since it had already been demonstrated that sperm enter the egg during fertilisation (Karr, 1991) and remain through development where they undergo morphological changes (Karr & Karr, 1996; Karr & Pitnick, 1996; Pitnick & Karr, 1998; Snook & Karr, 1998), the aim was to ensure the protocol developed was effective at each of these stages. The entrance and subsequent persistence of sperm within the egg suggests that sperm contribute more than genetic material to the egg and/or developing zygote. It has been suggested that sperm enter the egg to correctly align the pronuclei for fusion (Karr, 1991). Whatever their function, these intracellular sperm-egg interactions are critical for fertilisation (Karr, 1991; Krawetz, 2005). If components of the sperm tail evolved in response to selection, these changes in sperm characteristics must be incorporated into the interactions between sperm and egg. As such, the egg would need to evolve

counteradaptations to accommodate the sperm tail for fertilisation to be successful. In *D. melanogaster*, females can accommodate sperm that are longer than the length of the egg, suggesting that some coevolution of sperm and egg traits has taken place (Karr & Pitnick, 1996). However, such coevolution can only occur between males and females that regularly interact and as such, the egg of one species may be unable to accommodate the sperm of another, potentially resulting in an isolating barrier between species.

I took a systems biology approach to develop a protocol by which various data on sperm-egg interactions could be collected. The data collected demonstrates that this protocol can be used throughout development. Distances between points of interest were also successfully quantified. For example, I quantified the distances between the sperm and egg pronuclei and the distances from the polar bodies to various points along the sperm tail length. The original hypothesis was that sperm tails enter the egg to align the pronuclei for fusion. As such, measuring the distances between the pronuclei for both conspecific and hybrid crosses represents an important component for this system.

It had been previously suggested that the sperm form a species-specific 3D folding and coiling structure inside the egg (Karr, 1991). The data collected demonstrated some potential species-specific differences in sperm morphology between species of the *Drosophila obscura* species group (Figure 1.2, chapter 1) through development (chapter 4; Figures 4.7-4.9). For example, aspect ratio was significantly smaller in *D. miranda* compared to *D. persimilis* at the 4n stage of development (chapter 4; Figure 4.8).

The results from chapter 4 also showed some potentially interesting patterns that warrant further investigation. For example, there are some similarities between *D. pseudoobscura* and *D. bogotana* hybrids to their conspecific crosses. In these cases, eggs of one species appear to be able to manage the sperm tail structure of another species and facilitate successful fertilisation (to a point). There is some suggestion that hybrid sperm-egg interactions align more closely to the sperm shape inside the egg of the female's conspecific species in either hybrid cross. For example, curvature was significantly higher in *D. pseudoobscura* sperm

inside the egg compared with sperm of the hybrid cross between *D. bogotana* and *D. pseudoobscura* (chapter 4; Figure 4.12). Sperm tail average curvature of the *D. pseudoobscura* x *D. bogotana* hybrid appeared to be more similar to the *D. pseudoobscura* conspecific sperm (chapter 4; Figure 4.12). Finally, I showed that the sperm-egg interactions of hybrid crosses, just like their conspecific counterparts, undergo changes through development.

Summary

Sexual selection and/or sexual conflict have acted on several reproductive traits in *D. pseudoobscura*. E females were courted more than M females and subsequently were more likely to be harassed by males (chapter 2). A potential cost of this to female fitness was the reduced progeny production by E females and premature death of E females. I suggested that premature death of E females could be associated with the action of SFPs. In light of the results of chapter 3, it seems unlikely that SFPs were responsible for these effects. I showed there was no significant difference in proteins from the accessory glands between M and E treatments. Females in these selection treatments seem to be very good at not remating. This could explain the results of chapter 3, that there was no difference in secretome proteins, because perhaps postcopulatory sexual selection is not a potent driving force in these populations. Therefore, a far more likely explanation for the progeny production outcome is that, either E females have disrupted oviposition and/or they expend excess energy avoiding rematings that they then do not have to invest in egg production. Alternatively, M pairings could have become more benign to one another (as predicted by sexual conflict theory) and therefore have increased fitness in terms of increased progeny production. The premature death of E females compared to M females could be explained by the increased courtship harassment by E males and the subsequent over expenditure of energy to avoid rematings and/or by male density alone, regardless of selection history.

Another finding of chapter 2 was that M and E females significantly differed in their remating interval (in 3 out of the 4 replicates), with M females remating earlier than E females. It is possible that E females were delayed in remating by

the action of SFPs, such as sex peptide or Acp62F (Lung et al., 2002; Wigby et al., 2009). This is unlikely given that there were no significant differences in abundance or presence/absence for any proteins of the secretome (chapter 3). However, sex peptide and Acp62F were not identified as such in the secretome of *D. pseudoobscura* accessory glands. As I have identified a suite of secretome proteins, an area for future study could be to target proteins directly using tagging (iTRAQ or TMT) and assess what effects they may have on mated females. Equipment and logistical constraints prevented this analysis during these experiments. It would also be of interest to analyse the SFPs from within the female reproductive tract to give a full description of how these proteins function in the interaction between the sexes. For example, Acp26Aa is processed into active forms after transfer to the female (Park & Wolfner, 1995).

Given the results of chapter 2 and 3, it seems that males may be making trade-offs in their reproductive trait evolution. Males of these *D. pseudoobscura* populations appear to be investing more in precopulatory traits (e.g. courtship) in response to selection, rather than investing in postcopulatory traits (e.g. SFP quantities). A previous study has also shown that sexual selection has not acted on testes mass or sperm traits in these males (Crudgington et al., 2009). To examine a complete suite of reproductive traits, I studied the sperm and its interactions with the egg during fertilisation and early development. Since sperm traits, (both sperm number and size), are not shaped by sexual selection history in the *D. pseudoobscura* populations (Crudgington et al., 2009), it was not possible to use these selection treatments to examine the effects of sexual selection on sperm evolution. As such, I investigated the interactions between sperm and egg using *D. pseudoobscura* and made interspecific comparisons with other species of the *Drosophila obscura* species group. I found some putative differences in sperm-egg interactions between species that warrant further investigation. Overall, I have demonstrated a novel methodology to utilise in revealing the role of sexual selection in sperm-egg interactions. I have also shown that sperm traits, potentially shaped by sexual selection can be accommodated by the egg between a variety of closely related species and

coevolution between the sexes must be responsible for the success in fertilisation shown.

Complex interactions between the sexes take place at every stage of reproduction, both pre- and post-copulatory. These interactions can include behavioural, morphological, physiological and/or biochemical traits, which could become a source of incompatibility. Sexual selection has the ability to drive the rapid evolution of those traits associated with reproduction. Since reproductive proteins are known to evolve faster than non-reproductive proteins (Swanson & Vacquier, 2002), selection can drive the divergence of traits over relatively short time scales. In chapters 2 and 3, I used experimentally evolved sexual selection treatments to assess any differences in a variety of both pre- and post-copulatory reproductive traits. In chapter 4, I examined differences in sperm and egg interactions both between species and with hybrid crosses of those species. In doing so, in this thesis I have covered a wider range of intra- and inter-specific interactions and covered traits from several stages of reproduction, both pre- and post-copulatory.

## References


Alipaz, J.A., Wu, C. and Karr, T.L., 2001. Gametic incompatibilities between races of *Drosophila melanogaster*. *Proceedings of the Royal Society of London B: Biological Sciences*, 268(1469), pp.789-795.

Alberts, B. Johnson, A. Lewis, J. Raff, M. Roberts, K. Walter, P., 2002. Molecular Biology of the Cell. 4th edition. *New York: Garland Science.*

Alonzo, S.H. and Pizzari, T., 2013. Selection on female remating interval is influenced by male sperm competition strategies and ejaculate characteristics. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 368(1613), pp.20120044.

Anderson, W.W., 1974. Frequent multiple insemination in a natural population of *Drosophila pseudoobscura*. *The American Naturalist*, 108(963), pp.709-711.

Andersson, M.B., 1994. Sexual Selection. *Princeton University Press*.

Arnqvist, G. and Nilsson, T., 2000. The evolution of polyandry: multiple mating and female fitness in insects. Animal Behaviour, 60(2), pp.145-164.

Arnqvist, G. and Rowe, L., 2005. Sexual Conflict. *Princeton University Press*.

Avila, F.W., Sirot, L.K., LaFlamme, B.A., Rubinstein, C.D. and Wolfner, M.F., 2011. Insect seminal fluid proteins: identification and function. *Annual Review of Entomology*, 56, pp.21.

Bacigalupe, L.D., Crudgington, H.S., Hunter, F., Moore, A.J. and Snook, R.R., 2007. Sexual conflict does not drive reproductive isolation in experimental populations of *Drosophila pseudoobscura*. *Journal of Evolutionary Biology*, *20*(5), pp.1763-1771.

Bacigalupe, L.D., Crudgington, H.S., Slate, J., Moore, A.J. and Snook, R.R., 2008. Sexual selection and interacting phenotypes in experimental evolution: a study of *Drosophila pseudoobscura* mating behavior. *Evolution*, 62(7), pp.1804-1812.

Baer, B., Heazlewood, J.L., Taylor, N.L., Eubel, H. and Millar, A.H., 2009. The seminal fluid proteome of the honeybee *Apis mellifera*. *Proteomics*, 9(8), pp.2085-2097.

Baker, R.H., Denniff, M., Futerman, P., Fowler, K., Pomiankowski, A. and Chapman, T., 2003. Accessory gland size influences time to sexual maturity and mating frequency in the stalk-eyed fly, *Cyrtodiopsis dalmanni*. *Behavioral Ecology*, 14(5), pp.607-611.

Bangham, J., Chapman, T. and Partridge, L., 2002. Effects of body size, accessory gland and testis size on pre-and postcopulatory success in *Drosophila melanogaster*. *Animal Behaviour*, 64(6), pp.915-921.

Bantscheff, M., Schirle, M., Sweetman, G., Rick, J. and Kuster, B., 2007. Quantitative mass spectrometry in proteomics: a critical review. *Analytical and Bioanalytical Chemistry*, 389(4), pp.1017-1031.

Bateman, A.J., 1948. Intra-sexual selection in *Drosophila*. *Heredity*, 2(3), pp.349-68.

Bateman, P.W., Ferguson, J.W.H. and Yetman, C.A., 2006. Courtship and copulation, but not ejaculates, reduce the longevity of female field crickets (*Gryllus bimaculatus*). *Journal of Zoology*, 268(4), pp.341-346.

Bates, D., Maechler, M., Bolker, B. and Walker, S., 2014. lme4: Linear mixed-effects models using Eigen and S4. R package version, 1(7).

Bennison, C., Hemmings, N., Slate, J. and Birkhead, T., 2015. Long sperm fertilize more eggs in a bird. *Proceedings of the Royal Society of London B: Biological Sciences*, 282(1799), pp.20141897.

Bindea, G., Mlecnik, B., Hackl, H., Charoentong, P., Tosolini, M., Kirilovsky, A., Fridman, W.H., Pagès, F., Trajanoski, Z. and Galon, J., 2009. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics*, 25(8), pp.1091-1093.

Birkhead, T.R. and Pizzari, T., 2002. Postcopulatory sexual selection. *Nature Reviews Genetics*, 3(4), pp.262-273.

Blanckenhorn, W.U., Hosken, D.J., Martin, O.Y., Reim, C., Teuschl, Y. and Ward, P.I., 2002. The costs of copulating in the dung fly *Sepsis cynipsea*. *Behavioral Ecology*, 13(3), pp.353-358.

Brent, A.E., MacQueen, A. and Hazelrigg, T., 2000. The *Drosophila* wispy gene is required for RNA localization and other microtubule-based events of meiosis and early embryogenesis. *Genetics*, 154(4), pp.1649-1662.

Chapman, T., Liddle, L.F., Kalb, J.M., Wolfner, M.F. and Partridge, L., 1995. Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature*, 373(6511), pp.241-244.

Chapman, T., Neubaum, D.M., Wolfner, M.F. and Partridge, L., 2000. The role of male accessory gland protein Acp36DE in sperm competition in *Drosophila melanogaster*. *Proceedings of the Royal Society of London B: Biological Sciences*, 267(1448), pp.1097-1105.

Chapman, T., 2001. Seminal fluid-mediated fitness traits in *Drosophila*. *Heredity*, 87(5), pp.511-521.

Chapman, T., Herndon, L.A., Heifetz, Y., Partridge, L. and Wolfner, M.F., 2001. The Acp26Aa seminal fluid protein is a modulator of early egg hatchability in *Drosophila melanogaster*. *Proceedings of the Royal Society of London B: Biological Sciences*, 268(1477), pp.1647-1654.

Chapman, T., Arnqvist, G., Bangham, J. and Rowe, L., 2003. Sexual conflict. *Trends in Ecology & Evolution*, 18(1), pp.41-47.

Chapman, T. and Davies, S.J., 2004. Functions and analysis of the seminal fluid proteins of male *Drosophila melanogaster* fruit flies. *Peptides*, 25(9), pp.1477-1490.

Chen, P.S., 1984. The functional morphology and biochemistry of insect male accessory glands and their secretions. *Annual Review of Entomology*, 29(1), pp.233-255.

Chen, P.S., Stumm-Zollinger, E., Aigaki, T., Balmer, J., Bienz, M. and Böhlen, P., 1988. A male accessory gland peptide that regulates reproductive behavior of female *D. melanogaster*. *Cell*, 54(3), pp.291-298.

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.

Civetta, A. and Reimer, A., 2014. Positive selection at a seminal fluid gene within a QTL for conspecific sperm precedence. *Genetica*, 142(6), pp.537-543.

Clark, A.G., Aguadé, M., Prout, T., Harshman, L.G. and Langley, C.H., 1995. Variation in sperm displacement and its association with accessory gland protein loci in *Drosophila melanogaster*. *Genetics*, 139(1), pp.189-201.

Clark, N.L., Aagaard, J.E. and Swanson, W.J., 2006. Evolution of reproductive proteins from animals and plants. *Reproduction*, 131(1), pp.11-22.

Clark, A.G., Eisen, M.B., Smith, D.R., Bergman, C.M., Oliver, B., Markow, T.A., Kaufman, T.C., Kellis, M., Gelbart, W., Iyer, V.N. and Pollard, D.A., 2007. Evolution of genes and genomes on the *Drosophila* phylogeny. *Nature*, 450(7167), pp.203-218.

Clutton-Brock, T.H. and Parker, G.A., 1995. Punishment in animal societies. *Nature*, 373(6511), pp.209-216.

Clutton-Brock, T. and Langley, P., 1997. Persistent courtship reduces male and female longevity in captive tsetse flies *Glossina morsitans morsitans* Westwood (Diptera: Glossinidae). *Behavioral Ecology*, 8(4), pp.392-395.

Cox, J. and Mann, M., 2008. MaxQuant enables high peptide identification rates, individualized ppb-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnology*, 26(12), pp.1367-1372.

Cox, J., Hein, M.Y., Luber, C.A., Paron, I., Nagaraj, N. and Mann, M., 2014. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Molecular & Cellular Proteomics*, 13(9), pp.2513-2526.

Coyne, J.A. and Orr, H.A., 2004. Speciation. *Sunderland, MA: Sinauer Associates*.

Crudgington, H.S., Beckerman, A.P., Brüstle, L., Green, K. and Snook, R.R., 2005. Experimental removal and elevation of sexual selection: does sexual selection generate manipulative males and resistant females? *The American Naturalist*, 165(S5), pp.S72-S87.

Crudgington, H.S., Fellows, S., Badcock, N.S. and Snook, R.R., 2009. Experimental manipulation of sexual selection promotes greater male mating capacity but does not alter sperm investment. *Evolution*, 63(4), pp.926-938.

Crudgington, H.S., Fellows, S. and Snook, R.R., 2010. Increased opportunity for sexual conflict promotes harmful males with elevated courtship frequencies. *Journal of Evolutionary Biology*, 23(2), pp.440-446.

Danilchik, M.V. and Black, S.D., 1988. The first cleavage plane and the embryonic axis are determined by separate mechanisms in *Xenopus laevis*: I. Independence in undisturbed embryos. *Developmental Biology*, 128(1), pp.58-64.

Debelle, A., Ritchie, M.G. and Snook, R.R., 2014. Evolution of divergent female mating preference in response to experimental sexual selection. *Evolution*, 68(9), pp.2524-2533.

Dorus, S., Busby, S.A., Gerike, U., Shabanowitz, J., Hunt, D.F. and Karr, T.L., 2006. Genomic and functional evolution of the *Drosophila melanogaster* sperm proteome. *Nature Genetics*, 38(12), pp.1440-1445.

