Olfaction and nutrition in reproduction in the fruit fly *Drosophila melanogaster*

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Abstract

The aim of this thesis is to investigate the role of olfaction in reproduction in the male fruit fly *Drosophila melanogaster*. The ability to recognise different odours is of fundamental importance. For example, animals and insects use odours to find food, potential mates, and to detect rivals of the same species. Odorants are used to perceive both internal and external cues concerning the prevailing reproductive environment.

Odorant receptors and odorant binding proteins can be expressed in the head and in the reproductive systems of both males and females. Many odorant genes occur in clusters in the genome. I surveyed the distribution of expression sites of these clustered odorant genes using the FlyAtlas expression database. Examples where there have been changes in expression patterns of duplicated genes within clusters indicate possible changes in function. I tested whether this might have consequences for the rate of change of these genes by conducting BLAST searches to test for matches of odorant genes across *D. melanogaster* and its close relatives. The data provided preliminary evidence that odorant receptors and odorant binding proteins expressed in the head may be more conserved than those expressed in reproductive tissue, especially the male accessory glands.

The presence of odorant receptors and odorant binding proteins expressed in the reproductive system could be indicative of a novel role for odorants in determining fertility, perhaps via chemotaxis between the sperm and the egg. I found that both the males lacking an internal odorant receptor *83b* and those with the external odorant receptor (antenna) removed showed slower courtship than the male wild-type *Drosophila*. However, both types of male recognised rivals and the absence of *Or83b* had no effect on fecundity. Hence there was no obvious role for internal expressed odorants in determining male fertility other than through effects on mating latency.

An important part of the ability to produce and respond to chemical cues is the amount of resources available to males to allocate towards reproduction. Hence male mating success and a male's ability to respond to odorants and other cues that indicate the social environment is expected to be dependent upon a male's nutritional status. Previous data suggest that male nutrition alters mating ability and furthermore that there is an optimal level of nutrition to maximise reproductive success. My results showed that the nutritional condition of the male did not affect his ability to respond to rivals. Strikingly, even at starvation levels of nutrition males still responded to rivals by extending mating duration. I showed that normal mating latency is dependent upon high levels of sugar in the diet. In contrast, high levels of yeast are necessary for males to mate at a high frequency. Both sugar and yeast are also required in the diet to achieve maximum offspring output. Adult male nutrition therefore plays an important part in determining male reproductive success, and different dietary components have effects on different components of a male's reproductive success.

Overall my thesis work highlights the evolutionary ability of odorants, their role in determining a male's reproductive success and the contribution of dietary components to determining male fertility.

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Declaration

I declare that the work in this thesis is my own except where duly noted

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Janet Mason

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Chapter 1 General Introduction

1.1] Olfaction

Olfaction, the sense of smell, plays an important role in a multitude of behaviours across many species of invertebrates and mammals. The ability to recognise odours enables an animal to react to its environment and respond appropriately. Food odours are followed so the animal can feed, and odours can also alert an animal to a potential mate or indicate the presence of a rival. An animal's behaviour can be influenced by the odours it experiences, its sense of smell can help it avoid substances that are harmful and help to differentiate between friend or foe (Anholt & Mackay 2001; Touhara & Vosshall 2009). Specialized odours are produced for a number of purposes for example for territorial demarcation and defence (Stoddart 1981). For many animals odorants are the most efficient means of interpreting their surroundings and communicating with others. Innate behaviour in response to smell is essential for survival and may result from an unconscious perception of odours. Olfaction is also involved in mate choice and maternal bonding, factors vital to reproductive success (Brennam 2004). It has been suggested that the sense of smell and sexual reproduction may often co-evolve (Stoddart 1981).

Odorants are the chemicals that activate the olfactory system, generally at very low concentrations. Aroma compounds occur in food, wine, spices etc and they can form from biochemical changes during the ripening of fruits and other foodstuffs. Pheromones are odours that are used for communication. A female moth can release a pheromone that will attract a male from several miles away (Vogt & Riddiford 1981; Roelofs et al. 2002). Queen honey bees use pheromones to regulate activity within the hive, influencing both behaviour and physiology in their nest companions, the worker bees (Beggs et al. 2007). It has been discovered that Wilson's storm petrels (*Oceanites oceanicus*) from Antarctica prefer the odours of their own nest and mate. Jouventin et al. (2007) suggests that the ability of storm petrels to recognise their mate through olfaction could be widespread in burrowing petrels and may help olfactory nest recognition (Jouventin et al. 2007).

In the fruit fly *Drosophila melanogaster*, olfactory information received by olfactory sensory neurons (OSNs) is transmitted to the central nervous system (CNS) where this information is processed, leading to behavioural and physiological responses (Clyne et al. 1999). It is thought that the antennae may detect volatile pheromones (Stocker 1994) and that the male maxillary palp may help to detect the receptivity status of a mated female (Stocker & Gendre 1989). The proboscis probably plays a chemosensory role during licking by males of females during courtship, and female cuticular hydrocarbons are also detected by gustatory receptors on the male tarsi, particularly the foretarsi on the two front legs (Venard & Jallon 1980; Cobb & Ferveur 1996).

An analysis by Feveur et al. (1997) found two aspects of individual sexual identity in *Drosophila melanogaster*: the perception of others and the presentation of self to others. These aspects of recognition are believed to be under separate genetic and anatomical control. It is suggested that there is unlikely to be a simple mechanism underlying these processes due to the complex nature of sexual identity, even in relatively simple organisms such as *Drosophila* (Ferveur et al. 1997).

Mating begins with a courtship ritual of the male. Courting males sample pheromones of the target female via olfactory sensilla on the antennae and maxillary palps and gustatory sensilla on the foretarsi and proboscis (Stocker & Gendre 1989; Siwicki et al. 2005). The male follows a prospective female mate tapping her with his forelegs, which allows him to detect non-volatile pheromones on the female abdomen. The male then extends and vibrates his wing to produce a species specific courtship song. If the female slows down she is receptive and the male will try to copulate (Greenspan & Ferveur 2000; Amrein & Thorne 2005; Billeter et al. 2006).

1.2] The evolution of odorant genes

The mechanisms of discrimination used by *Drosophila* for detecting odours have been conserved from at least the separation of insects and mammals that occurred about 500 million years ago (Gaillard et al. 2004). Odorants are used in the perception of both the external and internal reproductive environment. Both males and female insects exude and respond to pheromones used in aggregation and in mate attraction (Chintapalli et al. 2007; Altschul et al. 1990; Hekmat-Scafe et al. 2002). It has taken nearly a decade since the characterization of the first odorant receptor (OR) genes in mammals to identify the olfactory receptor genes of *Drosophila*. A total of 57-60 OR *Drosophila* genes have been identified. In comparison to rodents, *Drosophila* detect fewer odorants, this could be because insects have a highly developed visual system or a very specialized OR repertoire. Most OR genes are expressed in the antennae of *Drosophila*; only 7 of the total 57 or 60 are expressed in the maxillary palp, and very few are expressed in both of these organs. The finding that OR genes are not expressed in other tissues such as the proboscis suggests that *Drosophila* ORs are dedicated to the perception of volatile odorants, rather than those perceived by direct contact (Gaillard et al. 2004).

Odorant binding proteins (Obps) are small proteins which are present at high levels in the fluid surrounding the OR neurons (Pelosi 1994). To date approximately 50 potential Obp genes have been discovered in the genome of *D. melanogaster* (Vogt et al. 1999). Their function is believed to be to convey odorants to the ORs (Xu et al. 2005; Pelosi 1994; Prestwich et al. 1995). Considerable divergence and variation in numbers has been found in Obps between different insect groups suggesting they may be important in species recognition (Hekmat-Scafe et al. 2002).

Recent bioinformatic analysis has shown that various ORs and Obps occur in gene clusters of which some members are restricted in expression to the head, while others are highly expressed in male and female reproductive organs (Chintapalli et al. 2007; Altschul et al. 1990; Hekmat-Scafe et al. 2002). This suggests the existence of duplication within gene families, which may have allowed new functions to develop in the reproductive system. The human odorant receptor hOR 17-4 is expressed in both the nose and the testes suggesting it may play a similar role in both organs (Vosshall 2004). However the role of odorants in the reproductive system is not yet known.

In chapter 3 of this thesis I addressed these issues, I catalogued the ORs and Obps which were expressed in the reproductive organs and examined in which other tissues they were also expressed. I examined whether there was evidence within families of odorant genes of new functions, as indicated by changes in expression patterns between different family members, coupled with evidence of higher rates of evolutionary change in odorant genes enriched for expression in the reproductive system.