Eberhard, W.G., 1996. Female Control: Sexual Selection by Cryptic Female Choice. *Princeton University Press*.

Findlay, G.D., Yi, X., MacCoss, M.J. and Swanson, W.J., 2008. Proteomics reveals novel *Drosophila* seminal fluid proteins transferred at mating. *PLoS Biol*, 6(7), p.e178.

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.886-896.

Findlay, G.D. and Swanson, W.J., 2010. Proteomics enhances evolutionary and functional analysis of reproductive proteins. *Bioessays*, 32(1), pp.26-36.

Fisher, R.A., 1930. The Genetical Theory of Natural Selection: A Complete Variorum Edition. *Oxford University Press*.

Fowler, K. and Partridge, L., 1989. A cost of mating in female fruitflies. *Nature*, 338(6218), pp.760-761.

Fricke, C., Wigby, S., Hobbs, R. and Chapman, T., 2009. The benefits of male ejaculate sex peptide transfer in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, 22(2), pp.275-286.

Fritzsche, K. and Arnqvist, G., 2013. Homage to Bateman: sex roles predict sex differences in sexual selection. *Evolution*, 67(7), pp.1926-1936.

Gage, M.J. and Morrow, E.H., 2003. Experimental evidence for the evolution of numerous, tiny sperm via sperm competition. *Current Biology*, 13(9), pp.754-757.

Gavrilets, S., 2000. Rapid evolution of reproductive barriers driven by sexual conflict. *Nature*, 403(6772), pp.886-889.

Gavrilets, S., Arnqvist, G. and Friberg, U., 2001. The evolution of female mate choice by sexual conflict. *Proceedings of the Royal Society of London B: Biological Sciences*, 268(1466), pp.531-539.

Gay, L., Eady, P.E., Vasudev, R.A.M., Hosken, D.J. and Tregenza, T., 2009. Costly sexual harassment in a beetle. *Physiological Entomology*, 34(1), pp.86-92.

Gillott, C., 2003. Male accessory gland secretions: modulators of female reproductive physiology and behavior. *Annual Review of Entomology*, 48(1), pp.163-184.

Gray, D., Plusa, B., Piotrowska, K., Na, J., Tom, B., Glover, D.M. and Zernicka-Goetz, M., 2004. First cleavage of the mouse embryo responds to change in egg shape at fertilization. *Current Biology*, 14(5), pp.397-405.

Green, L.H., Raff, E.C. and Raff, R.A., 1979. Tubulin from a development insect embryo undergoing rapid mitosis: Factors regulating in vitro assembly of tubulin from *Drosophila melanogaster*. *Insect Biochemistry*, 9(5), pp.489-495.

Gromko, M.H., Newport, M.E.A. and Kortier, M.G., 1984. Sperm dependence of female receptivity to remating in *Drosophila melanogaster*. *Evolution*, pp.1273-1282.

Gromko, M.H. and Markow, T.A., 1993. Courtship and remating in field populations of *Drosophila*. *Animal Behaviour*, 45(2), pp.253-262.

Harcourt, A.H., Harvey, P.H., Larson, S.G. and Short, R.V., 1981. Testis weight, body weight and breeding system in primates. *Nature*, 293(5827), pp.55-57.

Hathout, Y., 2007. Approaches to the study of the cell secretome. *Expert Review of Proteomics*, 4(2), pp.239-248.

Heifetz, Y., Lung, O., Frongillo, E.A. and Wolfner, M.F., 2000. The *Drosophila* seminal fluid protein Acp26Aa stimulates release of oocytes by the ovary. *Current Biology*, *10*(2), pp.99-102.

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.

Holland, B. and Rice, W.R., 1998. Perspective: chase-away sexual selection: antagonistic seduction versus resistance. *Evolution*, pp.1-7.

Holland, B. and Rice, W.R., 1999. Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. *Proceedings of the National Academy of Sciences*, 96(9), pp.5083-5088.

Hosken, D.J. and Ward, P.I., 2001. Experimental evidence for testis size evolution via sperm competition. *Ecology Letters*, 4(1), pp.10-13.

Howard, D.J., 1999. Conspecific sperm and pollen precedence and speciation. *Annual Review of Ecology and Systematics*, pp.109-132.

Hunt, J., Snook, R.R., Mitchell, C., Crudgington, H.S. and Moore, A.J., 2012. Sexual selection and experimental evolution of chemical signals in *Drosophila pseudoobscura*. *Journal of Evolutionary Biology*, 25(11), pp.2232-2241.

Immonen, E., Snook, R.R. and Ritchie, M.G., 2014. Mating system variation drives rapid evolution of the female transcriptome in *Drosophila pseudoobscura*. *Ecology and Evolution*, 4(11), pp.2186-2201.

Jennions, M.D. and Petrie, M., 2000. Why do females mate multiply? A review of the genetic benefits. *Biological Reviews of the Cambridge Philosophical Society*, 75(01), pp.21-64.

Karr, T.L., 1991. Intracellular sperm/egg interactions in *Drosophila*: a three-dimensional structural analysis of a paternal product in the developing egg. *Mechanisms of Development*, 34(2), pp.101-111.

Karr, T.L., 1996. Paternal investment and intracellular sperm-egg interactions during and following fertilization in *Drosophila*. *Current Topics in Developmental Biology*, 34, pp.3.

Karr, T.L., 2007. Fruit flies and the sperm proteome. *Human Molecular Genetics*, 16(R2), pp.R124-R133.

Karr, T.L., 2008. Application of proteomics to ecology and population biology. *Heredity*, 100(2), pp.200-206.

Kirkpatrick, M. and Ryan, M.J., 1991. The evolution of mating preferences and the paradox of the lek. *Nature*, 350(6313), pp.33-38.

Kokko, H., Jennions, M.D. and Brooks, R., 2006. Unifying and testing models of sexual selection. *Annual Review of Ecology, Evolution, and Systematics*, 37, pp.43-66.

Krawetz, S.A., 2005. Paternal contribution: new insights and future challenges. *Nature Reviews Genetics*, 6(8), pp.633-642.

Lassy, C.W. and Karr, T.L., 1996. Cytological analysis of fertilization and early embryonic development in incompatible crosses of *Drosophila simulans*. *Mechanisms of Development*, 57(1), pp.47-58.

Lung, O., Tram, U., Finnerty, C.M., Eipper-Mains, M.A., Kalb, J.M. and Wolfner, M.F., 2002. The *Drosophila melanogaster* seminal fluid protein Acp62F is a protease inhibitor that is toxic upon ectopic expression. *Genetics*, 160(1), pp.211-224.

Magurran, A.E. and Seghers, B.H., 1994. A cost of sexual harassment in the guppy, *Poecilia reticulata*. *Proceedings of the Royal Society of London B: Biological Sciences*, 258(1351), pp.89-92.

Markow, T.A., 2002. Perspective: female remating, operational sex ratio, and the arena of sexual selection in *Drosophila* species. *Evolution*, 56(9), pp.1725-1734.

Martin, O.Y. and Hosken, D.J., 2003a. Costs and benefits of evolving under experimentally enforced polyandry or monogamy. *Evolution*, 57(12), pp.2765-2772.

Martin, O.Y. and Hosken, D.J., 2003b. The evolution of reproductive isolation through sexual conflict. *Nature*, 423(6943), pp.979-982.

Matute, D.R. and Coyne, J.A., 2010. Intrinsic reproductive isolation between two sister species of *Drosophila*. *Evolution*, 64(4), pp.903-920.

Miller, G.T. and Pitnick, S., 2002. Sperm-female coevolution in *Drosophila*. *Science*, 298(5596), pp.1230-1233.

Moomjy, M., Colombero, L.T., Veeck, L.L., Rosenwaks, Z. and Palermo, G.D., 1999. Sperm integrity is critical for normal mitotic division and early embryonic development. *Molecular Human Reproduction*, 5(9), pp.836-844.

Morrow, E.H. and Gage, M.J., 2001. Sperm competition experiments between lines of crickets producing different sperm lengths. *Proceedings of the Royal Society of London B: Biological Sciences*, 268(1482), pp.2281-2286.

Mueller, J.L., Ripoll, D.R., Aquadro, C.F. and Wolfner, M.F., 2004. Comparative structural modeling and inference of conserved protein classes in *Drosophila* seminal fluid. *Proceedings of the National Academy of Sciences of the United States of America*, 101(37), pp.13542-13547.

Mueller, J.L., Ram, K.R., McGraw, L.A., Qazi, M.B., Siggia, E.D., Clark, A.G., Aquadro, C.F. and Wolfner, M.F., 2005. Cross-species comparison of *Drosophila* male accessory gland protein genes. *Genetics*, 171(1), pp.131-143.

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.

Ohsako, T., Hirai, K. and Yamamoto, M.T., 2003. The *Drosophila* misfire gene has an essential role in sperm activation during fertilization. *Genes & Genetic Systems*, 78(3), pp.253-266.

Orteiza, N., Linder, J.E. and Rice, W.R., 2005. Sexy sons from re-mating do not recoup the direct costs of harmful male interactions in the *Drosophila melanogaster* laboratory model system. *Journal of Evolutionary Biology*, 18(5), pp.1315-1323.

Ostermeier, G.C., Miller, D., Huntriss, J.D., Diamond, M. and Krawetz, S.A., 2005. Delivering spermatozoon RNA to the oocyte. *Nature*, 429, p.154.

Park, M. and Wolfner, M.F., 1995. Male and female cooperate in the prohormone-like processing of a *Drosophila melanogaster* seminal fluid protein. *Developmental Biology*, 171(2), pp.694-702.

Panhuis, T.M., Butlin, R., Zuk, M. and Tregenza, T., 2001. Sexual selection and speciation. *Trends in Ecology & Evolution*, 16(7), pp.364-371.

Panhuis, T.M., Clark, N.L. and Swanson, W.J., 2006. Rapid evolution of reproductive proteins in abalone and *Drosophila*. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 361(1466), pp.261-268.

Parker, G.A., 1970. Sperm competition and its evolutionary consequences in the insects. *Biological Reviews*, 45(4), pp.525-567.

Parker, G.A., 1979. Sexual Selection and Sexual Conflict. Sexual Selection and Reproductive Competition in Insects, pp.123-166.

Partridge, L., Hoffmann, A. and Jones, J.S., 1987. Male size and mating success in *Drosophila melanogaster* and *D. pseudoobscura* under field conditions. *Animal Behaviour*, 35(2), pp.468-476.

Partridge, L. and Fowler, K., 1990. Non-mating costs of exposure to males in female *Drosophila melanogaster*. *Journal of Insect Physiology*, 36(6), pp.419-425.

Pavlou, M.P. and Diamandis, E.P., 2010. The cancer cell secretome: a good source for discovering biomarkers?. *Journal of Proteomics*, 73(10), pp.1896-1906.

Pedersen, R.A., 2001. Developmental biology: Sperm and mammalian polarity. *Nature*, 409(6819), pp.473-474.

Petersen, T.N., Brunak, S., von Heijne, G. and Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods*, 8(10), pp.785-786.

Piotrowska, K. and Zernicka-Goetz, M., 2001. Role for sperm in spatial patterning of the early mouse embryo. *Nature*, 409(6819), pp.517-521.

Pitnick, S. and Markow, T.A., 1994. Large-male advantages associated with costs of sperm production in *Drosophila hydei*, a species with giant sperm. *Proceedings of the National Academy of Sciences*, 91(20), pp.9277-9281.

Pitnick, S., Spicer, G.S. and Markow, T.A., 1995a. How long is a giant sperm? *Nature*, 375(6527), pp.109-109.

Pitnick, S., Markow, T.A. and Spicer, G.S., 1995b. Delayed male maturity is a cost of producing large sperm in *Drosophila*. *Proceedings of the National Academy of Sciences*, 92(23), pp.10614-10618.

Pitnick, S. and Karr, T.L., 1996. Sperm caucus. *Trends in Ecology & Evolution*, 11(4), pp.148-151.

Pitnick, S. and Karr, T.L., 1998. Paternal products and by–products in *Drosophila* development. *Proceedings of the Royal Society of London B: Biological Sciences*, 265(1398), pp.821-826.

Pitnick, S., Markow, T. and Spicer, G.S., 1999. Evolution of multiple kinds of female sperm-storage organs in *Drosophila*. *Evolution*, pp.1804-1822.

Pitnick, S., Miller, G.T., Reagan, J. and Holland, B., 2001a. Males' evolutionary responses to experimental removal of sexual selection. *Proceedings of the Royal Society of London B: Biological Sciences*, 268(1471), pp.1071-1080.

Pitnick, S., Brown, W.D. and Miller, G.T., 2001b. Evolution of female remating behaviour following experimental removal of sexual selection. *Proceedings of the Royal Society of London B: Biological Sciences*, 268(1467), pp.557-563.

Pitnick, S., Miller, G.T., Schneider, K. and Markow, T.A., 2003. Ejaculate-female coevolution in *Drosophila mojavensis*. *Proceedings of the Royal Society of London B: Biological Sciences*, 270(1523), pp.1507-1512.

Pizzari, T., Dean, R., Pacey, A., Moore, H. and Bonsall, M.B., 2008. The evolutionary ecology of pre-and post-meiotic sperm senescence. *Trends in Ecology & Evolution*, 23(3), pp.131-140.

Punzalan, D., Rodd, F.H. and Rowe, L., 2008. Contemporary sexual selection on sexually dimorphic traits in the ambush bug *Phymata americana*. *Behavioral Ecology*, 19(4), pp.860-870.

Python Software Foundation. Python Language Reference, version 2.7. Available at http://www.python.org

R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/

Ram, K.R., Sirot, L.K. and Wolfner, M.F., 2006. Predicted seminal astacin-like protease is required for processing of reproductive proteins in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, 103(49), pp.18674-18679.

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.

Rice, W.R., 1996. Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature*, 381(6579), pp.232-234.

Rice, W.R. and Holland, B., 1997. The enemies within: intergenomic conflict, interlocus contest evolution (ICE), and the intraspecific Red Queen. Behavioral *Ecology and Sociobiology*, 41(1), pp.1-10.

Rice, W.R., 2000. Dangerous liaisons. *Proceedings of the National Academy of Sciences*, 97(24), pp.12953-12955.

Richards, S., Liu, Y., Bettencourt, B.R., Hradecky, P., Letovsky, S., Nielsen, R., Thornton, K., Hubisz, M.J., Chen, R., Meisel, R.P. and Couronne, O., 2005. Comparative genome sequencing of *Drosophila pseudoobscura*: chromosomal, gene, and cis-element evolution. *Genome Research*, 15(1), pp.1-18.

Ridley, M., 1988. Mating frequency and fecundity in insects. *Biological Reviews*, 63(4), pp.509-549.

Ritchie, M.G., 2007. Sexual selection and speciation. *Annual Review of Ecology, Evolution, and Systematics*, pp.79-102.

Rowe, L., Arnqvist, G., Sih, A. and Krupa, J.J., 1994. Sexual conflict and the evolutionary ecology of mating patterns: water striders as a model system. *Trends in Ecology & Evolution*, 9(8), pp.289-293.

Rowe, L., Cameron, E. and Day, T., 2003. Detecting sexually antagonistic coevolution with population crosses. *Proceedings of the Royal Society of London B: Biological Sciences*, 270(1528), pp.2009-2016.

Rowe, L. and Day, T., 2006. Detecting sexual conflict and sexually antagonistic coevolution. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 361(1466), pp.277-285.

Sakurai, G. and Kasuya, E., 2008. The costs of harassment in the adzuki bean beetle. *Animal Behaviour*, 75(4), pp.1367-1373.

Schärer, L., Rowe, L. and Arnqvist, G., 2012. Anisogamy, chance and the evolution of sex roles. *Trends in Ecology & Evolution*, 27(5), pp.260-264.

Shapiro, B.M., Schackmann, R.W. and Gabel, C.A., 1981. Molecular approaches to the study of fertilization. *Annual Review of Biochemistry*, 50(1), pp.815-843.

Simerly, C., Wu, G.J., Zoran, S., Ord, T., Rawlins, R., Jones, J., Navara, C., Gerrity, M., Rinehart, J., Binor, Z. and Asch, R., 1995. The paternal inheritance of the centrosome, the cell's microtubule-organizing center, in humans, and the implications for infertility. *Nature Medicine*, 1(1), pp.47-52.

Simmons, L.W., 2001. Sperm Competition and Its Evolutionary Consequences in the Insects. *Princeton University Press*.

Simmons, L.W., Tan, Y.F. and Millar, A.H., 2013. Sperm and seminal fluid proteomes of the field cricket *Teleogryllus oceanicus*: identification of novel proteins transferred to females at mating. *Insect Molecular Biology*, 22(1), pp.115-130.