1.3] Potential functions of internally expressed odorant genes

The presence of odorant receptors and binding proteins in the reproductive system could suggest a novel role for odorants in determining fertility, perhaps via chemotaxis between the sperm and the egg. In order to test this, functional information is sorely needed on the roles of odorant genes expressed in the reproductive system.

There is a growing body of evidence that suggests OR expression is not just restricted to the external tissues (e.g. the nose, vomeronasal organ or antennae). For example it is suggested that mammalian ORs could be important in sperm to egg chemical communication. OR transcripts have been found to be expressed in various tissues in mammals, for example in the brainstem, (Raming et al. 1998; Conzelmann et al. 2000) prostate and colon (Yuan et al. 2001). Parmentier et al. (1992) demonstrated the transcription of about 20 mammalian OR genes most notably during the late stages of spermatogenesis in cells of the male germ line (Parmentier et al. 1992). Zhang et al. confirmed using a high-throughput oligonucleotide microarray approach, the enriched expression of 66 OR genes in the mouse testis (Zhang et al. 2004). Branscomb et al. (2000) found that the percentage of intact sequences among testicular ORs and their average levels of amino acid conservation to be higher than in nasal receptors, which suggests a probable physiological function (Branscomb et al. 2000).

Pheromones and food odours can act together to attract flies to food (Bartelt et al. 1985). Pheromones can have a profound effect on the reproductive physiology and behaviour in mammals and odours elicit a variety of behavioural responses from *Drosophila* via a relatively simple, but sensitive, olfactory system (Carlson 1996). An individual odorant receptor is not dedicated to the recognition of a single odour but can be activated by a multitude of different chemical stimuli (Anholt & Mackay 2001). It has been found *Or83b* encodes a broadly expressed odorant receptor that is essential for *Drosophila* olfaction. Larsson et al (2004) showed by cellular, physiological and behavioural analysis that *Or83b* was essential for olfaction in *Drosophila. Or83b* is highly conserved across insect species and is expressed in large numbers of OSNs with different odour specificities. The onset of *Or83b* expression is late in both the larval and adult olfactory systems ruling out any developmental role for this protein (Larsson et al. 2004). An increasing number of mutants have been found to be defective in olfactory function (Carlson 1996), but the fact that this *Or83b* acts in concert with conventional ORs to respond to many different odours argues against an independent function for *Or83b* in recognising a particular odorant (Larsson et al. 2004).

The aim of the work described in chapter 4 of my thesis was to use males carrying a loss of function mutation for an odorant receptor (*Or83b*) with widespread expression to determine whether such males suffered lowered fertility. Such males were also tested for the ability to detect the presence of rivals.

1.4] Odorants, the detection of rivals and potential mates

It is thought that courtship behaviour in *D. melanogaster* is largely dependent on pheromones (Stocker & Gendre 1989). Stimulatory pheromones are volatile and can be detected by the fly before any physical contact, from a distance of less than a few centimetres. Behavioural tests on males show an increase in courtship towards each other when exposed to female pheromones (Tompkins et al. 1980; Venard & Jallon 1980).

Drosophila have two main olfactory organs the antennae and the maxillary palp (Shanbhag et al. 1999). The antenna has 6 segments, with different functions. For example, segment 2 determines audition (Si Dong et al. 2002) while the third segment (also called the funiculus) determines olfaction. The antennae and maxillary palp have odorant receptors that are responsible for odorant detection. Most smells activate more than one odorant receptor and the system can detect and distinguish between an almost infinite number of smells (Gaillard et al. 2004). In both the antenna and maxillary palp olfactory receptor neurons (ORNs) are expressed in sensory hairs called sensillae (Gaillard et al. 2004). These sensillae are distributed over the surface of the antennae and the maxillary palp, and enable olfactory recognition (Carlson 1996; Clyne et al. 1997; Vosshall et al. 1999) through exposure to the environment (Spletter & Luo 2009). There are about 450 olfactory sensillae on the funiculus and 80 on the maxillary palp (Carlson 1996), which is consistent with the observation that the maxillary palp has a decreased sensitivity to odours in comparison to the antennae (Charro & Alcorta 1994). Different types of sensillae on these organs, known as basiconic, coeloconic and trichoid sensillae are thought to respond to different types of odorants in the environment (Spletter & Luo 2009). The funiculus is sexually dimorphic in sensillum type with males having 30% more sensilla trichodea than females, which could suggest a role in courtship (Venkatesh & Singh 1984; Stocker & Gendre 1989; Shanbhag et al. 1999). Flies with mutations affecting the development of olfactory sensillae have defective odorant receptors or none at all and so have defective olfactory perception (Riesgo-Escovar et al. 1997). Begg and Hogben (1946) found that the *Drosophila* mutant called *antennaless*, had a loss of olfactory reception to food odours and so suggested a correlation between these two traits; the loss of the olfactory organ and the loss of reception to food odours (Begg & Hogben 1946).

In chapter 5 of my thesis I tested whether the loss of olfactory reception would also affect a male's ability to detect rivals and also affect his general fertility levels. In previous experiments *D. melanogaster* responded to variations in levels of sperm competition by increasing their mating duration and the transfer of seminal fluid proteins during mating, ultimately resulting in an increase in their share of paternity. Although how males perceive the presence of rivals is not yet known it is likely to involve at least in part the detection of male specific volatile pheromones (Bretman et al. 2010). I tested this idea by removing the odorant receptors on the male antennae and testing whether males could still respond to the presence of rivals.

1.5] Dietary resources and detection of socio-sexual environment

Ingested food components are essential for normal metabolism and development (House 1962). Restricting the diet of the fruit fly *Drosophila* is a well known method to extend lifespan (Bass et al. 2007). There has been significant progress towards understanding the effects of diet and reproduction on lifespan by studying female *Drosophila*. Different food levels affect fecundity and lifespan and there are effects of dietary restriction on female fertility (Chapman & Partridge 1996). The fruit fly uses carbohydrates and proteins as a major food source. Salts and acids are also integral to the diet and the proper uptake of these chemicals is crucial for electrolyte homeostasis (Amrein & Thorne 2005). However nutrient consumption affects male and female lifespan differently in *Drosophila.* There is significant evidence suggesting that a male's nutrition can impact on his ability to gain a mating and that an optimal level of nutrition is required to maximise reproductive success (Fricke et al. 2008).

Pheromones can be detected from afar as well as on contact. For example, chemical cues can be detected via an ant tapping another ant with its antenna, and by doing this it can detect a complex mixture of chemicals in the cuticle that differ between colonies. This allows a sophisticated differentiation between friends and strangers (Wyatt 2003). Pheromones can also be transferred directly from the signaller to the receiver. The way in which a male responds to his environment and how well he detects pheromones may depend on his condition, i.e. food resources accrued. Post eclosion nutrition is important in order for *Drosophila* to become sexually mature (Droney 1996) and it is probable that it has effects on a males reproductive investment (Engels & Sauer 2007; Fricke et al. 2008). Fricke et al. (2008) found novel effects in which expression of a male's reproductive success was maximized at intermediate food levels. They found that females mated to males fed low and a high quality diets were quicker to mate again, suggesting that intermediate fed males were better at reducing receptivity in their mates. They also found males on the intermediate diets fathered the most offspring and also provided evidence that nutritional status affected a male's ability to court. It is thought that increasing nutritional levels even further may not in fact decrease nutritional stress because of potential costs due to detoxifying excess nutrients. Therefore both high and low nutrition could represent stressful environments (Fricke et al. 2008).

In chapter 6 of my thesis I subjected wild-type adult male *Drosophila* to differing diets that varied in their concentrations of sugar or yeast. I then determined the effects of the different diets on reproduction and fecundity and more importantly on a male's ability to detect and respond to rivals. I did this to test the idea that males under severe nutritional stress (of either sugar or yeast) would be less likely to be able to respond to rivals.

1.6] The utility of the *Drosophila* model system

The fruit fly *Drosophila melanogaster* is one of the most intensely studied organisms in biology and the known genome sequence and well characterised genetics of this organism offer many advantages. Sequencing of the entire genome of *Drosophila* was completed in 2000 and a large genomic database is available at http://flybase.bio.indiana.edu/. Through the sequencing of the *Drosophila* genome, the molecular study of olfaction has been made possible (Gaillard et al. 2004). *Drosophila* serves as a model system for the investigation of many cellular and developmental processes common to higher eukaryotes including humans (Gaillard et al. 2004). It has been discovered that there are many genes functionally equivalent between humans and *Drosophila* and many of these are genes that cause a broad spectrum of human disease. These include developmental defects, neurological disorders, cancers, cardiovascular diseases and metabolic disorders. Genes required for function of the visual, auditory and immune systems are also highly conserved from flies to humans (Reiter et al. 2001).

There is a vast store of accumulated knowledge concerning *Drosophila*, it is easily cultured, has a short life cycle and one can obtain large numbers of experimental subjects over a short generation time (Roberts 2006). Under ideal laboratory conditions a minimum of 11 days is required to go from egg to egg, this is the shortest generation time known for any *Drosophila* species. After eggs are hatched the first larval moult occurs after 25 hours and the second 24 hours later. Puparia form 48 hours after that, and the formation of adult organs and structures in the pupal stage requires about 100 hours in total. After eclosion and the emergence of adult flies, it takes two to three days for the females to develop mature eggs (Powell 1997). There are detailed bioinformatic data and genetic reagents available, for example loss of function mutant stocks are available for key odorant receptors such as *Or83b*.

1.7] Thesis outline

In this thesis I used the model organism *Drosophila melanogaster* to determine the role of olfaction in reproduction. The aim was to investigate the role odorants play in reproduction of *D. melanogaster* by using the experimental techniques outlined in the four data chapters of this thesis.

- Chapter 2 provides general materials, methods and techniques used throughout the thesis.
- Chapter 3 used bioinformatic analysis to determine in which tissues odorant receptors and odorant binding proteins are expressed. Then went on to investigate the odorant genes expressed in the reproductive tissues, especially those found in gene clusters. The predicted functions of these genes were surveyed and their evolutionary relationships tested in comparison with orthologues in closely related species. This was to test the idea that the duplication of a gene into clusters allows some members of the cluster to take on new functions and also to take on a more rapid pattern of evolutionary change.
- Chapter 4 tested the possible role of internal odorants in males by using a loss of function mutation for the odorant receptor *Or83b*. The role of lack of functional odorant receptors on male fertility, rivalry, mating behaviour and offspring viability was tested.
- Chapter 5 tested the effect of excluding external odorants from males by removing the antennae on a male’s mating behaviour and ability to detect rivals.
- Chapter 6 determined how nutrition of the male can affect mating behaviour, rivalry, and reproduction and offspring viability by varying the amounts of yeast and sugar available in the diet.
- Chapter 7 then summarised the results and discussed the general themes that emerged from this work. Potentially fruitful avenues for taking the research forward in the future were also proposed.

Chapter 2 General Materials and Method

2.1] Materials

All flies were maintained in a humidified room at 25°C on a 12:12 hour light:dark cycle.

2.1.1 Cages, bottles and vials

Cages in which wild-type *Drosophila* were held measured 45x25x25cm. Each cage contained 12 bottles filled with 70ml of SYA food (for recipe see Appendix 4). Each week the oldest 3 bottles were removed and new ones added, allowing ample time for the flies to lay eggs, larvae to develop and for pupae to eclose. Each bottle remained in the cage for approximately 4 weeks allowing plenty of time for adult development which takes approximately 12 days at 25°C

Glass bottles – 189ml (1/3 pint) bottles were used in stock cages and also for maintaining mutant stocks. Mutant stocks were held on ASG medium (Appendix 4) and were tipped over onto new food every two or three weeks, giving ample time for flies to develop.

Glass vials – 73mm high x 23mm diameter vials containing 7ml of food medium were used in mating experiments (as specified in relevant chapters). Vials containing ASG medium were also used to maintain new stocks in quarantine in the 25°C incubator while establishing that the new stocks were healthy and free from mite infestation.

Cotton wool bungs were used to contain flies in vials and bottles.

Food recipes and methods are shown in Appendix 4.

2.1.2 *Drosophila* wild-type stock

The *Drosophila* wild-type stock was originally collected from Dahomey, now the Republic of Benin, West Africa in 1970. These stocks have been maintained in the laboratory since collection in cages as above (section 2.1.1).

2.1.3 *Or83b* mutant stock

The *Or83b* loss of function mutant stock was obtained from the Bloomington stock centre (http://flystocks.bio.indiana.edu/; stock number 23129). The *Or83b* gene in this stock has been replaced by *w[+]* by ends out targeting. The genotype is *w[*]; w[+*] Or83b[1]*. *Or83b* is a broadly expressed odorant receptor essential for olfaction in *Drosophila,* and its removal enables the study of the significance of olfaction (Larsson et al. 2004). *Or83b* has been found to be expressed in the head, brain and larval midgut (http://130.209.54.32/atlas/atlas.cgi).

2.2] Method

2.2.1 Standard larval density culture

To collect eggs for standard larval density culture, petri dishes containing grape juice medium as above (Appendix 4) were supplemented with a small amount of yeast paste added to the middle of the plate and were placed in one of the Dahomey cages for 12-24 hours. At the end of the egg laying period the yeast was removed and the plate left for 24 hours in the 25°C room in order for the first instar larvae to emerge. These emerging larvae were then picked from the grape juice plate using a larval pick (a mounted needle) and placed into vials containing SYA food medium at a density of 100 larvae per vial. A standard number of drops of liquid yeast were added to each vial (Appendix 4).

Having a standard density equalises any competition between larvae, it minimises competition for food and each larval cohort therefore shares the same environment. There are many environmental factors that can affect larval morphology or behaviour for which we need to control (Gage 1995; Hosken et al. 2000). For example larval density, temperature or nutrition can affect body size. Standardising larval density is therefore essential when measuring adult fitness traits in *Drosophila*.

2.2.2 Fly handling and virgin collection

Flies that had to be handled for transfer or experimental procedures were anaesthetized using either ice or exposure to carbon dioxide (Bretman et al. 2010). Virgin flies were always collected using ice anaesthesia because exposure to carbon dioxide in flies less than 3 hours old can cause damage to the adult gut and premature death.

2.2.3 Mating

For mating trials vials were placed on a specially designed viewing rack, which allows large numbers of vials to be observed easily. When each fly was added to its prospective mate in the vial, I recorded the time of introduction and the time that mating began and finished. Barron (2000) states that copulations lasting less than 1 minute are considered possible pseudo-copulations so I only recorded matings of at least 5 minutes and over (Barron 2000). Virgin females were used in all experiments in this thesis.

2.2.4 Antennal removal

Antennae were removed while flies were anaesthetized under CO_2. In the first section of Chapter 5 the funiculus (segment 3) of the antenna was removed and during the second experiment the arista was also removed as an extra control. Each section of the antenna was removed by viewing under a stereo microscope and using small tweezers to remove the appropriate structures.

2.2.5 Egg and Offspring Counts

Eggs from the mated females were counted 24 hours after mating. Egg counting was facilitated by increasing the contrast between egg and medium using charcoal (Appendix 4).

Some vials were retained to allow offspring to emerge; these were kept for 12 days to ensure all offspring had eclosed. The vials were then inverted and frozen at -80°C for progeny counting at a later date. Inverting the vials made it easier to remove the flies for counting.

2.2.6 Statistical analysis

All data was entered into Microsoft Office Excel version 2003 and 2007. All statistical analysis were run in SPSS version 16.0.