Sirot, L.K., Wolfner, M.F. and Wigby, S., 2011. Protein-specific manipulation of ejaculate composition in response to female mating status in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, 108(24), pp.9922-9926.

Sirot, L.K., Wong, A., Chapman, T. and Wolfner, M.F., 2015. Sexual conflict and seminal fluid proteins: a dynamic landscape of sexual interactions. *Cold Spring Harbor Perspectives in Biology*, 7(2), pp.017533.

Skerget, S., Rosenow, M.A., Petritis, K. and Karr, T.L., 2015. Sperm Proteome Maturation in the Mouse Epididymis. *PloS One*, 10(11), pp.0140650.

Snook, R.R., Markow, T.A. and Karr, T.L., 1994. Functional nonequivalence of sperm in *Drosophila pseudoobscura*. *Proceedings of the National Academy of Sciences*, 91(23), pp.11222-11226.

Snook, R.R., 1997. Is the production of multiple sperm types adaptive? *Evolution*, pp.797-808.

Snook, R.R., 1998. The risk of sperm competition and the evolution of sperm heteromorphism. *Animal Behaviour*, 56(6), pp.1497-1507.

Snook, R.R. and Karr, T.L., 1998. Only long sperm are fertilization-competent in six sperm-heteromorphic *Drosophila* species. *Current Biology*, 8(5), pp.291-294.

Snook, R.R. and Hosken, D.J., 2004. Sperm death and dumping in *Drosophila*. *Nature*, 428(6986), pp.939-941.

Snook, R.R., 2005. Sperm in competition: not playing by the numbers. *Trends in Ecology & Evolution*, 20(1), pp.46-53.

Snook, R.R., Robertson, A., Crudgington, H.S. and Ritchie, M.G., 2005. Experimental manipulation of sexual selection and the evolution of courtship song in *Drosophila pseudoobscura*. *Behavior Genetics*, 35(3), pp.245-255.

Snook, R.R., Brüstle, L. and Slate, J., 2009a. A test and review of the role of effective population size on experimental sexual selection patterns. *Evolution*, 63(7), pp.1923-1933.

Snook, R.R., Chapman, T., Moore, P.J., Wedell, N. and Crudgington, H.S., 2009b. Interactions between the sexes: new perspectives on sexual selection and reproductive isolation. *Evolutionary Ecology*, 23(1), pp.71-91.

Snook, R.R., Bacigalupe, L.D. and Moore, A.J., 2010. The quantitative genetics and coevolution of male and female reproductive traits. *Evolution*, 64(7), pp.1926-1934.

Snook, R.R., Gidaszewski, N.A., Chapman, T. and Simmons, L.W., 2013. Sexual selection and the evolution of secondary sexual traits: sex comb evolution in *Drosophila*. *Journal of Evolutionary Biology*, 26(4), pp.912-918.

Stockley, P., 1997. Sexual conflict resulting from adaptations to sperm competition. *Trends in Ecology & Evolution*, 12(4), pp.154-159.

Sutovsky, P. and Schatten, G., 1999. Paternal contributions to the mammalian zygote: fertilization after sperm-egg fusion. *International Review of Cytology*, 195, pp.1-65.

Swanson, W.J., Clark, A.G., Waldrip-Dail, H.M., Wolfner, M.F. and Aquadro, C.F., 2001. Evolutionary EST analysis identifies rapidly evolving male reproductive proteins in *Drosophila*. *Proceedings of the National Academy of Sciences*, 98(13), pp.7375-7379.

Swallow, J.G. and Wilkinson, G.S., 2002. The long and short of sperm polymorphisms in insects. *Biological Reviews of the Cambridge Philosophical Society*, 77(02), pp.153-182.

Swanson, W.J. and Vacquier, V.D., 2002. The rapid evolution of reproductive proteins. *Nature Reviews Genetics*, 3(2), pp.137-144.

Therneau, T.M., 2014. A Package for Survival Analysis in S, 2014. R package version, pp.2-37.

Till-Bottraud, I., Joly, D., Lachaise, D. and Snook, R.R., 2005. Pollen and sperm heteromorphism: convergence across kingdoms? *Journal of Evolutionary Biology*, 18(1), pp.1-18.

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.

Tregenza, T. and Wedell, N., 2000. Genetic compatibility, mate choice and patterns of parentage: invited review. *Molecular Ecology*, 9(8), pp.1013-1027.

Tremblay, E. and Caltagirone, L.E., 1973. Fate of polar bodies in insects. *Annual Review of Entomology*, 18(1), pp.421-444.

Trevitt, S., Fowler, K. and Partridge, L., 1988. An effect of egg-deposition on the subsequent fertility and remating frequency of female *Drosophila melanogaster*. *Journal of Insect Physiology*, 34(8), pp.821-828.

Turner, M.E. and Anderson, W.W., 1983. Multiple mating and female fitness in *Drosophila pseudoobscura*. *Evolution*, pp.714-723.

Ursprung, C., Den Hollander, M. and Gwynne, D.T., 2009. Female seed beetles, *Callosobruchus maculatus*, remate for male-supplied water rather than ejaculate nutrition. *Behavioral Ecology and Sociobiology*, 63(6), pp.781-788.

Wagstaff, B.J. and Begun, D.J., 2005. Comparative genomics of accessory gland protein genes in *Drosophila melanogaster* and *D. pseudoobscura*. *Molecular Biology and Evolution*, 22(4), pp.818-832.

Watson, P.J., Stallmann, R.R. and Arnqvist, G., 1998. Sexual conflict and the energetic costs of mating and mate choice in water striders. *The American Naturalist*, 151(1), pp.46-58.

Wei, D., Li, H.M., Tian, C.B., Smagghe, G., Jia, F.X., Jiang, H.B., Dou, W. and Wang, J.J., 2015. Proteome analysis of male accessory gland secretions in oriental fruit flies reveals juvenile hormone-binding protein, suggesting impact on female reproduction. *Scientific Reports*, 5, pp. 16845.

West-Eberhard, M.J., 1984. Sexual selection, competitive communication and species-specific signals in insects. *Insect Communication*, pp.283-324.

Wigby, S. and Chapman, T., 2004. Female resistance to male harm evolves in response to manipulation of sexual conflict. *Evolution*, 58(5), pp.1028-1037.

Wigby, S. and Chapman, T., 2005. Sex peptide causes mating costs in female *Drosophila melanogaster*. *Current Biology*, 15(4), pp.316-321.

Wigby, S., Sirot, L.K., Linklater, J.R., Buehner, N., Calboli, F.C., Bretman, A., Wolfner, M.F. and Chapman, T., 2009. Seminal fluid protein allocation and male reproductive success. *Current Biology*, 19(9), pp.751-757.

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.

Wolfner, M.F., Harada, H.A., Bertram, M.J., Stelick, T.J., Kraus, K.W., Kalb, J.M., Lung, Y.O., Neubaum, D.M., Park, M. and Tram, U., 1997. New genes for male accessory gland proteins in *Drosophila melanogaster*. *Insect Biochemistry and Molecular Biology*, 27(10), pp.825-834.

Wolfner, M.F., 2002. The gifts that keep on giving: physiological functions and evolutionary dynamics of male seminal proteins in *Drosophila*. *Heredity*, 88(2), pp.85-93.

Wolfner, M.F., 2009. Battle and ballet: molecular interactions between the sexes in *Drosophila*. *Journal of Heredity*, 100(4), pp.399-410.

Wolfram Research, Inc. Title: Mathematica Edition: Version 10.0 Publisher: Wolfram Research, Inc. Place of publication: Champaign, Illinois Date of publication: 2014.

Wong, A., Albright, S.N. and Wolfner, M.F., 2006. Evidence for structural constraint on ovulin, a rapidly evolving *Drosophila melanogaster* seminal protein. *Proceedings of the National Academy of Sciences*, 103(49), pp.18644-18649.

Wong, A., Christopher, A.B., Buehner, N.A. and Wolfner, M.F., 2010. Immortal coils: Conserved dimerization motifs of the *Drosophila* ovulation prohormone ovulin. *Insect Biochemistry and Molecular Biology*, 40(4), pp.303-310.

Yapici, N., Kim, Y.J., Ribeiro, C. and Dickson, B.J., 2008. A receptor that mediates the post-mating switch in *Drosophila* reproductive behaviour. *Nature*, 451(7174), pp.33-37.

# Appendices

## Appendix 1: Chapter 3

### Appendix 1.1 Materials and Methods

*Solutions for proteomics sample preparation:*

| Key: | ABC = Ammonium Bicarbonate |
|---|---|
| | ACN = Acetonitrile |
| | FA = Formic Acid |
| | DTT = Dithiothreitol |
| | HCl = Hydrochloric acid |
| | IAA = Iodoacetamide |
| | TFA = Trifluroacetic acid |

Solution 1: 200mM ABC, 40% ACN

Solution 2: 50mM ABC

Solution 3: 50mM ABC, 50% ACN

Solution 4: 40mM ABC, 9% ACN

Solution 5: 100% ACN

Solution 6: 5% FA

Solution 7: 50% ACN, 5%FA

Solution 8: 1mM HCl

Reduction Buffer: 10mM DTT, 50mM ABC (Solution 2).

Alkylation Buffer: 55mM IAA, 50mM ABC (Solution 2).

Trypsin solution: 1 vial of proteomic grade trypsin (TPCK treated) (20µg), add 100µL of Solution 8, then add 900µL of Solution 4 (= 0.02µg/µL)

## Appendix 1.2 **Results**

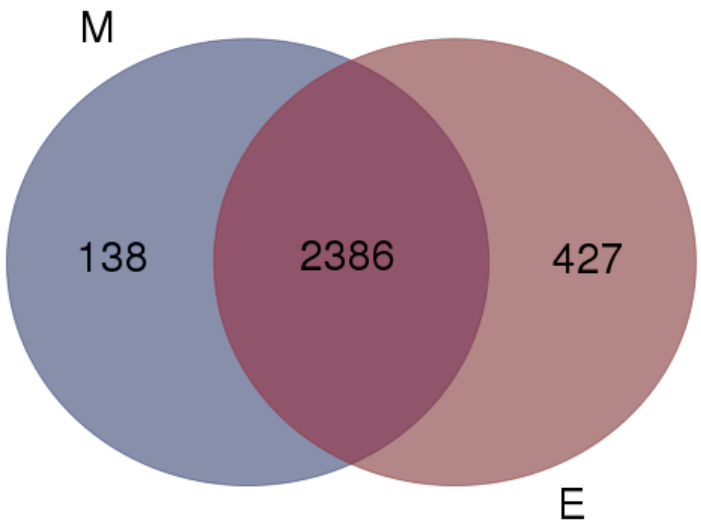



**Figure 1.2.1: Venn diagram illustrating the overlap of protein identification between the two treatments (M and E) of the proteome. 2386 proteins were found to be common to both treatments. The proteome dataset was filtered so that each protein identified had to be present in 2 out of the 4 replicates (with at least two unique peptides to identify it in each replicate) within a treatment.**

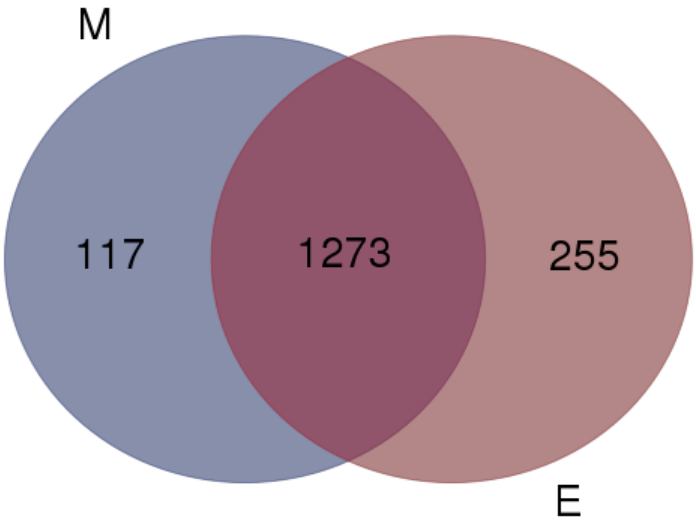



**Figure 1.2.2: Venn diagram illustrating the overlap of protein identification between the two treatments (M and E) of the proteome. 1273 proteins were found to be common to both treatments. The proteome dataset was filtered so that each protein identified had to be present in 4 out of the 4 replicates (with at least two unique peptides to identify it in each replicate) within a treatment.**

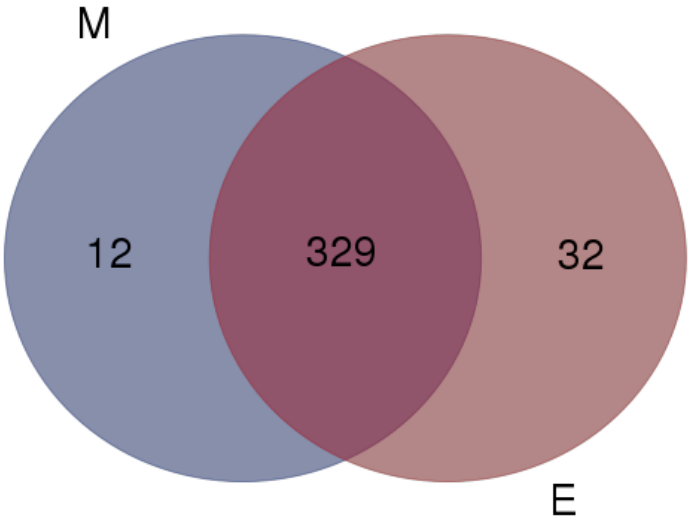



**Figure 1.2.3: Venn diagram illustrating the overlap of protein identification between the two treatments, M and E of the secretome. 329 proteins were found to be common to both treatments. The secretome dataset was filtered so that each protein identified had to be present in 2 out of the 4 replicates (with at least two unique peptides to identify it) within a treatment.**

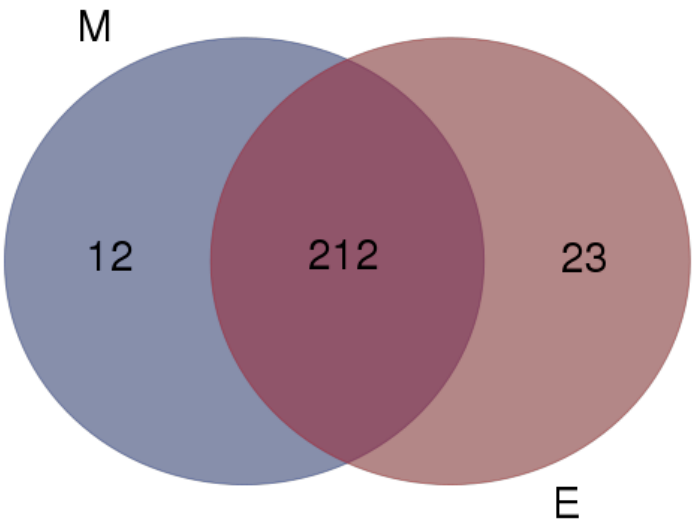



**Figure 1.2.4: Venn diagram illustrating the overlap of protein identification between the two treatments, M and E of the secretome. 212 proteins were found to be common to both treatments. The secretome dataset was filtered so that each protein identified had to be present in 4 out of the 4 replicates (with at least two unique peptides to identify it) within a treatment.**

**Table 1.2.1: Protein identifications for the secretome that are "unique" to M and E, based on each protein being identified by at least two unique peptides in 3 out of the 4 replicates (data from Figure 8 in the main text of Chapter 3).**

| | Flybase Id M Unique | Flybase Id E Unique |
|---|---|---|
| **1** | FBgn0077610 | FBgn0078170 |
| **2** | FBgn0074745 | FBgn0080919 |
| **3** | FBgn0073695 | FBgn0249459 |
| **4** | FBgn0077784 | FBgn0071146 |
| **5** | FBgn0248233 | FBgn0250574 |
| **6** | FBgn0074998 | FBgn0081035 |
| **7** | FBgn0247364 | FBgn0075256 |
| **8** | FBgn0073529 | FBgn0074211 |
| **9** | FBgn0076349 | FBgn0074893 |
| **10** | FBgn0245904 | FBgn0262049 |
| **11** | FBgn0075669 | FBgn0075128 |
| **12** | - | FBgn0248203 |
| **13** | - | FBgn0247400 |
| **14** | - | FBgn0071522 |
| **15** | - | FBgn0074345 |
| **16** | - | FBgn0075363 |