2.3] Figures

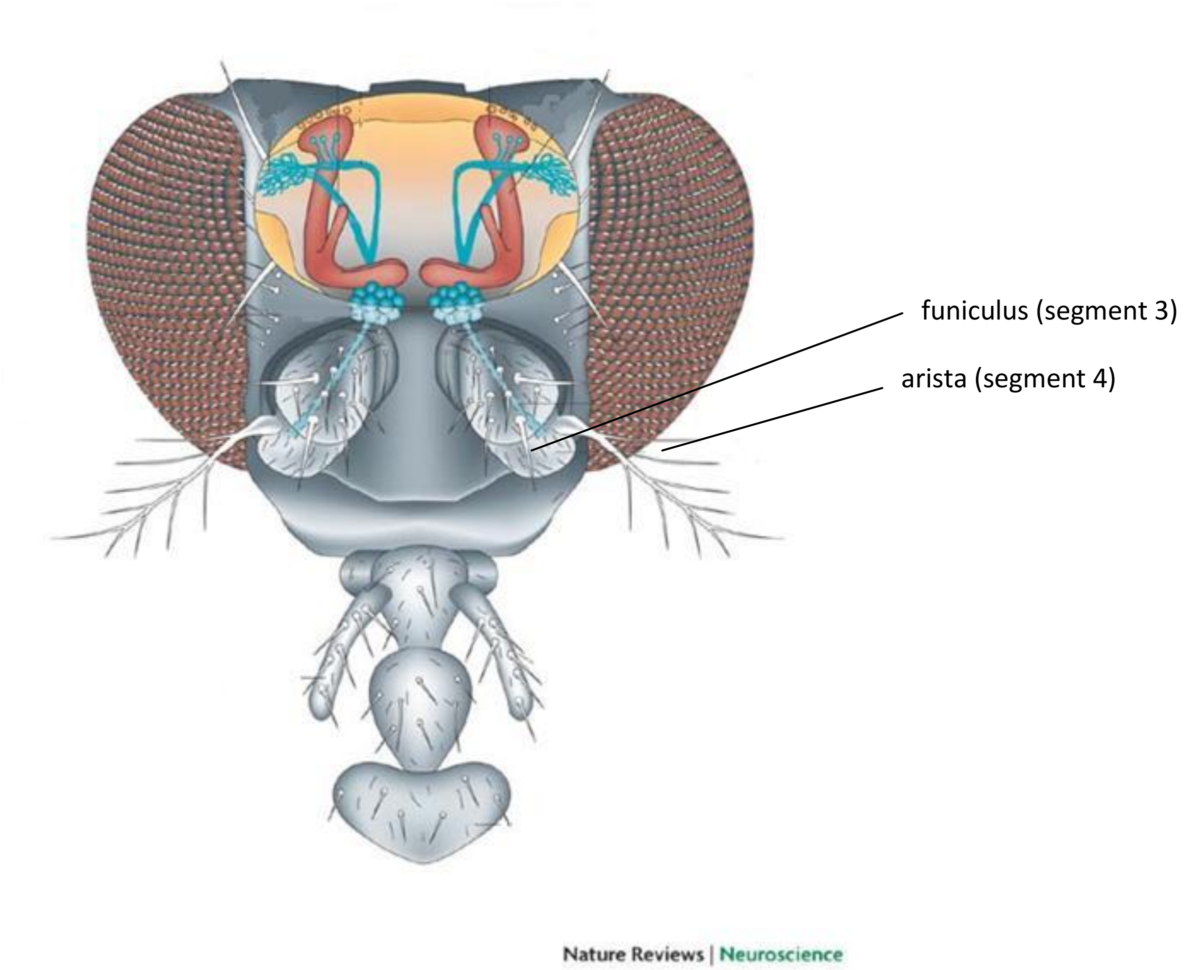

Figure 2.3.1 The head of *Drosophila melanogaster*. The funiculus and arista were removed under CO_2 anaesthesia, in the work described in Chapter 5 of this thesis. Figure reproduced from (Keene & Waddell 2007)

Chapter 3 New functions and rapid evolution of odorant genes expressed in the reproductive system of the fruit fly

3.1] Abstract

Odorant receptors recognise airborne odorants. Odorant binding proteins are thought to make hydrophobic odorants soluble and convey them to odorant receptors, and in this role they play an important part in olfaction. The research in this chapter surveyed the expression patterns of odorant receptors and odorant binding proteins and showed that both are expressed not just in the olfactory system but also in the reproductive tissues of *D. melanogaster*. The survey showed that some odorant receptors and odorant binding proteins are expressed only in the reproductive system. Many odorant genes have arisen by duplication and are found in gene clusters. Duplication of genes within clusters could allow odorant genes to take on new functions, however the specific functions of most odorant genes are not yet known. The adoption of new functions by odorant genes could also allow genes with different expression patterns to exhibit differing rates of evolutionary change, I tested this by examining the degree of conservation of odorant genes across closely related *Drosophila* species. The results suggested that odorant genes expressed in the head were more highly conserved across the *Drosophila* species than those expressed in the male reproductive accessory glands.

3.2] Introduction

The ability to distinguish friend from foe, male from female and to determine members of the same group or species is a crucially important ability for organisms across a huge variety of different taxa. Recognition is a major component in establishing mate choice and also in maternal bonding, and both are vital for reproductive success. In terms of mate choice, recognition may facilitate the choosing of mates that are genetically different or otherwise 'compatible' so as to reduce inbreeding and increase fertility and reproductive success (Brennam 2004; Meagher et al. 2000). In some species, including humans, recognition may rely primarily on sight, but in many other taxa, it is olfaction that is the main means to distinguish relatedness, to detect mating status and to initiate courtship during reproduction (Snyder et al. 1988).

Insects detect odorants with olfactory neurons that are sequestered in chemosensory sensilla located mostly on the antenna (Xu et al. 2005). Odorants are foreign molecules that are widely varied in structure, and the mechanism by which odorants are identified is still partially unknown (Snyder et al. 1988). Vertebrates and invertebrates can detect and distinguish between many thousands of chemically distinct odorants and, at first sight both have evolved similar mechanisms to detect these odorants, namely odorant receptors (ORs) (Benton et al. 2006). It has, however, recently been discovered that the organisation and structure of the invertebrate ORs is strikingly different to that of vertebrates. Insect ORs have evolved independently in parallel but it appears they may be more evolutionary conserved than the ORs of vertebrates. There is, strikingly, no similarity in the sequences of vertebrate in comparison to insect ORs (Kazushige & Vosshall 2009). Individual olfactory sensory neurons (OSNs) normally express a single odorant receptor, which is exposed to the environment on the surface of ciliated endings of OSN dendrites (Barnea et al. 2004). In many mammals there are large numbers of different ORs which are thought to correspond to the number of odorants that need to be detected. In contrast, the *D. melanogaster* genome has revealed far fewer ORs than expected based on these results from other species. To date approximately only 60 ORs have been discovered in *D. melanogaster* whereas *Caenorhabditis elegans* has approximately 800, and mice and rats have over 1000 (Hekmat-Scafe et al. 2002). Evidence now also suggests that OR expression is not tightly restricted to sensory neurons in the nose or antennae (Spehr et al. 2006). The transcription of about 20 mammalian ORs has been demonstrated in the male germ line, most notably during the late stages of spermatogenesis (Parmentier et al. 1992). In addition, it is also now clear that many insect ORs and associated genes are highly enriched for their expression in the reproductive system (Chintapalli et al. 2007). These and other findings suggest that olfactory receptors could potentially be involved in chemotaxis during fertilization.

As well as the ORs themselves, odorant binding proteins (Obps) also play a significant role in odorant detection in invertebrates (Hekmat-Scafe et al. 2002). Obps are a large and diverse family (Xu et al. 2005) and were first identified as pheromone binding proteins in moths (Vogt & Riddiford 1981). They are small proteins present at high levels in the fluid surrounding the OR neurons (Pelosi 1994; Vogt et al. 1999). To date approximately 50 potential Obp genes have been discovered in *D. melanogaster.* It is believed that Obps function to convey odorants to the ORs by rendering the hydrophobic odorant molecules soluble, hence they play an important role in olfaction (Xu et al. 2005; Pelosi 1994; Prestwich et al. 1995). One theory suggests that insect Obps and ORs are expressed within different but overlapping subsets of sensilliae, resulting in a multitude of sensillae with different odorant thresholds. This would mean that only a limited number of odorant receptors would be needed to greatly increase the discriminating ability of the olfactory system. This perhaps explains why the number of ORs in invertebrates may be fewer than in mammals but with no loss of odorant discrimination. As for the ORs described above, there is also evidence that odorant receptors are not only found in the olfactory system, but that they are also present in many different organs (Spehr et al. 2006). This suggests that they not only recognise airborne odorants but that they also play an important role in chemotaxis or even in organ construction in the embryo during development (Itakura et al. 2006). Well characterised developmental genes are often found to have different functions in adult tissues (Chintapalli et al. 2007). Similarly, based on sites of expression, Obps also appear to have many diverse functional roles. They have been found in male mouse urine, associated with pheromones, as well as in the nasal passage (Cavaggioni & Mucignat-Caretta 2000). Odorant binding proteins have been found in the trunks of the Asian elephant, and a significant fraction of Obps are also bound to elephant serum albumin (ESA) in the urine. This provides key evidence of roles for Obps in transport and in reproductive signalling (Lazar et al. 2002). Insects also express Obps in other tissues, for example in the legs and wings of the wasp *Polistes dominulus* (Calvello et al. 2003), and in the wings of the locust *Locusta migratoria* (Ban et al. 2003). This suggests that Obps have a key role in chemical communication in many different species. The fact that there is considerable divergence in Obps and variation in their numbers between insect groups suggests that they may also be important in within species recognition.

The role of odorants in mediating internal reproductive processes is based upon the idea that the same signalling pathways are used in external communication. One hypothesis for how this has occurred is via neofunctionalisation of odorant genes, to allow new patterns of tissues specificity to arise. This process may have been greatly facilitated by the duplication of odorant related genes, allowing some members to retain their original functions and some to take on new roles within the reproductive tract. Consistent with this hypothesis it is found that, of the Obp genes dispersed across the genome of *D. melanogaster,* 73% occur in tight clusters. A particular example is for chromosome 2R, where 29 of the 51 Obps are found in several clusters (Hekmat-Scafe et al. 2002).

This study was designed to survey, using the published FlyAtlas database, the expression patterns of odorant receptors and odorant binding proteins in the reproductive tissues of *D. melanogaster*. I focussed on odorant genes occurring in chromosomal clusters, to test the idea that duplication of OR and functionally related Obp genes allowed specialization and new functions of odorant genes in the reproductive system to evolve. I predicted that this would be accompanied by a switch in the site of expression of such genes from head/olfactory system to reproductive tissues. In addition, given that in general, reproductive genes show high rates of evolutionary change, I tested the prediction that the release of evolutionary constraint that followed the duplication of such genes, led to higher rates of evolutionary change in those odorant related genes that became limited in their expression in the reproductive system, in comparison to the putative ancestral genes from which they duplicated.

3.3] Materials and Method

3.3.1 Survey of expression patterns of ORs and Obps in the head and reproductive tissues

Expression data for odorant receptors and odorant binding proteins were obtained using the FlyAtlas database of *Drosophila* adult gene expression (www.flyatlas.org/). FlyAtlas reports the expression of 27 distinct tissues from larvae, adults and *Drosophila* S2 cells. We focussed on the 18 adult tissues currently surveyed: brain, head, eye, thoracicoabdominal ganglion, salivary gland, crop, midgut, malpighian tubule, hindgut, heart, fat body (from the thorax and abdomen), testis, ovary, male accessory glands (MAG), virgin spermatheca, mated spermatheca, adult carcass and whole adults. I searched for expression patterns of all known odorant receptors and odorant binding protein genes and recorded those with an above background mRNA signal and a 4 out of 4 present call (meaning that each of the 4 replicate microarrays in the database gave detectable expression in that tissue). The signal conveys mRNA abundance in each tissue and for odorant receptors this was expected to be lower than for odorant binding proteins, given the often very localized expression of ORs to only a few cells. Signal values of 100 are classed as abundant and over 1000 remarkably abundant (as sited on website http://www.flyatlas.org/). I summarised the data for ORs and Obps expressed in head, ovary, testis and male accessory glands (table 3.6.1).

3.3.2 Molecular function and biological processes of odorant receptors and odorant binding proteins expressed in the head, ovary, testis and male accessory glands

I then surveyed the known and predicted roles of ORs and Obps expressed in the head and reproductive tissues. I searched for functional information using Flybase data (Wilson et al. 2008) for individual genes and also the Biological General Repository for Interaction Datasets (BioGRID) database, which provides details on the function of genes (Stark et al. 2006).

3.3.3 Survey of chromosomal clusters of odorant receptors and odorant binding proteins

I mapped the chromosomal location of ORs and Obps to investigate which of them occurred in genomic clusters. I obtained this information by searching chromosomal location data from Flybase (Wilson et al. 2008), NCBI (National Centre for Biotechnology Information, www.ncbi.nlm.nih.gov/) and published information (Hekmat-Scafe et al. 2002). I then combined this with expression site and functional information on the members of those gene clusters, to test the idea that gene duplications within these clusters led to changes in tissue specificity which could be accompanied by changes in function.

3.3.4 Evolutionary change in odorant receptor and odorant binding protein cluster genes

I tested the idea that putative new functions for ORs or Obps within the reproductive system would relax previous functional constraint and potentially allow those genes with new functions to be subject to more rapid evolutionary change. I tested this idea by examining divergence in OR and Obp sequence across the 12 *Drosophila* species genomes databases. I used nucleotide searches in the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) to probe the *D. melanogaster* OR and Obp sequences of interest against the other species of *Drosophila*. I then tabulated BLAST E-value hits. I used the following general criteria for homology searching.

i] Exact and very closely matched hits had an E-value of ~ 0
ii] A hit was also considered very similar to the query sequence if the E-value was between $1x10^{-50}$ and 0.

The aim was to look for quantum steps in the degree of similarity across species, to indicate distant versus more recent divergence consistent with the idea of change of function following duplication.

3.4] Results

3.4.1 Survey of expression patterns of odorant receptors and odorant binding proteins in the head and reproductive tissues

Odorant receptors and odorant binding proteins are co-expressed in the antennae (the antennae are included in the head tissue in FlyAtlas (Chintapalli et al. 2007)) and maxillary palp (Charro & Alcorta 1994). It is only relatively recently these genes have been discovered to be expressed in other tissues including the reproductive organs (Graham & Davies 2002). To test whether different members of an OR or Obp family had taken on different functional roles, I examined expression patterns for whole gene clusters to look for examples where there was a marked change in expression pattern. It is likely that these genes found in clusters have arisen via duplication. This is indicated because genes in clusters are each other's closest homologs (as seen in BLAST searches, data not shown).

I recorded as 'expressed' any ORs or Obps with an mRNA signal and a present call of 4 out of 4 in any tissue and then focussed on determining the patterns for genes expressed in the head and/or the reproductive tissues (ovary, testis and male accessory glands shown in table 3.6.1).

3.4.2 Survey of expression patterns of clusters of odorant receptors and odorant binding proteins and their molecular and biological functions

ORs and especially Obps can occur in gene clusters, for example the Or 59 cluster has 3 members and there are gene clusters for Obps 19, 50, 56, 57 and 99. I examined each cluster for evidence of changes in expression patterns, which could indicate a switch in functional roles between gene duplicates. I discuss the findings for each of the clusters below.

Odorant receptors - Or59

The Or59 cluster is located in a region of chromosome 2R. All three genes in the Or59 cluster are expressed in the male accessory glands, while Or59b is also expressed in the head (table 3.6.1). There is little evidence from the Gene Ontology (GO) designation of functional data so far of a distinct functional profile for head versus head and male reproductive system expression. All the Or59 genes in the cluster share the olfactory receptor activity and sensory perception of smell GO categories (table 3.6.2). However, it should be noted here and below that the functions of most of the odorant genes are not yet known, hence GO categories are relatively uninformative with respect to exact type of chemosensation for each gene.

Odorant binding proteins

Several of the clusters of Obp genes showed evidence of changes in tissue specific expression, as follows

i] Obp19 genes are X linked and Obp19a, b and c are all expressed in the head, while Obp19c was also expressed in the ovary. There are no distinct roles yet detected in the GO categories (table 3.6.2) for the different duplicate members.

ii] Obp50 genes are located on chromosome 2R. I found no signal for Obp50a expression, however, Obp50b, c, d, and e are all abundantly expressed in the testis.

iii] Obp56 is a cluster of 9 genes mostly showing expression in the head (table 3.6.1). Obp56a and d are also expressed in the testis, while e, f, and g are also expressed in the male accessory glands. The expression of Obp56i is restricted to the testis and MAG and not the head. Obp56a, d and h, which are all expressed in the head, have extra predicted biological functions of olfactory behaviour and response to pheromone, according to the GO system. Only Obp56a and h have the biological function sensory perception of smell. However, as noted above, these GO functions are relatively uninformative for this purpose. The expression of Obp56h is abundant in the head only while in Obp56a the expression is abundant in the head but not in the testis (table 3.6.1).

iv] Obp57a, b and c are all expressed in the head. Obp57a is also expressed in the ovary and testis.

v] All the Obp99 cluster genes are expressed in the head, but Obp99a is also expressed in the ovaries and the testis.

3.4.3 Evolutionary change in odorant receptor and odorant binding protein cluster genes

As noted above to determine the rates of sequence change in the OR and Obp cluster genes, I used BLAST searches. I queried each OR and Obp *D. melanogaster* sequence against the 12 *Drosophila* species genomes. I tested the hypothesis that genes within odorant clusters that had taken on different patterns of tissue specificity might be subject to relaxed functional constraint leading to higher rates of evolutionary change. I determined whether all members of a gene cluster were present in close relatives and determined sequence similarity of orthologues from E values returned in the BLAST searches. I examined whether there were step changes (i.e. large changes in the order of magnitude of a match) across a phylogeny, as described below.