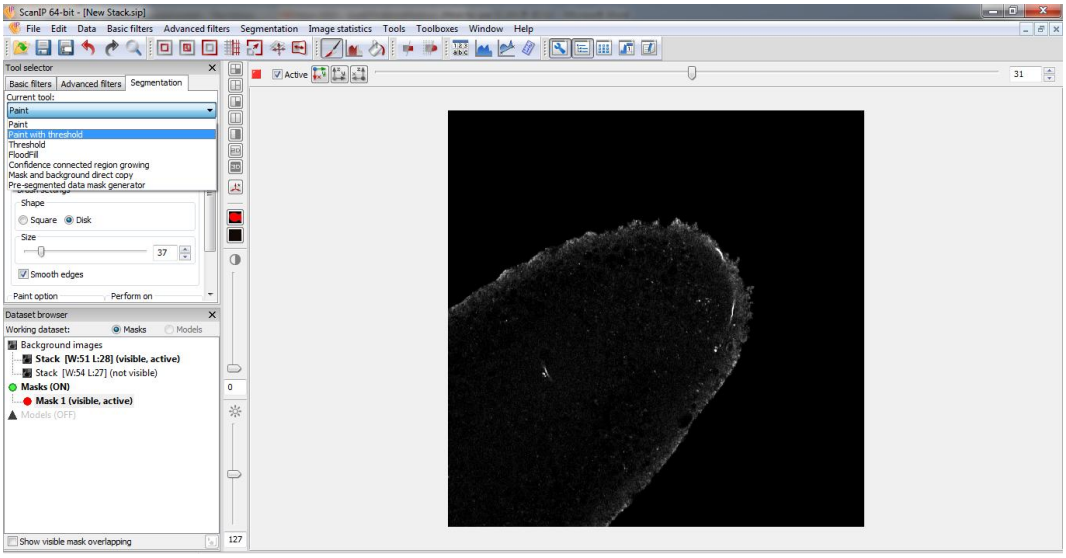
**Table 1.2.2: Flybase identifiers for proteins that are annotated as enriched in the "female receptivity, post mating" GO category.**

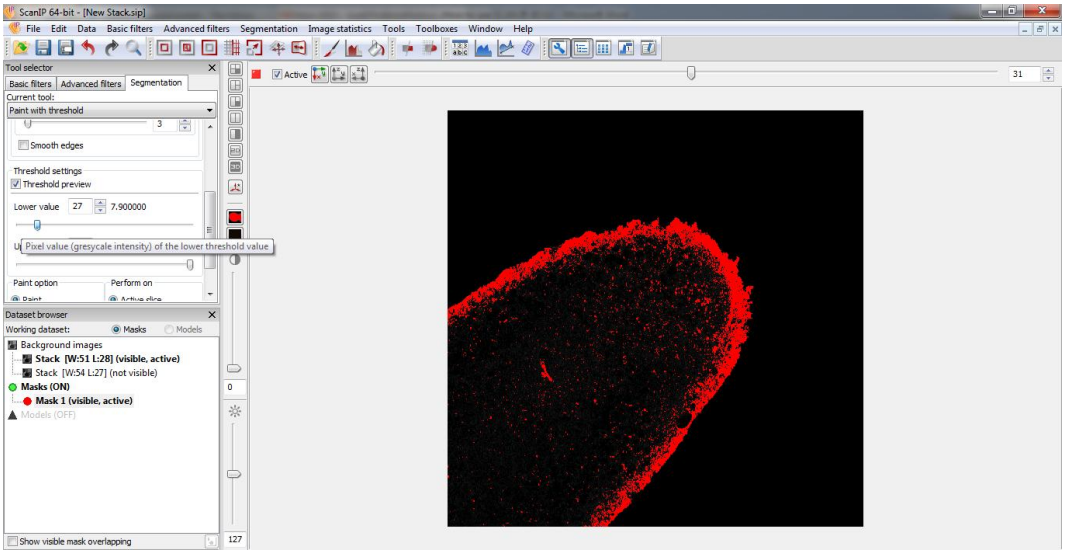
| Flybase Id enriched for female receptivity, post mating | | | |
|---|---|---|---|
| **1** | FBgn0070474 | **5** | FBgn0082155 |
| **2** | FBgn0071743 | **6** | FBgn0245599 |
| **3** | FBgn0074106 | **7** | FBgn0245732 |
| **4** | FBgn0074591 | **8** | FBgn0248361 |

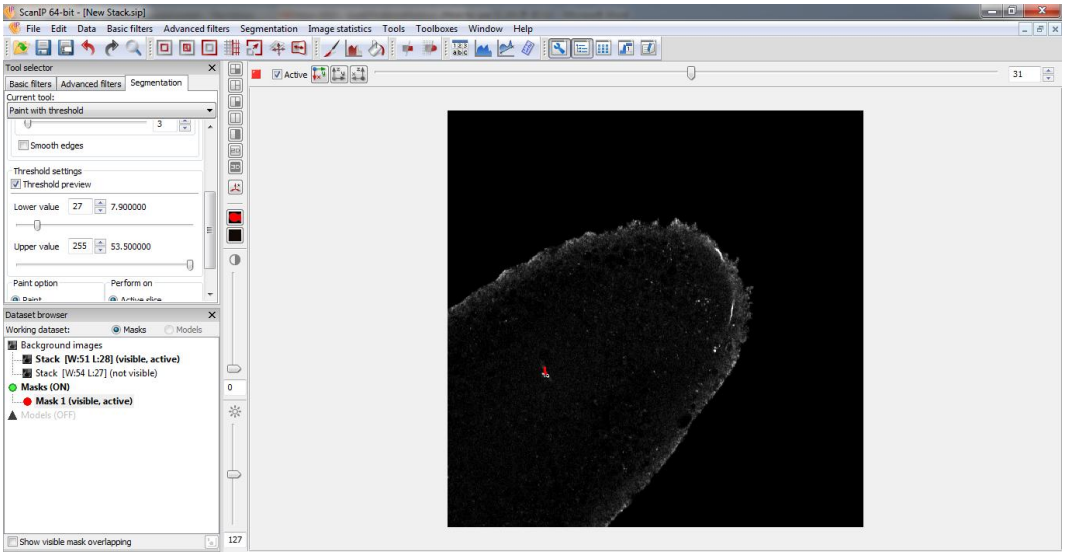
## Appendix 2: Chapter 4

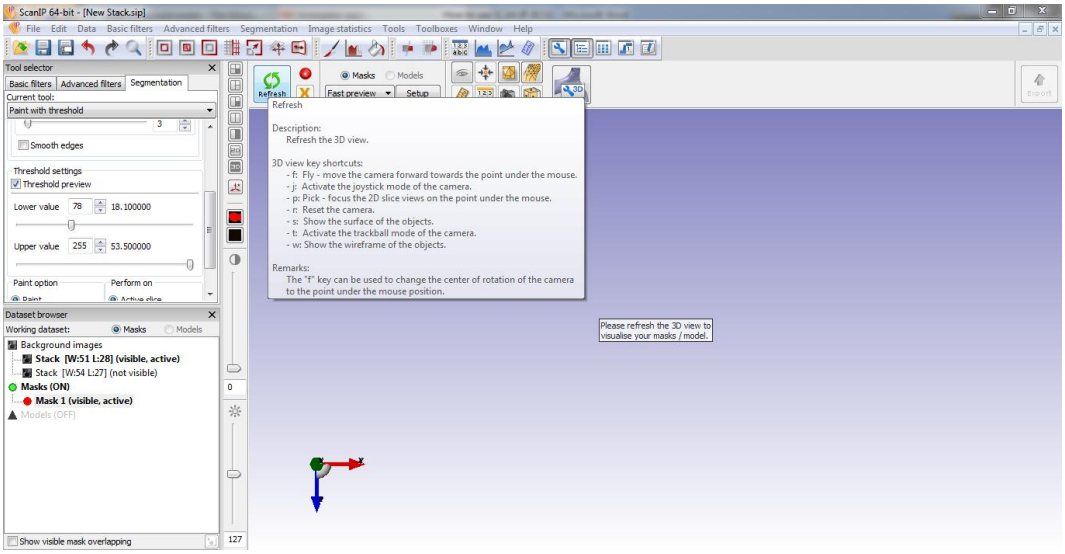
### Appendix 2.1 Materials and Methods

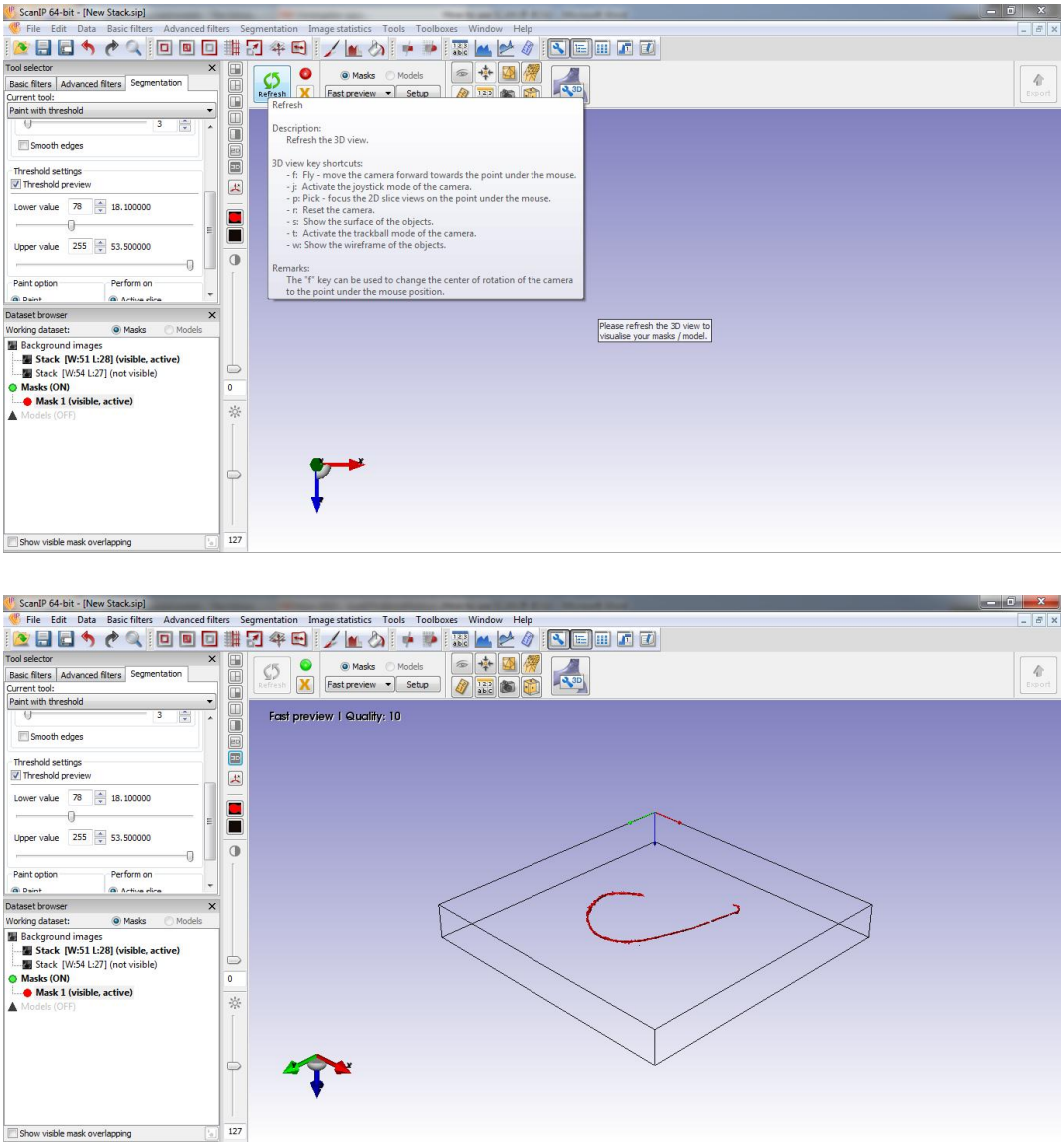
**The following screenshots demonstrate how I created masks to construct a 3D model of the sperm shape within the egg.** The entire sperm tail and all pronuclei/nuclei and polar bodies (where present) were “masked” using the paint with threshold function and reconstructed into a 3D model in ScanIP.




ScanIP 64-bit - [New Stack.sip]
File Edit Data Basic filters Advanced filters Segmentation Image statistics Tools Toolboxes Window Help
Tool selector
Basic filters Advanced filters Segmentation
Current tool:
Paint with threshold
Smooth edges
Threshold settings
Threshold preview
Lower value 27 7.900000
Upper value 255 53.500000
Paint option
Perform on
Dataset browser
Working dataset:
Masks Models
Background images
Stack [W:51 L:28] (visible, active)
Stack [W:54 L:27] (not visible)
Masks (ON)
Mask 1 (visible, active)
Models (OFF)
Show visible mask overlapping
Active
31
0
127


ScanIP 64-bit - [New Stack.sip]
File Edit Data Basic filters Advanced filters Segmentation Image statistics Tools Toolboxes Window Help
Refresh
Masks Models
Fast preview
Setup
Export
Refresh
Description:
Refresh the 3D view.
3D view key shortcuts:
- f: Fly - move the camera forward towards the point under the mouse.
- j: Activate the joystick mode of the camera.
- p: Pick - focus the 2D slice views on the point under the mouse.
- r: Reset the camera.
- s: Show the surface of the objects.
- t: Activate the trackball mode of the camera.
- w: Show the wireframe of the objects.
Remarks:
The "f" key can be used to change the center of rotation of the camera to the point under the mouse position.
Please refresh the 3D view to visualise your masks / model.
Tool selector
Basic filters Advanced filters Segmentation
Current tool:
Paint with threshold
Smooth edges
Threshold settings
Threshold preview
Lower value 78 18.100000
Upper value 255 53.500000
Paint option
Perform on
Dataset browser
Working dataset:
Masks Models
Background images
Stack [W:51 L:28] (visible, active)
Stack [W:54 L:27] (not visible)
Masks (ON)
Mask 1 (visible, active)
Models (OFF)
Show visible mask overlapping
0
127




**Appendix Figure 2.1: The workflow for paint with threshold in ScanIP. The mask shown in the screenshots above show how to paint the sperm tail using ScanIP.**

**The following pages show screenshots of the Wolfram Mathematica script to quantify sperm shape parameters, i.e. arc length, curvature and aspect ratio. Courtesy of Professor Mitchell Berger.**

```
SetDirectory[U:\spermwork[]];
```

# 0. Setup

## Load Packages

The tuba package draws tubes around curves so that they can be viewed more easily. It has more options than the intrinsic mathematica Tube function - but that can also be used if Tuba does not load. The writhe calculator package calculates writhe for one of the statistical tests.

```
SetDirectory[NotebookDirectory[]];
Needs["Tuba`"];
SetOptions[tube, nalongtube → 100, naroundtube → 6];
SetOptions[Graphics3D, PlotRange → All];
```

```
M A Berger, U Exeter, April 2009. Comments to m.berger@exeter.ac.uk
```

```
<< "WritheCalculator.m";
```

```
Writhe Calculator written by Prof M A Berger and Chris Prior, UCL Mathematics,
  University of London. Last complete revision December 2007 to fit version 6.
Please send comments and suggestions to m.berger@exeter.ac.uk.
  writhe[curve] calculates open or closed writhe of a curve.
The curve should be defined on interval [0,1]. Default resolution:
  1000 points on the curve. If writhe[curve] does not work,
  try alternatives which use slightly different algorithms:
  closedwrithe[curve] assumes curve is closed. loopwrithe[curve]
  assumes endpoints are at same height z.
```

## Curve Parameterization by Arclength

- Vector Utilities
- normalize, unitvector
  Purpose : = creates unit vectors.
  input : = single vector or two vectors.
  Output : = single vector.

```
unitvector[x1_, x2_] := normalize[x2 - x1];
```

- arclength
  Purpose : = find total arclength of the curve.
  input : = curve as a list of np points in 3D.
  Output : = A number giving total arclength.
- arcfinder
  Purpose : = append arclength information to the curve.

input : = curve as a list of np points in 3D.
Output : = A list of {x,y,z,s} values, where s is the arclength and x,y,z the position on the curve at that arclength (with s(first point)=0). This list is of form {{x values},{y values},{z values},{s values}}.

- arclengthparametrization
  Purpose : = create an arclength parametrization of the curve.
  input : = curve as a list of np points in 3D.
  Output : =A new list of points on the same curve, but with equal distance between points.

- zparametrization
  Purpose : = create a parametrization of the curve according to one coordinate (the third coordinate).
  input : = curve as a list of np points in 3D.
  Output : =A new list of points on the same curve, but with equal z distance between points.