3.4.4 Presence/absence of orthologues

ORs and Obps from gene clusters were recorded, although some fell below the detection levels for expression in the tissues required in the FlyAtlas database (e.g. Obp56b and c are listed as not expressed). The presence of detectable orthologues across species was determined (table 3.6.3). In some cases there was a pattern of loss of different members of genes within odorant clusters. For example from the cluster Obp19, Obp19a and c were conserved across the *melanogaster* species subgroup while Obp19b was found more widely across the whole *melanogaster* group. All three were expressed in the head but Obp19c was also expressed in the ovaries. While the Obp50 cluster of genes were found to be conserved across the *melanogaster* species subgroup and all were confined in expression to the testis.

The grouping of *Drosophila* species are as follows:
i] The *melanogaster* subgroup includes *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba* and *D. erecta*
ii] The *melanogaster* group is the *melanogaster* subgroup above and *D. ananassae*
iii] The *obscura* group includes *D. pseudoobscura* and *D.persimilis*

3.4.5 Evidence for changes in the rates of evolutionary change in odorant genes

Data from Obps occuring in clusters and expressed in the head and/or reproductive tissues
Previous data suggested that genes expressed in the reproductive organs are less highly conserved than those expressed in the head. I found that for the Obp56 cluster this was true: Obp56h which is expressed in only the head is highly conserved across many *Drosophila* species (8 in total) while Obp56i which is expressed in only the reproductive organs is less divergent and found in only the 3 closest species of *Drosophila* to *D. melanogaster* (table 3.6.4). This would seem to indicate that Obp cluster genes expressed in the reproductive organs perhaps evolve more rapidly than those expressed in the head. However other clusters in this study do not support this hypothesis (table 3.6.4) and a more extensive and sophisticated analysis is needed to determine if Obps found in the reproductive organs are diverging faster than those found in the head. For example, it would be possible to run models of adaptive site divergence.

Data from single Obps not occurring in clusters and expressed in the head and/or reproductive tissue
I examined whether there was any evidence that Obps occurring singly in the genome, and expressed in the reproductive system, were less evolutionary conserved than those expressed in the head. I searched through the data base of all the Obps that were not in clusters looking for those that were present in the just the head and just the MAG. There was only one expressed in the male accessory glands (MAG) alone and so I included two expressed in the head + MAG together (table 3.6.5).

Obp22a is specific to the MAG and was found to less conserved. It was found in only three of the *Drosophila* species in the *melanogaster* subgroup in comparison to Obp59a, which is expressed only in the head and was highly conserved across the whole of the *melanogaster* group. Two Obps expressed in the head and in the MAG (Obp83cd and Obp8a) were conserved across the five species of the *melanogaster* subgroup. These data suggest that genes expressed in the reproductive organs, especially the MAG, may evolve more rapidly than those found in the head, although further work is needed to confirm this suggestion.

3.5] Discussion

My survey of the expression patterns of OR and Obp genes in adults showed clearly that they can be expressed in the reproductive system as well as in the olfactory system (i.e. in the head which includes antennal and maxillary palp expression). Some genes, for example Or59c and Obp56i, were restricted in their expression to the reproductive system. There were several instances of olfactory genes occurring in clusters, and there was evidence within clusters for the evolution of differences in tissue specificity in expression and hence potentially also function, for example according to GO data, Obp99band d are involved in autophagic cell death, a category which was not indicated for Obp99a or c. I also found some evidence that odorant genes found in the head can be more highly conserved across species than those expressed in the MAG. For example Or59b found in the head and MAG was highly conserved across the *Drosophila melanogaster* group and *obscura* group while Or59c found only in the MAG was conserved only across the *melanogaster* subgroup.

3.5.1 Odorant receptors

I investigated whether OR genes are restricted to functions in olfaction or whether there is any evidence for novel roles within the reproductive system. ORs that become restricted in their expression to the reproductive system could function via the same signalling pathways as in external olfaction or via novel ones (Spehr et al. 2006). Here I showed that Or59a and Or59c were both expressed in only the MAG whereas Or59b although expressed in the MAG was also expressed in other tissues including the head. The mRNA signal of Or59b in the MAG was extremely high for an OR, as was its enrichment. The sensory perception of smell is a series of events required for an organism to receive an olfactory stimulus, convert it to a molecular signal and to recognise it (Stark et al. 2006). It is probable that Or59b has functions in both the head and the MAG as it is expressed in both but whether all functions are retained in both tissues is not known. ORs tend to show very low levels of expression as recorded in the FlyAtlas database. Obps on the other hand can be detected at much higher expression levels. This suggests that high levels of Obp are necessary to detect, capture and transport odorants to ORs. In contrast ORs that receive the delivery of odorants by Obps can function through expression at much lower levels. Overall, this difference in expression levels gave a much lower signal among the OR in comparison to the Obp data. Hence most of my analysis focussed on Obps.

My results are consistent with the emerging view that ORs can be expressed in many internal tissues. For example, in mammals there is ectopic expression of ORs in the spleen (Blache et al. 1998), brainstem (Raming et al. 1998; Conzelmann et al. 2000), colon and prostrate (Yuan et al. 2001), but as yet none have been assigned a physiological function. In 1991, Parmenteir et al. demonstrated the transcription of approximately 20 mammalian OR genes in the male germ line most notably during the late stages of spermatogenesis. The ectopic (i.e. in locations other than the nose) expression of up to 50 ORs has been reported (Parmentier et al. 1992). Similar numbers were found in 2004 by Zhang et al. who used a high-throughput oligonucleotide microarray approach to detect 66 OR genes with enriched expression in mouse testis (Zhang et al. 2004). These findings suggest that a common receptor gene family encodes olfactory receptors and also sperm cell receptors that may be involved in chemotaxis during fertilization.

Many of the ORs have multiple functions which may change over evolutionary time. In many cases the evolutionary requirement for ORs is met by the recruitment of novel gene families rather than using pre-existing OR families in the ancestral genome (Vosshall et al. 1999). Here I started to probe this question in *D. melanogaster* by testing whether some of the gene recruitment to new functions could occur following gene duplication in odorant genes. Or59b, which is expressed in the head and the MAG, was more highly conserved across the *Drosophila melanogaster* and *obscura* groups than Or59a and c which were expressed only in the MAG (see section 3.4.4 for *Drosophila* species grouping). This is preliminary evidence that ORs expressed in different tissues may evolve at different rates.

3.5.2 Odorant binding proteins

As noted above odorant binding protein genes showed a higher level of expression overall than did ORs. It is believed that Obp genes could have been the first specific biochemical step in odorant reception (Vogt et al. 1999). Obps were initially identified by their ability to bind [^{3}H]2-isobutyl-3-methoxypyrazine. Obps are thought to be homogenous proteins which can bind ^{3}H-odorants of differing chemical structures. This would indicate that they show only a low level of specificity (Snyder et al. 1988). However the selective binding of odorants to different Obps derived from different species has now been demonstrated. Many Obps that have been identified in a single species have been shown to interact differently with functionally distinct classes of olfactory sensilla. This differential expression and diversity of Obps has led to the suggestion that Obps may act as selective filters which influence a range of odorant molecules that can gain access to the ORs of a given sensillium (Vogt et al. 1999). A few of the Obps investigated here are also expressed in larval tissues, which suggests they may play a common role in olfaction at both stages (Park et al. 2000).

Odorant binding was a common GO functional category for all Obps. All Obps are described as having sensory perception to chemical stimulus and transport. However some, for example Obp56a and h, are also described with the category ‘sensory perception of smell’. The clusters that are located together on a chromosome arm seem at present to have less varied biological processes as indicated by GO categories, for example the Obp50 cluster are all found together on the 2R chromosome and are all conserved across the *melanogaster* subgroup. Obp50a and b have the biological function ‘sensory perception of chemical stimulus’ as do Obp 50c, d and e but they also have the biological process ‘transport’. This is the same for the Obp57 cluster where all members have the same biological processes. Obp57a, b and c are grouped and are conserved across the *melanogaster* subgroup while Obp57d and e are grouped and both have no detectable expression values in the FlyAtlas database. Obp57d is highly conserved across the *melanogaster* and *obscura* groups and also appears in the more distantly related *D. virilis* (see section 3.4.4). To date the GO category data summarised here give only a very approximate picture of the roles of different odorant genes. Future work will focus on defining roles for ORs and Obps and this will greatly illuminate the possibility of new functions for duplicated odorant genes.