## Curve finding

- getcurve
  Purpose : = import curve data from a file.
  input : = name of file. Also, nignore gives number of points to ignore (e.g. polar bodies).
  Output : =list of points on the same curve in an n by 3 array where n is the total number of points.

- tailcurve: same, but assuming nignore=3.

- tailandpro: same, but assuming the file ends with "_curvePoints.txt".

```
getcurve[file_, nignore_] := Module[{ell, data}, data = Import["file", "CSV"];
   ell = data[[1, 1]] + 1 - nignore;
   Table[{data[[i, 2]], data[[i, 3]], data[[i, 4]]}, {i, 2, ell}]];
```

```
tailandpro[name_, nignore_] :=
  Module[{ell, data}, data = Import[name <> "_curvePoints.txt", "CSV"];
   ell = data[[1, 1]] + 1 - nignore;
   Table[{data[[i, 2]], data[[i, 3]], data[[i, 4]]}, {i, 2, ell}]];
tailcurve[name_] := Module[{ell, data, nignore = 3}, data = Import[name, "CSV"];
   ell = data[[1, 1]] + 1 - nignore;
   Table[{data[[i, 2]], data[[i, 3]], data[[i, 4]]}, {i, 2, ell}]];
```

- curveplusvector: adds a constant vector v to each point on a curve c.

- fixedcurve, standingcurve
  Purpose : = rotate a curve so that beginning and end points are directly over each other (useful for z parametrization).
  input : = curve.
  Output : =curve of same shape which moves more or less vertically, with maximum wandering aligned to x direction.

```
standingcurve[curve_] :=
  Module[{startpoint = curve // First, endpoint, cl, c2, m, ell = Length[curve],
    cofs, rr, maxr, maxii, m2, c3}, cl = curveplusvector[curve, -startpoint];
   endpoint = cl[-3]; m = RotationMatrix[{endpoint, {0, 0, 1}}];
   c2 = Table[m.cl[i], {i, ell}];
   (* Now rotate so maximum wandering is in x direction *)
   rr = Table[Norm[{c2[i, 1], c2[i, 2]}], {i, ell}]; maxr = Max[rr];
   maxii = Ordering[rr, -1][1];
   m2 = RotationMatrix[{c2[maxii], {maxr, 0, c2[maxii, 3]}}];
   Table[m2.c2[i], {i, ell}]];

fixedcurve[curve_] :=
  Module[{cc, cofs}, cc = standingcurve[curve]; cofs = arcparametrization[cc[1 ;; -3]];
   AppendTo[cofs, cc[-2]]; AppendTo[cofs, cc[-1]]; cofs];
```

- blow
  Purpose : = Some of the curves are almost two dimensional. This procedure widens the curve in the flattest (y) direction, enhancing the 3D structure.
  input : = curve.
  Output : =curve with same number of wiggles, but more movement in the y direction.

- Curve drawing
  Purpose : = Draw the curve
  input : = curve, npoints along curve (default 300).
  Output : =picture of curve with small spheres at end and nuclei.

```
showcurve[c_, nalong_] :=
  Module[{endpt = c[-3], t, d = Max[Abs[c[3, All] - (c[3, 1])]], sph, fnucleus,
    tuberadius = 0.004, nucleusradius = 0.008},
   sph = Sphere[endpt, nucleusradius]; fnucleus = Sphere[c[-1], 1.5 nucleusradius];
   t = tube[c[1 ;; -3], {EdgeForm[], Cyan}, tuberadius, nalongtube → nalong];
   Show[t, Graphics3D[{Yellow, sph, Pink, fnucleus}]]];
showcurve[c_] := showcurve[c, 300];
```

## Statistics

- getaverages, getsd
  Purpose : = obtain average values of each statistic for one of the fly types
  input : = data for particular sperm type
  Output : = average or Standard Deviation.

```
getaverages[type_, col_] := Mean[type[[All, col]]];
getsd[type_, col_] := StandardDeviation[type[[All, col]]];
```

- stats, statstables, allstats
  Purpose : = combine all of the type averages and type S.D.s
  input : = column tells you which diagnostic is being analysed.
  Output : = all the averages and S.D.s for that diagnostic.

## Diagnostics

- tailwrithe
  Purpose : = obtain writhe of a curve

```
tailwrithe[c_] := writhe[c[[1 ;; -2]]];
```

- Simpsons
  Purpose : = integrate a function along a curve using Simpson's rule

```
Simpsons[v_] := Module[{n = Length[v], simpson}, simpson = v[[1]] + v[[-1]];
   simpson += 4 Sum[v[[i]], {i, 2, n - 1, 2}]; simpson += 2 Sum[v[[i]], {i, 3, n - 2, 2}];
   simpson / 3];
```

- getfourier routines
  Purpose : = get first few Fourier modes of a curve. getFourierrho uses the cylindrical radius of a curve as a scalar function (cylindrical radius is distance away from axis, where the axis is a line segment from the startpoint to the end point). getfourrierxx and getfourrieryy just use the x or y coordinates as scalar functions.

```
getfourierrho[c_] :=
  Module[{rho, fff, n = Length[c]}, rho = Table[Norm[{c[[i, 1]], c[[i, 2]]}], {i, n}];
    fff = Abs[Fourier[rho, FourierParameters → {-1, 1}]]^2; fff[[1 ;; 4]];
getfourierxx[c_] := Module[{xx, fff, n = Length[c]}, xx = c[[All, 1]];
    fff = Abs[Fourier[xx, FourierParameters → {-1, 1}]]^2; fff[[1 ;; 4]];
getfourieryy[c_] := Module[{yy, fff, n = Length[c]}, yy = c[[All, 2]];
    fff = Abs[Fourier[yy, FourierParameters → {-1, 1}]]^2; fff[[1 ;; 4]];
```

- diagnostics
  Purpose : = combine all of the curve diagnostics
  input : = first, a name which will be printed along with the diagnostics, second, a curve
  Output : = all the diagnostics for a curve, e.g. {name},{"arc length","aspect ratio","curvature", "torsion","writhe", "Fourier0", "Fourier1", "Fourier2", "Fourier3"}

```
diagnostics[name_, cc_] := Module[{c, ttwrithe, ints, arc, aspect, curvature, torsion, a,
    nn = Length[cc] - 2, curvdata, torsiondata, fourier, ds, i}, c = cc[[1 ;; -3]];
  ttwrithe = tailwrithe[c // blow];
  arc = arclength[c];
  aspect = arc / Norm[Last[c] - First[c]];
  ints = intrinsics[c // blow, nalongtube → Length[c]];
  ds = Table[(Norm[c[[i]] - c[[i - 1]]] + Norm[c[[i + 1]] - c[[i]]]) / 2, {i, 2, nn - 1}];
  PrependTo[ds, Norm[c[[2]] - c[[1]]]]; AppendTo[ds, Norm[c[[nn]] - c[[nn - 1]]]];
  curvdata = ints[[1]] * ds; torsiondata = ints[[2]] * ds;
  curvature = Simpsons[curvdata] / arc;
  torsion = Simpsons[torsiondata] / arc;
  fourier = 2 * (getfourierxx[c] + getfourieryy[c]);
  a = Grid[{{name}, {"arc length", "aspect ratio", "curvature", "torsion", "writhe",
      "Fourier0", "Fourier1", "Fourier2", "Fourier3"}, {arc, aspect, curvature,
      torsion, ttwrithe, fourier[[1]], fourier[[2]], fourier[[3]], fourier[[4]]}}, Frame → All];
  Print[a]; {name, arc, aspect, curvature, torsion, ttwrithe,
   fourier[[1]], fourier[[2]], fourier[[3]], fourier[[4]]}
 ];
```

- analysis
  Purpose : = combine all of the curve diagnostics
  input : = set of names of files
  Output : = Table of all the diagnostics for a curve, e.g. {name},{"arc length","aspect ratio","curvature", "torsion","writhe", "Fourier0", "Fourier1", "Fourier2", "Fourier3"}

```
analysis[names_] :=
  Table[diagnostics[names[[i]], fixedcurve[tailcurve[names[[i]]]]], {i, Length[names]}];
analysis[names_, files_] :=
  Table[diagnostics[names[[i]], fixedcurve[tailcurve[files[[i]]]]], {i, Length[names]}];
getname[fff_, a_, n_] := StringTake[fff, {a, n}];
```

## 2. Curve Drawing Example

```
Directory[]
```

```
D:\Exeter\Perseus\Sperm\Feb 2014\ALL TOGETHER
```

```
curveBogA = tailcurve["Bog\\CURVE POINTS\\BogA-shrink_curvePoints.txt"];
```

```
curveBogA
```

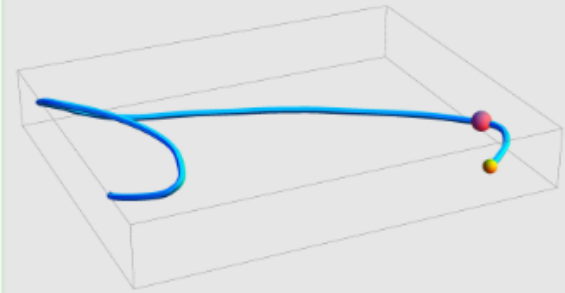
```
{{0.214964, 0.506018, 0.0395091}, {0.217623, 0.499961, 0.0398831},
 {0.231115, 0.476269, 0.0480385}, {0.250652, 0.44913, 0.0598478},
 {0.262474, 0.440263, 0.0685439}, {0.274462, 0.443923, 0.0784231},
 {0.282769, 0.480538, 0.0881538}, {0.2765, 0.5175, 0.09225}, {0.2598, 0.5538, 0.0954},
 {0.24169, 0.584793, 0.0961034}, {0.225703, 0.609703, 0.0952162},
 {0.206231, 0.636169, 0.0937692}, {0.194952, 0.663333, 0.0882381},
 {0.194041, 0.695575, 0.0743151}, {0.197945, 0.708011, 0.0672637},
 {0.229144, 0.723054, 0.0488559}, {0.241929, 0.720619, 0.042619},
 {0.275697, 0.705879, 0.034697}, {0.308095, 0.688524, 0.0338571},
 {0.3429, 0.6734, 0.0364}, {0.378872, 0.658489, 0.038617},
 {0.415978, 0.643044, 0.0394889}, {0.454095, 0.627095, 0.0409048},
 {0.48875, 0.611375, 0.0413125}, {0.509063, 0.60125, 0.0414375},
 {0.542577, 0.581385, 0.0415}, {0.576116, 0.558721, 0.042814},
 {0.605143, 0.534762, 0.0450476}, {0.626067, 0.513444, 0.0474889},
 {0.647522, 0.483087, 0.0513478}, {0.660308, 0.449538, 0.0584615},
 {0.657091, 0.418636, 0.0641818}, {0.641581, 0.389323, 0.069129},
 {0.625308, 0.369077, 0.0706923}, {0.598733, 0.351133, 0.065}, {0.5958, 0.3498, 0.065},
 {0.583772, 0.343904, 0.060982}, {0.653448, 0.460497, 0.0588276}}
```

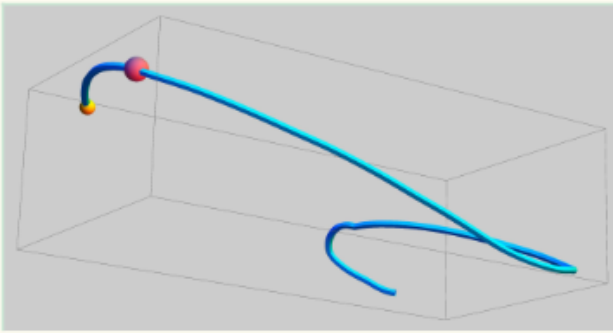
```
standingcurveBogA = fixedcurve[curveBogA];
diagnostics["BogA", standingcurveBogA]
```

| BogA | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| arc length | aspect ratio | curvature | torsion | writhe | Fourier0 | Fourier1 | Fourier2 | Fourier3 |
| 1.10192 | 2.67184 | 9.13888 | -6.74668 | -0.1806-42 | 0.03766-85 | 0.00788-497 | 0.00072-7434 | 0.00026-9146 |

```
{BogA, 1.10192, 2.67184, 9.13888, -6.74668,
 -0.180642, 0.0376685, 0.00788497, 0.000727434, 0.000269146}
```

```
showcurve[curveBogA]
showcurve[standingcurveBogA]
```





## 3. Analysis of Data

```
bogfiles = FileNames["*.txt", {"Bog"}, 3]
bognames = Map[getname[#, 18, 21] &, bogfiles]
```

```
{Bog\CURVE POINTS\BogA-shrink_curvePoints.txt,
 Bog\CURVE POINTS\BogB-shrink_curvePoints.txt,
 Bog\CURVE POINTS\BogC-shrink_curvePoints.txt,
 Bog\CURVE POINTS\BogD-shrink_curvePoints.txt,
 Bog\CURVE POINTS\BogE-shrink_curvePoints.txt,
 Bog\CURVE POINTS\BogF-shrink_curvePoints.txt,
 Bog\CURVE POINTS\BogG-shrink_curvePoints.txt,
 Bog\CURVE POINTS\BogH-shrink_curvePoints.txt,
 Bog\CURVE POINTS\BogI-shrink_curvePoints.txt,
 Bog\CURVE POINTS\BogJ-shrink_curvePoints.txt}
```

```
{BogA, BogB, BogC, BogD, BogE, BogF, BogG, BogH, BogI, BogJ}
```

```
bogpseudofiles = FileNames["*.txt", {"BogPseudo"}, 3];
bogpseudonames = Map[getname[#, 24, 33] &, bogpseudofiles]
```

```
perfiles = FileNames["*.txt", {"Per"}, 3];
pernames = Map[getname[#, 18, 21] &, perfiles]
```

```
pseudofiles = FileNames["*.txt", {"Pseudo"}, 3];
pseudonames = Map[getname[#, 21, 27] &, pseudofiles]
```

```
perpseudofiles = FileNames["*.txt", {"PerPseudo"}, 3]
perpseudonames = Map[getname[#, 24, 33] &, perpseudofiles]
```

```
pseudobogfiles = FileNames["*.txt", {"PseudoBog"}, 3];
pseudobognames = Map[getname[#, 24, 33] &, pseudobogfiles]
```

```
pseudoperfiles = FileNames["*.txt", {"PseudoPer"}, 3];
pseudopernames = Map[getname[#, 24, 33] &, pseudoperfiles]
```

```
bog = analysis[bognames, bogfiles]
```

```
bogpseudo = analysis[bogpseudonames, bogpseudofiles]
```

```
per = analysis[pernames, perfiles]
```

```
pseudo = analysis[pseudonames, pseudofiles]
```

```
perpseudonames
```

```
perpseudo = analysis[perpseudonames[{2, 3, 4, 5, 6, 7, 8, 10}],
  perpseudofiles[[{2, 3, 4, 5, 6, 7, 8, 10}]]
```

```
pseudobog = analysis[pseudobognames, pseudobogfiles]
```

```
pseudoper = analysis[pseudopernames, pseudoperfiles]
```

```
allofit = Join[bog, bogpseudo, per, perpseudo, pseudo, pseudobog, pseudoper]
```

```
Export["spermtails.xls", allofit]
```

```
spermtails.xls
```

```
allstats
```

```
allstats
```

```
goo = statstables[4];
goo
```

```
statstables[4]
```

## Stats

```
statstables[2]
```

| Arc Length | bog | bogpseudo | per | perpseudo | pseudo | pseudobog | pseudoper |
|---|---|---|---|---|---|---|---|
| Mean | 0.828 | 0.934 | 0.922 | 0.922 | 0.828 | 0.867 | 0.941 |
| Standard Deviation | 0.204 | 0.0816 | 0.0857 | 0.121 | 0.0864 | 0.0681 | 0.0541 |

```
statstables[3]
```

| Aspect Ratio | bog | bogpseudo | per | perpseudo | pseudobog | pseudoper | |
|---|---|---|---|---|---|---|---|
| Mean | 2.31 | 3.41 | 2.28 | 2.8 | 2.91 | 2.84 | 2.71 |
| Standard Deviation | 1.11 | 1.95 | 0.814 | 1.23 | 1.4 | 1.58 | 1.22 |

```
statstables[4, 3]
```

| Curvature | bog | persimilis | pseudo | pseudobog |
|---|---|---|---|---|
| Mean | 8.82 | 9.48 | 14.8 | 11.3 |
| Standard Deviation | 2.01 | 3.55 | 10.8 | 3.9 |

```
statstables[5, 3]
```

| Torsion | bog | persimilis | pseudo | pseudobog |
|---|---|---|---|---|
| Mean | -0.0718 | -0.975 | 9.22 | 0.997 |
| Standard Deviation | 2.99 | 12.9 | 20.2 | 4.32 |

```
statstables[6, 3]
```

| Writhe | bog | persimilis | pseudo | pseudobog |
|---|---|---|---|---|
| Mean | -0.188 | -0.147 | -0.0992 | 0.0205 |
| Standard Deviation | 0.509 | 0.39 | 0.335 | 0.219 |

```
statstables[7, 3]
```

| Fourier 0 | bog | persimilis | pseudo | pseudobog |
|---|---|---|---|---|
| Mean | 0.0423 | 0.0426 | 0.0228 | 0.0639 |
| Standard Deviation | 0.0282 | 0.0342 | 0.026 | 0.0347 |

```
statstables[8, 3]
```

| Fourier 1 | bog | persimilis | pseudo | pseudobog |
|---|---|---|---|---|
| Mean | 0.0116 | 0.0136 | 0.00418 | 0.0127 |
| Standard Deviation | 0.00611 | 0.00718 | 0.00427 | 0.00377 |

```
statstables[9, 2]
```

| Fourier 2 | bog | persimilis | pseudo | pseudobog |
|---|---|---|---|---|
| Mean | 0.001 | 0.0015 | 0.0004 | 0.0013 |
| Standard Deviation | 0.0013 | 0.0014 | 0.00039 | 0.001 |

```
statstables[10, 2]
```

| Fourier 3 | bog | persimilis | pseudo | pseudobog |
|---|---|---|---|---|
| Mean | 0.00041 | 0.00072 | 0.00015 | 0.00056 |
| Standard Deviation | 0.00044 | 0.00073 | 0.00019 | 0.00051 |

```
Export["spermtails.xls", allofit]
```

```
spermtails.xls
```

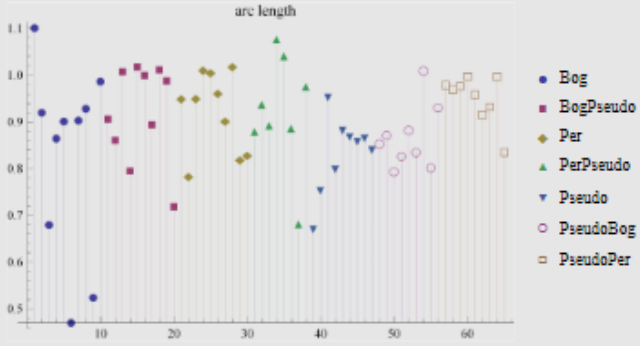
```
addhorizontal[q_, start_] := Module[{n = Length[q]}, Table[{start + i, q[[i]]}, {i, n}]];
```

```
names = {"arc length", "aspect ratio", "curvature",
   "torsion", "writhe", "Fourier0", "Fourier1", "Fourier2", "Fourier3"};
showdata[col_] := Module[{
    bogt = (bog // Transpose)[[col]],
    bogpseudot = (bogpseudo // Transpose)[[col]],
    pert = (per // Transpose)[[col]],
    perpseudot = (perpseudo // Transpose)[[col]],
    pseudot = (pseudo // Transpose)[[col]],
    pseudobogt = (pseudobog // Transpose)[[col]],
    pseudopert = (pseudoper // Transpose)[[col]], a, b, c, d, e, f, q, plotdat},
   a = Length[bogt]; b = Length[bogpseudot]; c = Length[pert]; d = Length[perpseudot];
   q = a + b + c + d; e = Length[pseudot]; f = Length[pseudobogt];
   plotdat = {addhorizontal[bogt, 0], addhorizontal[bogpseudot, a] , addhorizontal[
      pert, a + b], addhorizontal[perpseudot, a + b + c], addhorizontal[pseudot, q],
     addhorizontal[pseudobogt, q + e], addhorizontal[pseudopert, q + e + f] };
   ListPlot[plotdat, Filling → Axis, PlotLabel → names[[col - 1]] ,
    PlotLegends → {"Bog", "BogPseudo", "Per", "PerPseudo",
      "Pseudo", "PseudoBog", "PseudoPer"}, PlotMarkers → Automatic]];
```

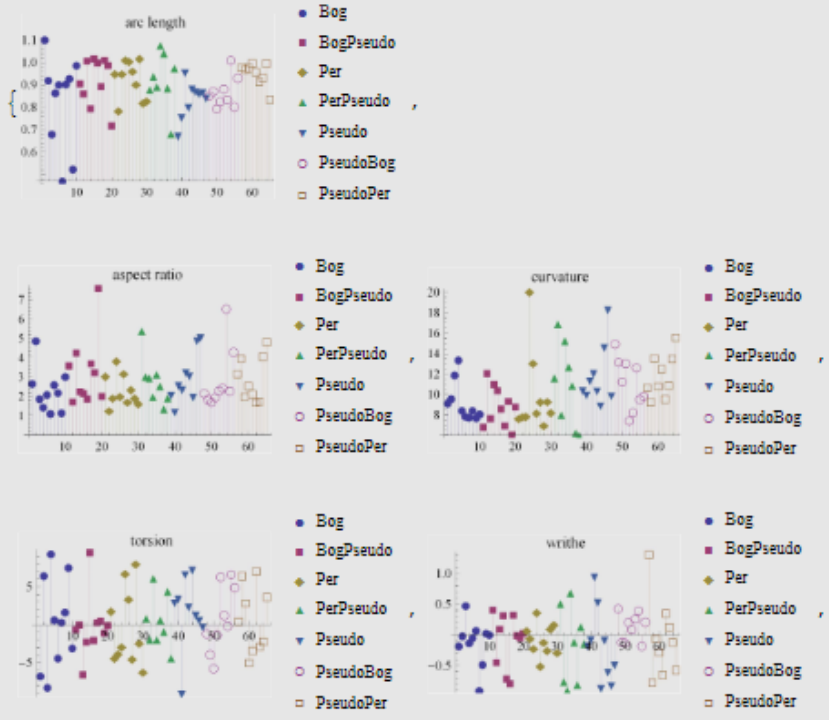
```
per
```

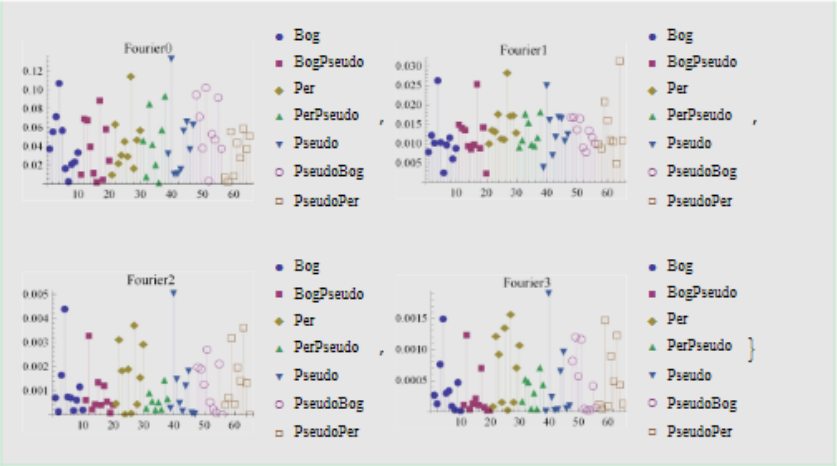
```
{{PerA, 0.949127, 3.04809, 7.65074, 1.77019, 0.0675136, 0.010041,
  0.010051, 0.000476437, 0.0000861604}, {PerB, 0.783206, 1.2527, 7.80977,
  -4.39794, -0.0589185, 0.0637004, 0.0135378, 0.00313054, 0.00121538},
 {PerC, 0.949579, 1.91446, 7.84743, -3.85955, -0.222458, 0.0221203,
  0.0131891, 0.00184342, 0.000926484}, {PerD, 1.01066, 3.82902, 20.046,
  -2.94105, 0.364099, 0.0309385, 0.0176494, 0.0000410775, 0.000155296},
 {PerE, 1.00523, 2.00062, 13.0419, 6.70439, -0.509511, 0.0455672, 0.0113127,
  0.00190456, 0.0013518}, {PerF, 0.960691, 3.1764, 8.1774, 3.34485,
  -0.121207, 0.0297082, 0.0111444, 0.0000790907, 0.0000276694},
 {PerG, 0.90168, 1.71132, 9.28253, -4.51575, -0.256452, 0.114663,
  0.0282898, 0.00372462, 0.00156817}, {PerH, 1.01787, 2.3474, 6.95782,
  7.97535, 0.0957914, 0.0169123, 0.0171837, 0.000451306, 0.000153039},
 {PerI, 0.81875, 1.90803, 9.30062, -2.42115, 0.165874, 0.0470619,
  0.0173088, 0.00156127, 0.000710729}, {PerJ, 0.828011, 1.62142, 8.21613,
  -6.2452, -0.288608, 0.057288, 0.0128363, 0.00293992, 0.00107144}}
```

```
showdata[2]
```




```
Table[showdata[i], {i, 2, 10}]
```




Fourier0
Fourier1
Fourier2
Fourier3
Bog
BogPseudo
Per
PerPseudo
Pseudo
PseudoBog
PseudoPer