It is likely that many Obps have multiple functions that may have changed over evolutionary time. A study by Vogt et al. in 1991 suggested the widespread distribution of Obp related genes throughout the *Endopterygota* and *Exopterygota* indicated that Obps genes are ancient and were present in the ancestors of these groups (Vogt et al. 1991). Precedents for changes in role between duplicated Obps are known. For example human lipocalins involved in odorant binding correspond to a gene family located on the human chromosome 9q34 and have been produced by gene duplications. Two Obps $hOBP_{IIa}$ and $hOBP_{IIb}$ are 95% similar in sequence but are differentially expressed in secretary structures. $hOBP_{IIa}$ is strongly expressed in the olfactory system, in nasal structures, the lung and the salivary and lachrymal glands. $hOBP_{IIb}$ on the other hand is more strongly expressed in the reproductive organs of the prostate and mammary glands (Lacazette et al. 2000). Obps have also been described as expressed in non olfactory tissues in other species. The Asian elephant has Obps in its urine (Lazar et al. 2002) and the mouse has Obps both in its urine and nasal passages. It is thought that major urinary proteins in the mouse bind to odorants in the bloodstream which are then conveyed to the urine. There is direct evidence to show that these major urinary proteins slowly release olfactory signals into the air once the urine has been released (Cavaggioni & Mucignat-Caretta 2000). These major urinary proteins have been attributed to acceleration of puberty in the female mouse (Vandenbergh 1969; Vandenbergh et al. 1976) and proteins have also been identified in some insects suggesting roles for Obps in reproduction. A protein identified as GOBP2 in the giant silk moth (*Antheraea polyphemus*) has been observed binding to a sex pheromone odorant (Vogt et al. 1991; Park et al. 2000).

Obp genes present in the same genomic cluster generally show differing patterns of expression in chemosensory organs (Hekmat-Scafe et al. 2002). For example the Obp56 cluster I investigated indicated that those Obps found in the head or head and testis have more biological processes than those that are either not expressed in any tissue or that are found in the head and MAG or the testis and MAG. My data suggests that Obps found only in the MAG can be less conserved across species of *Drosophila* than those Obps expressed in different tissues. For example Obp56h is expressed only the head and is highly conserved across both the *melanogaster* and *obscura* groups, while Obp56i is expressed in the testis and MAG and is conserved across just three species of *Drosophila* (table 3.6.4).

Genes in the reproductive system may often evolve more rapidly than non reproductive genes (Swanson et al. 2001). It is thought that this may result from selection arising from sperm competition, from cryptic female choice or from sexual conflict (Holland & Rice 1999; Chapman et al. 2003; Nuzhdin et al. 2004). In future work it will be interesting to probe further the comparative rates of change of odorant genes expressed in the reproductive system versus elsewhere, both within species and between species, and to narrow down explanations for any differences by using functional tests. Many of the functions of ORs and Obp genes are not yet known. Their discovery will further enhance our understanding of the way in which OR and Obp genes work. Whether reproductive ORs and Obps are restricted to reproductive functions or also perform conventional tasks in olfaction is still to be determined. The information presented in this thesis provides preliminary data to suggest that over time some ORs and Obp genes have changed expression from the olfactory system to the reproductive tissues and in doing so may have evolved new functions. This process may have been facilitated by gene duplication. More investigation is needed to determine how these clusters evolved and whether their functions have changed across species.

3.6] Tables

Table 3.6.1 The expression patterns for all odorant genes occurring in clusters and which are expressed in either the head or the reproductive organs. The data were collected using the FlyAtlas database.

Odorant receptor or Odorant binding protein	**Tissue where gene is expressed**			
	Head	**Ovary**	**testis**	**Male Accessory Glands**
Or59a	--	--	--	y
Or59b	y	--	--	y*
Or59c		--	--	y
Obp19a	y**	--	--	--
Obp19a	y**	--	--	--
Obp19b	y*	--	--	--
Obp19c	y	y*	--	--
Obp50b	--	--	y	--
Obp50c	--	--	y*	--
Obp50d	--	--	y	--
Obp50e	--	--	y	--
Obp56a	y*	--	y	--
Obp56d	y**	--	y*	--
Obp56e	y**	--	--	y**
Obp56f	y	--	--	y**
Obp56g	y**	--	--	y*
Obp56h	y*	--	--	--
Obp56i	--	--	y	y**
Obp57a	y*	y	y	--
Obp57b	y*	--	--	--
Obp57c	y**	--	--	--
Obp99a	y**	y	y**	--
Obp99b	y*	--	--	--
Obp99c	y**	--	--	--
Obp99d	y	--	--	--

Key

y = Expression in this tissue has an mRNA signal of 100 or under.
y* = Expression in this tissue is abundant with an mRNA signal of over 100.
y** = Expression in this tissue is remarkable with an mRNA signal of over 1000.

Table 3.6.2 The molecular functions and biological processes for odorant genes occurring in clusters. The data were collected from FlyAtlas and functional categories determined by using the BioGrid database

OR	**Molecular function**	**Biological process**
59a	Olfactory receptor activity Electron carrier activity Oxidorductase activity	Sensory perception of smell Detection of chemical stimulus involved in sensory perception
59b	Olfactory receptor activity Odorant binding	Sensory perception of smell
59c	Olfactory receptor activity Odorant binding	Sensory perception of smell
Obp	**Molecular function**	**Biological process**
19a	Odorant binding	Olfactory behaviour Response to pheromone Sensory perception to chemical stimulus Transport
19b	Odorant binding	Sensory perception to chemical stimulus Transport
19c	Odorant binding	Sensory perception to chemical stimulus Transport
50a	Odorant binding	Sensory perception to chemical stimulus
50b	Odorant binding	Sensory perception to chemical stimulus
50c	Odorant binding	Sensory perception to chemical stimulus Transport
50d	Odorant binding	Sensory perception to chemical stimulus Transport
50e	Odorant binding	Sensory perception to chemical stimulus Transport
56a	Odorant binding	Sensory perception of smell Olfactory behaviour Response to pheromone Sensory perception to chemical stimulus Transport
56b	Odorant binding	Sensory perception to chemical stimulus Transport
56c	Odorant binding	Sensory perception to chemical stimulus Transport
56d	Odorant binding	Olfactory behaviour Response to pheromone Sensory perception to chemical stimulus Transport
56e	Odorant binding	Sensory perception to chemical stimulus Transport
56f	Odorant binding	Sensory perception to chemical stimulus Transport
56g	Odorant binding	Sensory perception to chemical stimulus Transport
56h	Odorant binding	Sensory perception of smell

		Olfactory behaviour Response to pheromone Sensory perception to chemical stimulus Transport
56i	Odorant binding	Sensory perception to chemical stimulus Transport
57a	Odorant binding	Sensory perception to chemical stimulus Transport
57b	Odorant binding	Sensory perception to chemical stimulus Transport
57c	Odorant binding	Sensory perception to chemical stimulus Transport
57d	Odorant binding	Sensory perception to chemical stimulus Transport
57e	Odorant binding	Sensory perception to chemical stimulus Transport
99a	Odorant binding	Olfactory behaviour Response to pheromone Sensory perception to chemical stimulus Transport
99b	Odorant binding	Olfactory behaviour Response to pheromone Sensory perception to chemical stimulus Transport Autophagic cell death Salivary gland cell autophagic cell death
99c	Odorant binding	Sensory perception to chemical stimulus Transport
99d	Odorant binding	Sensory perception to chemical stimulus Transport Autophagic cell death Salivary gland cell autophagic cell death

Table 3.6.3 Odorant receptor and odorant binding proteins found in clusters, sites of expression and species in which there are putative orthologues. I used Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) to find OR and Obp sequences of interest in *D. melanogaster* and test for matches against other species of *Drosophila*. Y indicates the presence of the odorant gene (OR or Obp) in that species with a significant E-value of ~ 0 or between $1x10^{-50}$ and 0. N/P shows the odorant gene was not considered present in this species with an E-value of $1x10^{-50}$ and below.