## Appendix 2.2 Results

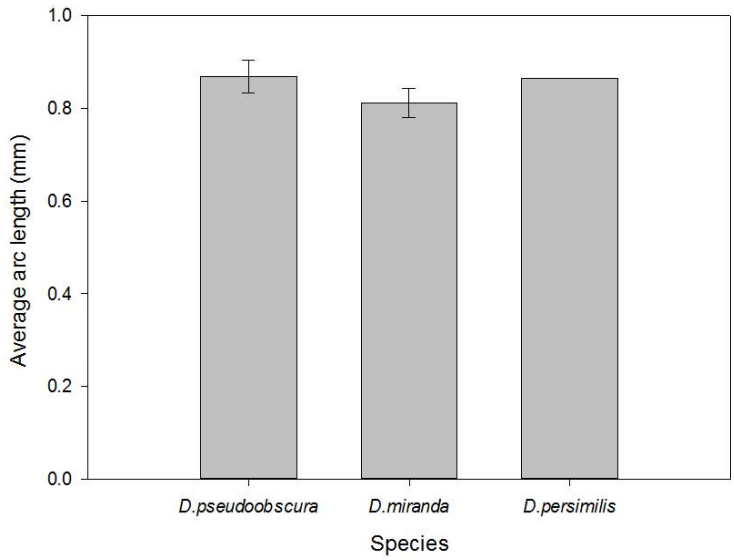



**Figure 2.2.1: Average (±SE) arc length for the conspecific crosses during the 2n stage of development.**

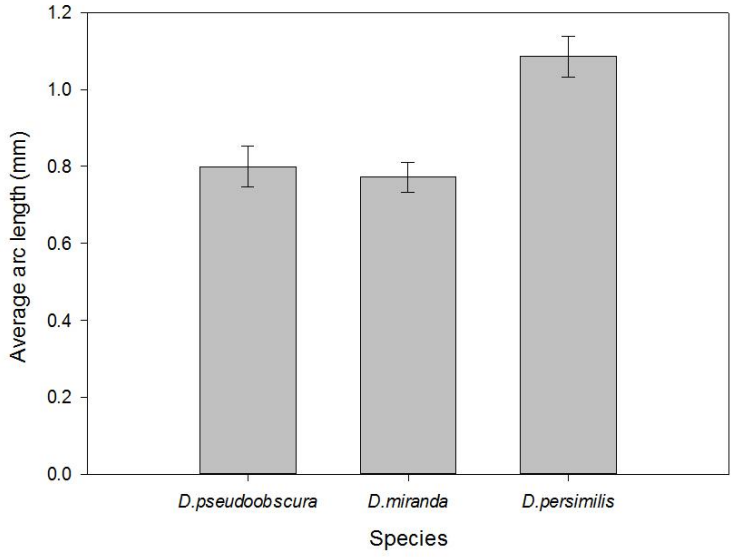



**Figure 2.2.2: Average (±SE) arc length for the conspecific crosses during the 4n stage of development.**

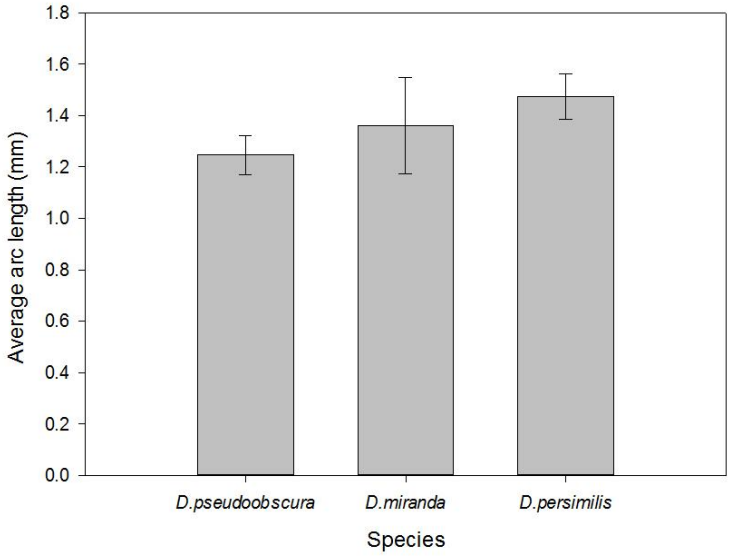



**Figure 2.2.3: Average (±SE) arc length for the conspecific crosses during the 8n stage of development.**

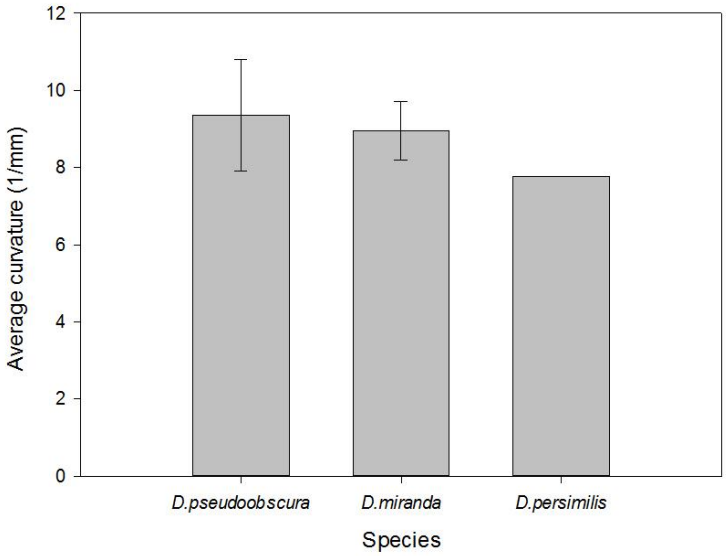



**Figure 2.2.4: Average (±SE) curvature for the conspecific crosses during the 2n stage of development.**

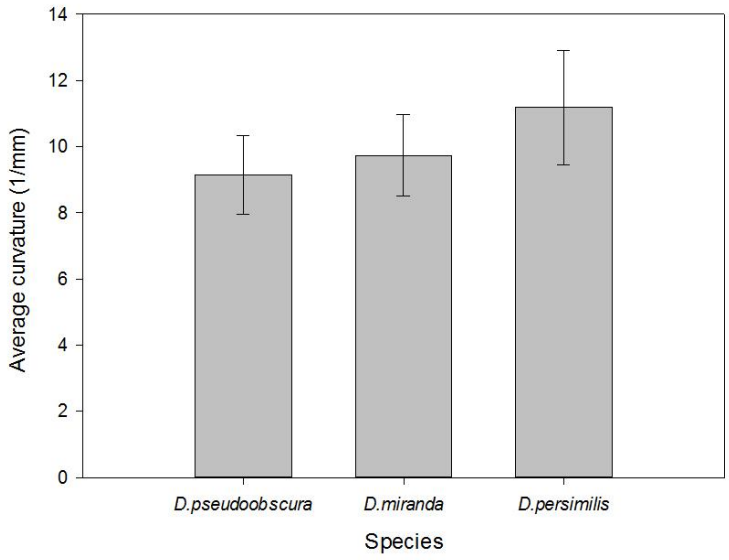



**Figure 2.2.5: Average (±SE) curvature for the conspecific crosses during the 4n stage of development.**

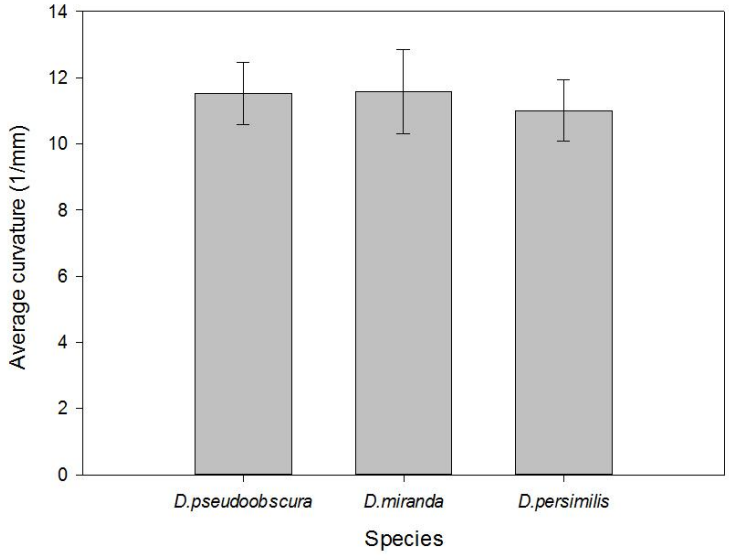



**Figure 2.2.6: Average (±SE) curvature for the conspecific crosses during the 8n stage of development.**

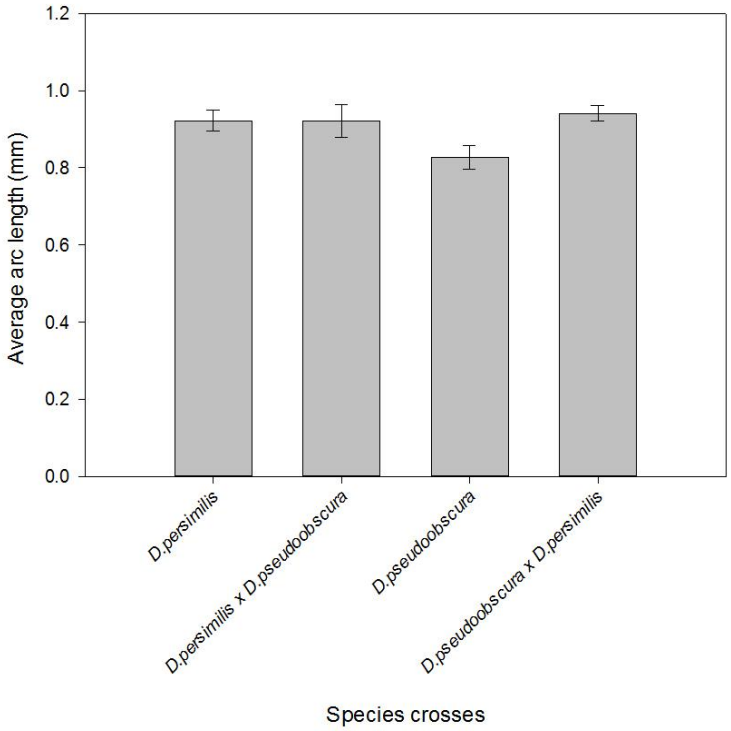



**Figure 2.2.7: Average (±SE) arc length for the *D. persimilis* and *D. pseudoobscura* conspecific compared to the hybrid crosses during the pronuclear (PN) stage.**

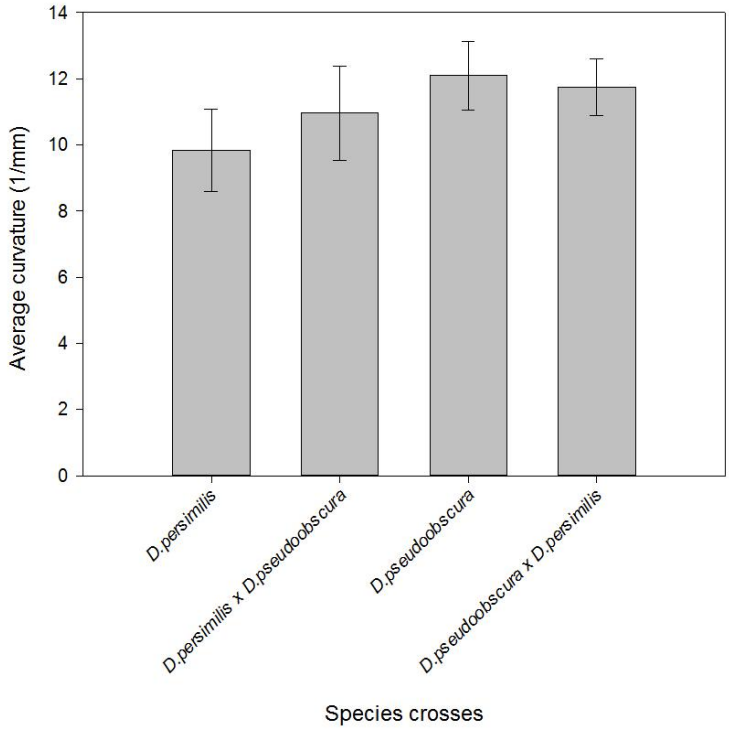



**Figure 2.2.8: Average (±SE) curvature for the *D. persimilis* and *D. pseudoobscura* conspecific compared to the hybrid crosses during the PN stage.**

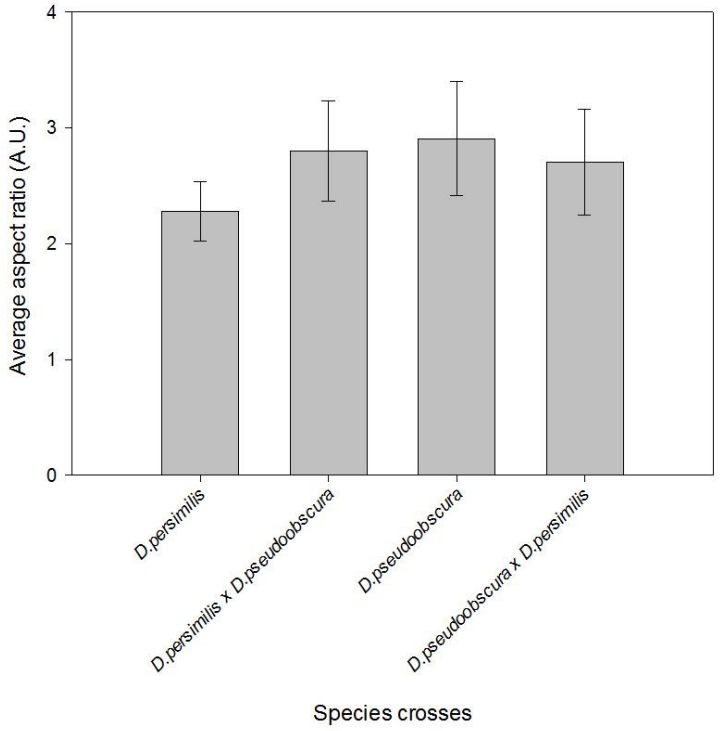



**Figure 2.2.9: Average (±SE) aspect ratio for the *D. persimilis* and *D. pseudoobscura* conspecific compared to the hybrid crosses during the PN stage.**

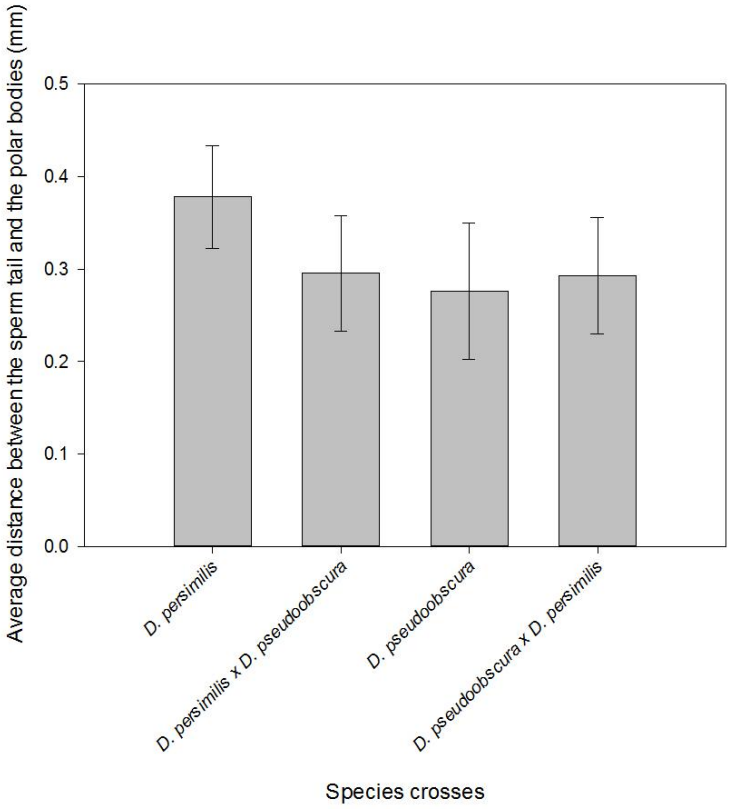



**Figure 2.2.10: Average (±SE) distance between points along the sperm tail and the polar bodies for the *D. persimilis* and *D. pseudoobscura* conspecific compared to the hybrid crosses during the PN stage.**

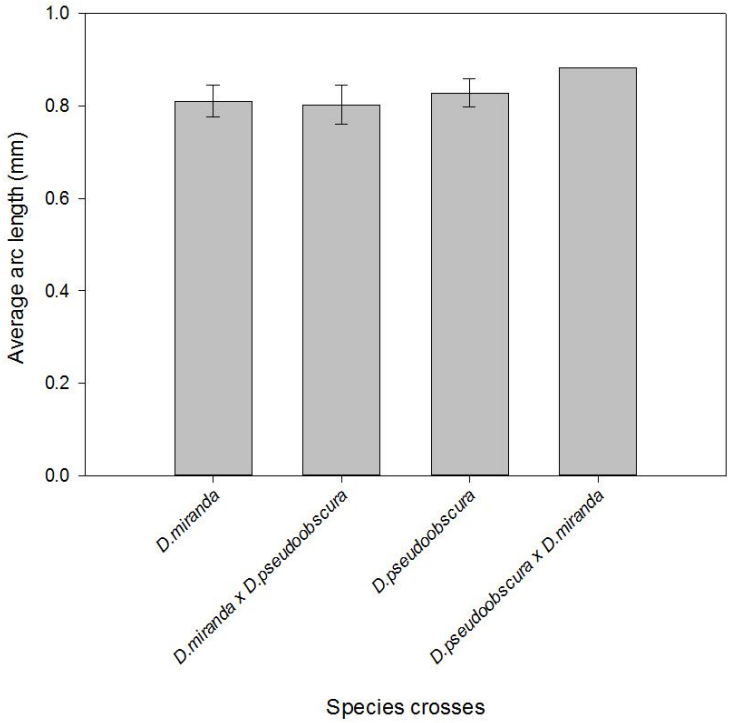



**Figure 2.2.11: Average (±SE) arc length for the *D. miranda* and *D. pseudoobscura* conspecific compared to the hybrid crosses during the PN stage.**

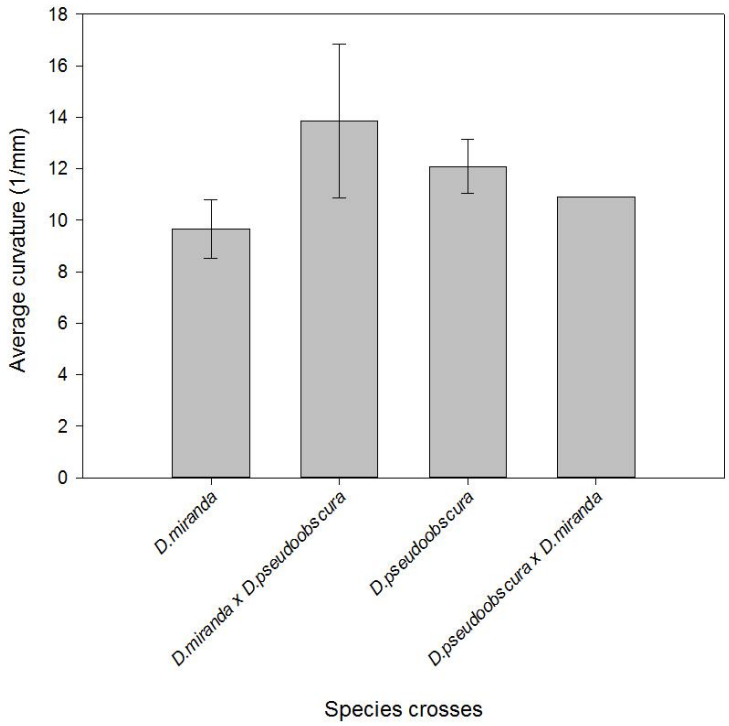



**Figure 2.2.12: Average (±SE) curvature for the *D. miranda* and *D. pseudoobscura* conspecific compared to the hybrid crosses during the PN stage.**

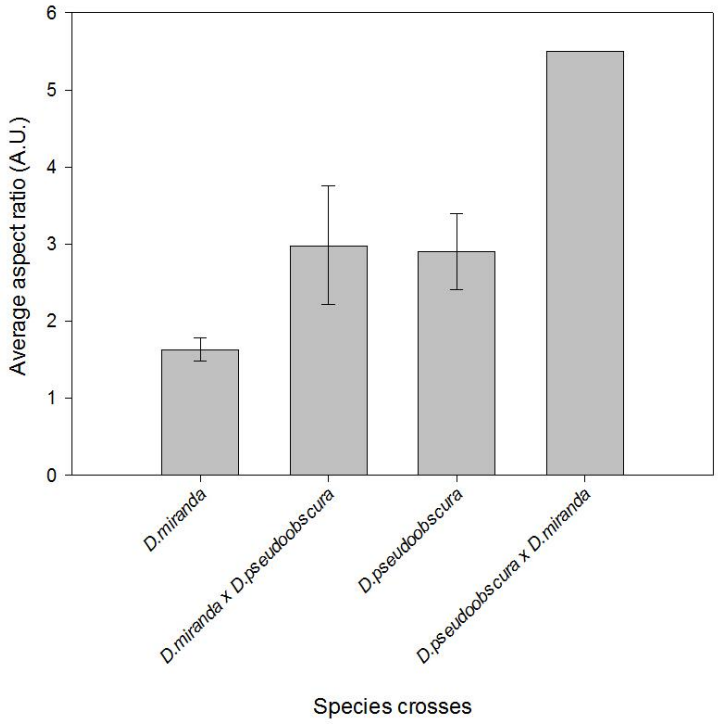



**Figure 2.2.13: Average (±SE) aspect ratio for the *D. miranda* and *D. pseudoobscura* conspecific compared to the hybrid crosses during the PN stage.**

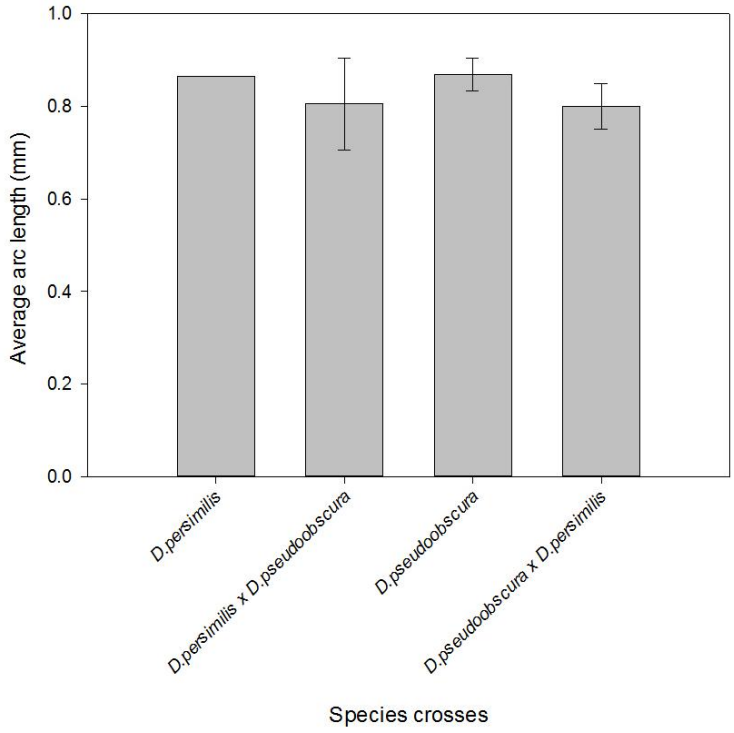



**Figure 2.2.14: Average (±SE) arc length for the *D. persimilis* and *D. pseudoobscura* conspecific compared to the hybrid crosses during the 2n stage.**

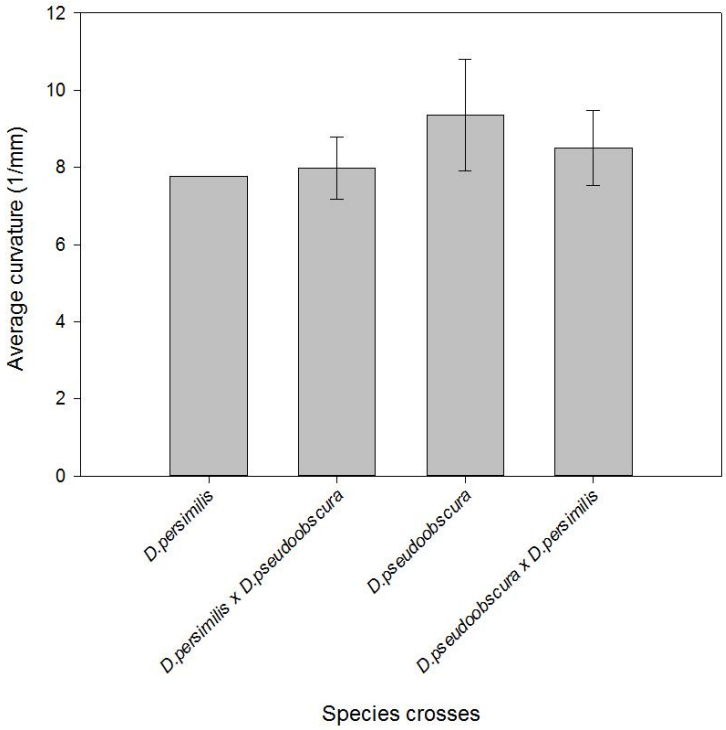



**Figure 2.2.15: Average (±SE) curvature for the *D. persimilis* and *D. pseudoobscura* conspecific compared to the hybrid crosses during the 2n stage.**

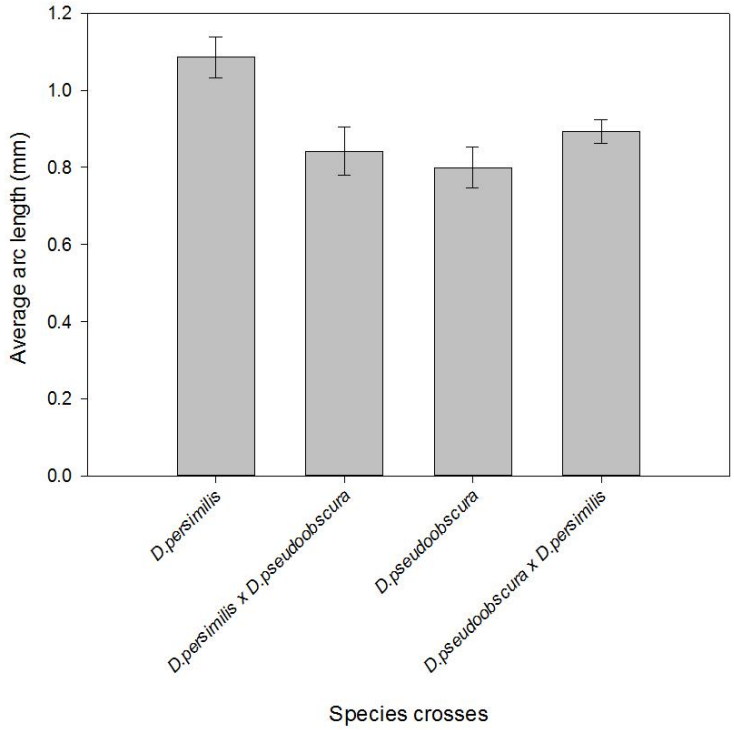



**Figure 2.2.16: Average (±SE) arc length for the *D. persimilis* and *D. pseudoobscura* conspecific compared to the hybrid crosses during the 4n stage.**

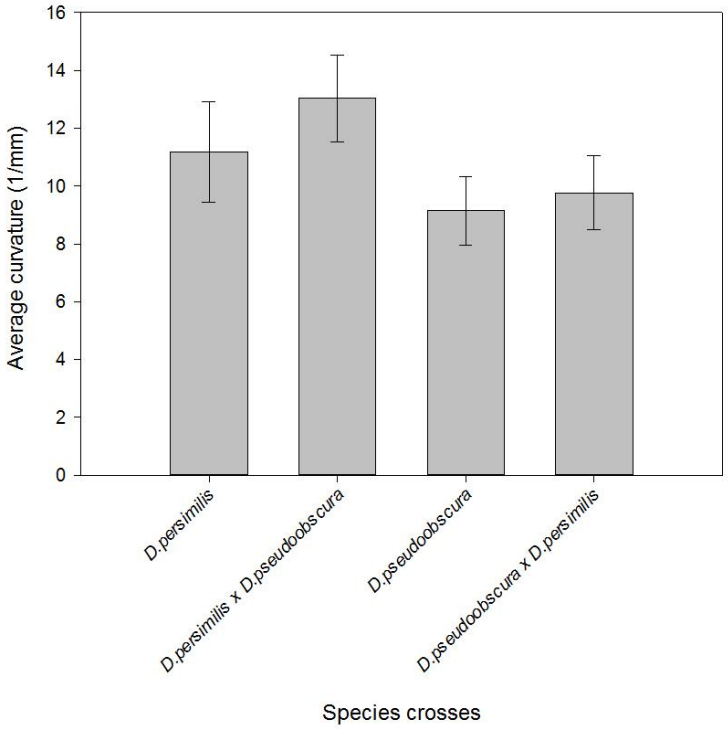



**Figure 2.2.17: Average (±SE) curvature for the *D. persimilis* and *D. pseudoobscura* conspecific compared to the hybrid crosses during the 4n stage.**

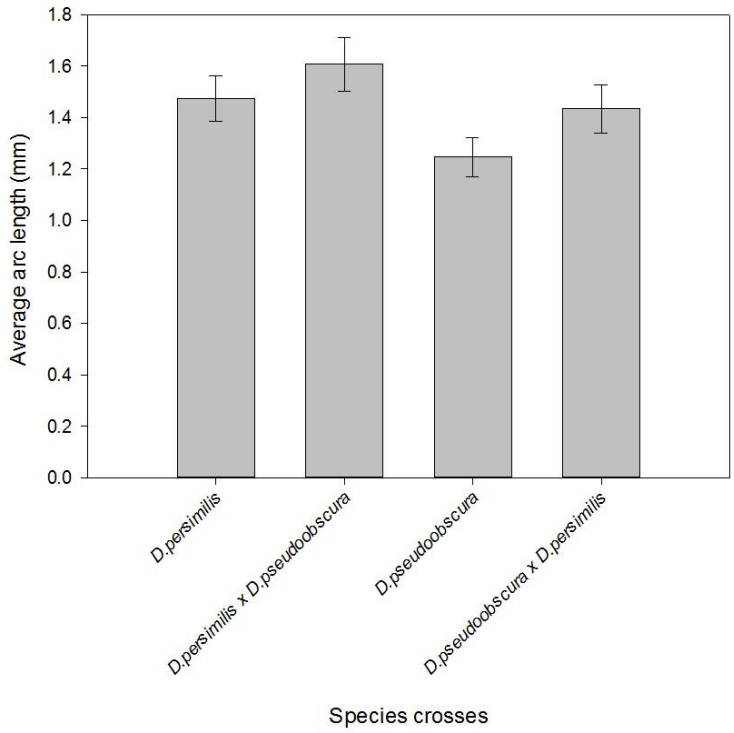



**Figure 2.2.18: Average (±SE) arc length for the *D. persimilis* and *D. pseudoobscura* conspecific compared to the hybrid crosses during the 8n stage.**

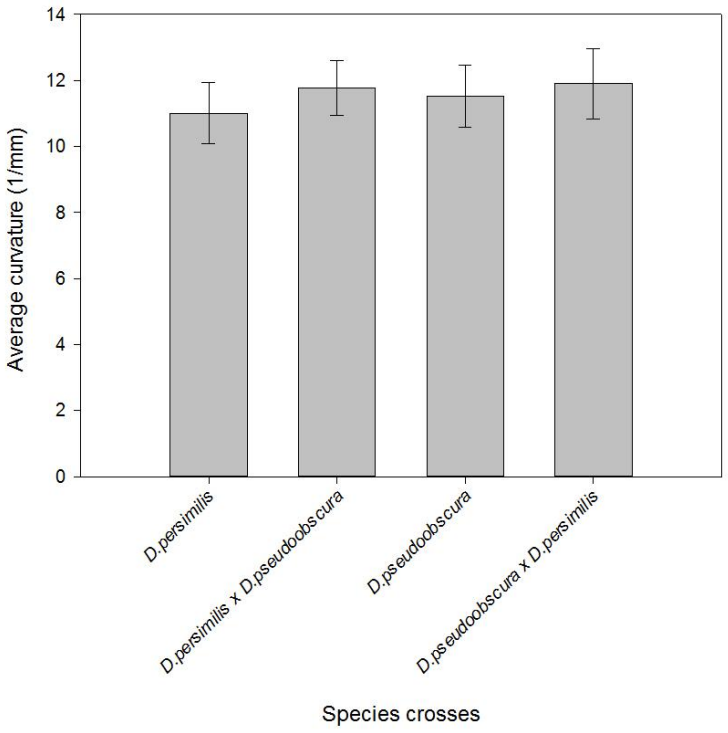



**Figure 2.2.19: Average (±SE) curvature for the *D. persimilis* and *D. pseudoobscura* conspecific compared to the hybrid crosses during the 8n stage.**

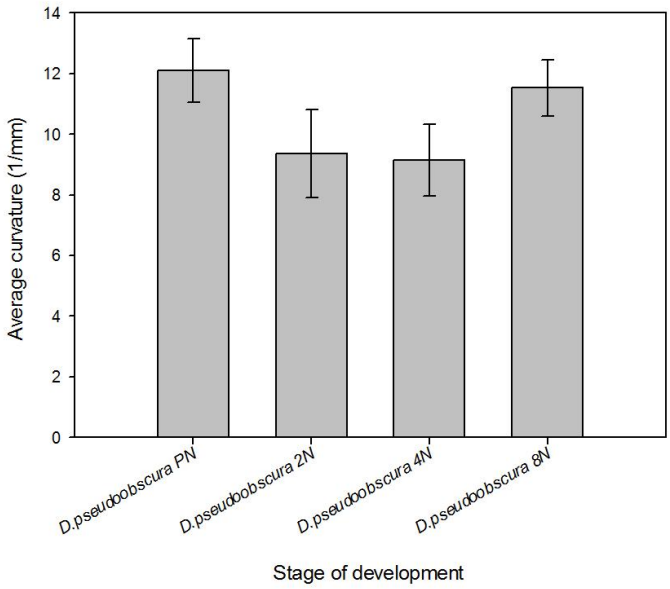



**Figure 2.2.20: Average curvature for *D. pseudoobscura* from the PN stage to the 8n stage of development to identify changes in sperm tail shape within a species during development.**

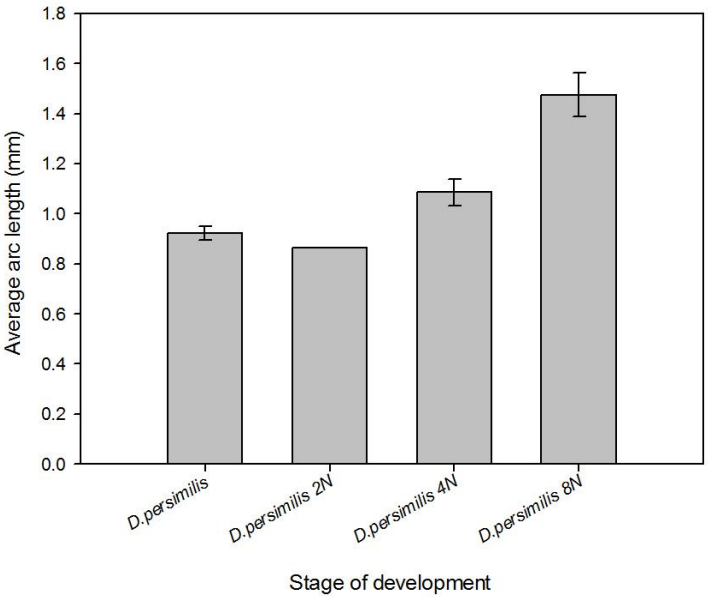



**Figure 2.2.21: Average arc length for *D. persimilis* from the PN stage to the 8n stage of development to identify changes in sperm tail shape within a species during development.**

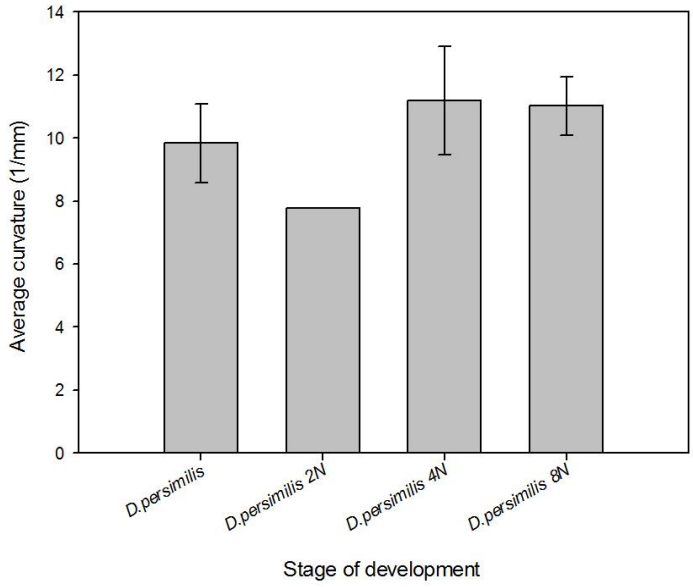



**Figure 2.2.22: Average curvature for *D. persimilis* from the PN stage to the 8n stage of development to identify changes in sperm tail shape within a species during development.**

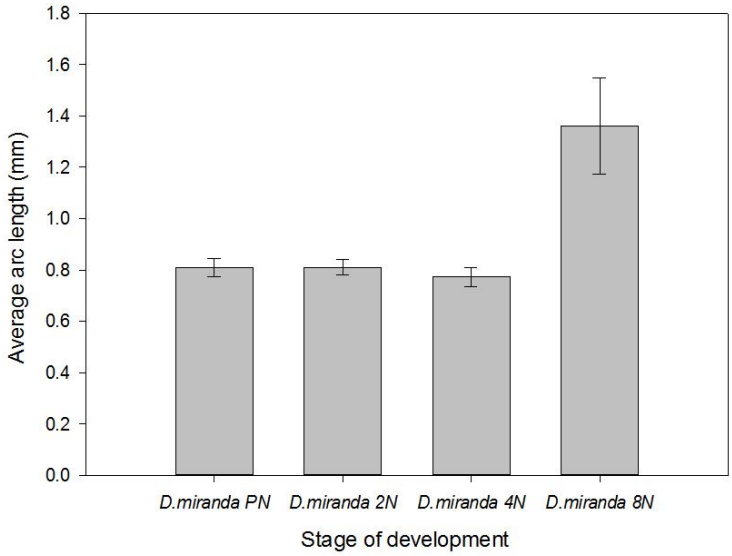



**Figure 2.2.23: Average arc length for *D. miranda* from the PN stage to the 8n stage of development to identify changes in sperm tail shape within a species during development.**

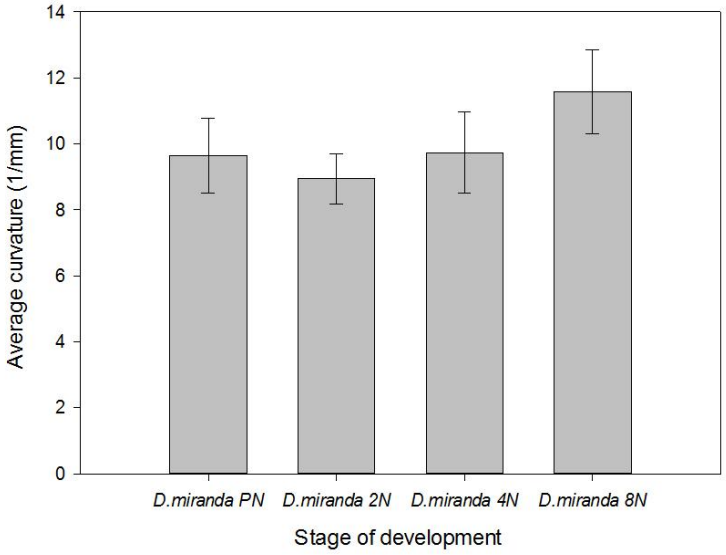



**Figure 2.2.24: Average curvature for *D. miranda* from the PN stage to the 8n stage of development to identify changes in sperm tail shape within a species during development.**