Gene	Tissue expressed	Species of *Drosophila* gene is found in								
		D.mel	*D.sim*	*D.sec*	*D.ere*	*D.yak*	*D.ana*	*D.per*	*D.pse*	*D.vir*
OR										
59a	MAG	y	y	y	y	y	y	N/P	N/P	N/P
59b	head + MAG	y	y	y	y	y	y	y	y	N/P
59c	MAG	y	y	y	y	y	N/P	N/P	N/P	N/P
Obp										
19a	head	y	y	y	y	y	N/P	N/P	N/P	N/P
19b	head	y	y	y	y	y	y	N/P	N/P	N/P
19c	head + ovary	y	y	y	y	y	N/P	N/P	N/P	N/P
50a	testis	y	y	y	y	y	N/P	N/P	N/P	N/P
50b	testis	y	y	y	y	y	N/P	N/P	N/P	N/P
50c	testis	y	y	y	y	y	N/P	N/P	N/P	N/P
50d	testis	y	y	y	y	y	N/P	N/P	N/P	N/P
56a	head + testis	y	y	y	y	y	y	N/P	N/P	N/P
56b	Not expressed	y	y	y	y	y	N/P	N/P	N/P	N/P
56c	Not expressed	y	y	y	y	y	y	N/P	N/P	N/P
56d	head + testis	y	y	y	y	y	N/P	N/P	N/P	N/P
56e	head + MAG	y	y	y	y	y	N/P	N/P	N/P	N/P
56f	head + MAG	y	y	y	N/P	y	N/P	N/P	N/P	N/P
56g	head + MAG	y	y	y	y	y	N/P	N/P	N/P	N/P
56h	head	y	y	y	y	y	y	y	y	N/P
56i	testis + MAG	y	y	y	y	N/P	N/P	N/P	N/P	N/P
57a	head + ovary + testis	y	y	y	y	y	N/P	N/P	N/P	N/P
57b	head	y	y	y	y	y	N/P	N/P	N/P	N/P
57c	head	y	y	y	y	y	N/P	N/P	N/P	N/P
57d	Not expressed	y	y	y	y	y	y	y	y	y
57e	Not expressed	y	y	y	y	y	N/P	N/P	N/P	N/P
99a	head + ovary + testis	y	y	y	y	y	y	y	y	N/P
99b	head	y	y	y	N/P	y	N/P	N/P	N/P	N/P
99c	head	y	y	y	y	y	y	y	N/P	N/P
99d	head	y	y	y	y	y	N/P	N/P	N/P	N/P

Phylogenetic tree of *Drosophila* as found on FlyBase http://flybase.org/blast/ for use with table 3.6.3 and 3.6.4 (shows a basic rooted tree with relatedness but not related evolutionary distance).

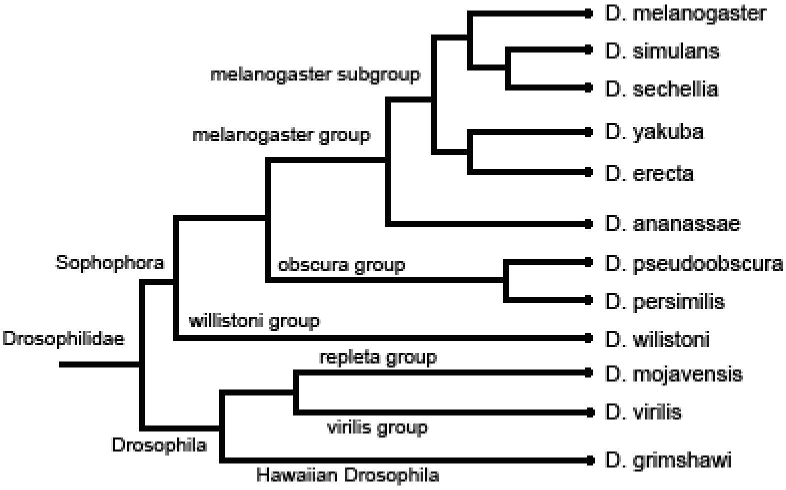

Table 3.6.4 Odorant binding proteins found expressed in the head and reproductive organs (repro) and in which species there are putative orthologues. I used nucleotide searches in the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) to test the *D. melanogaster* OR and Obp sequences of interest against the other species of *Drosophila*. I then tabulated BLAST E-value hits. I used the following general criteria for homology searching.

i] Exact and very closely matched hits had an E-value of ~ 0
ii] A hit was also considered very similar to the query sequence if the E-value was between $1x10^{-50}$ and 0
iii] N/P shows the odorant gene was not present in this species with an E-value of $1x10^{-50}$ and below

Drosophila species are from left to right, *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. erecta, D. yakuba*, *D. ananassae*, *D.persimilis* and *D. pseudoobscura*

Obp	Tissue expressed in	Species of *Drosophila* Obp is found in							
		D.mel	*D.sim*	*D.sec*	*D.ere*	*D.yak*	*D.ana*	*D.per*	*D.pse*
19a	head	0	0	0	1.80e-121	4.40e-119	N/P	N/P	N/P
19b	head	0	2.20e-88	0	6.20e-135	1.10e-142	9.10e-51	N/P	N/P
19c	head + repro	0	0	0	0	0	N/P	N/P	N/P
56a	head + repro	0	0	0	3.70e-157	2.80e-136	5.10e-73	N/P	N/P
56d	head + repro	0	0	0	0	0	N/P	N/P	N/P
56e	head + repro	0	0	0	1.40e-100	7.90e-130	N/P	N/P	N/P
56f	head + repro	0	4.40e-162	4.00e-156	N/P	1.20e-57	N/P	N/P	N/P
56g	head + repro	0	0	0	1.10e-140	3.40e-122	N/P	N/P	N/P
56h	head	0	0	0	1.80e-142	8.10e-154	9.20e-55	3.50e-88	3.50e-88
56i	repro	0	0	0	1.10e-66	N/P	N/P	N/P	N/P
57a	head + repro	0	0	0	1.60e-152	1.20e-128	N/P	N/P	N/P
57b	head	0	0	0	4.40e-162	1.20e-165	N/P	N/P	N/P
57c	head	0	0	0	0	0	N/P	N/P	N/P
99a	head + repro	0	0	0	0	5.90e-171	3.60e-58	1.10e-70	2.60e-68
99b	head	0	0	0	N/P	0	N/P	N/P	N/P
99c	head	0	0	0	9.90e-177	3.90e-176	5.40e-55	3.40e-53	N/P
99d	head	0	5.30e-167	1.00e-152	1.20e-124	1.80e-154	N/P	N/P	N/P

Table 3.6.5 – Odorant binding proteins not occurring in clusters that have expression in the head, the male accessory glands and in both together, and the species of *Drosophila* with putative orthologues.

Organ expressed is the tissue of the organ where the Opb is expressed either the head and/or the male accessory gland (MAG).

i] Exact and very closely matched hits had an E-value of ~ 0
ii] A hit was also considered very similar to the query sequence if the E-value was between $1x10^{-50}$ and 0
iii] N/P shows the odorant gene was not present in this species with an E-value of $1x10^{-50}$ and below

Drosophila species are from left to right, *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. erecta, D. yakuba* and *D. ananassae*.

Obp	Tissue	*D.mel*	*D.sim*	*D.sec*	*D.ere*	*D.yak*	*D.ana*
59a	Head	0	0	0	0	0	3.30e-87
83cd	Head & MAG	0	0	0	1.50e-139	1.70e-146	N/P
22a	MAG	0	0	4.40e-125	N/P	N/P	N/P
8a	Head & MAG	0	0	0	9.80e-124	5.20e-147	N/P

Chapter 4 Silencing internal odorants affects mating latency but not responses to rivals or offspring viability.

4.1] Abstract

Olfaction is an important sense and this type of chemosensory communication plays a vital role in conveying information from the environment to the individual. The ability to engage in courtship may be an indicator of a male's general viability, and mating latency has also been suggested as a good indicator of mating propensity. The odorant receptor *Or83b* is expressed in approximately two thirds of all olfactory receptor neurons in *Drosophila* and acts as a type of odorant co-receptor. My results suggest that males lacking *Or83b* show altered mating latency and mating duration, but also suggest that the lack of *Or83b* does not affect a male's responses to rivals or a male's overall fertility.

4.2] Introduction

Behaviour that is shaped by the perception of odours can be essential for an organism's survival, reproduction and therefore overall fitness (Mackay & Fry 1996). Olfaction and chemosensory communication play vital roles in conveying information from the environment to the individual (Brennam 2004). For example mice can be trained to discriminate between the odours in the urine of congenic versus wild-type mice (Yamazaki et al. 1979). This is consistent with the idea that there is genetic variation for the receipt and emission of chemical signals. It is thought that genes encoding proteins involved in stimulus recognition and processing represent only a tiny fraction of those involved in controlling olfactory behaviour (Anholt et al. 1996). It is predicted that mutations in olfactory loci will have phenotypic consequences ranging from the virtually undetectable to major effects, and that such mutations may also have pleiotropic effects. For example mutations in odorant receptor 83b (*Or83b*) of *D. melanogaster* have been shown to lead to severe olfactory defects by altering adult metabolism, but also to enhance stress resistance and extend lifespan in healthy animals (Libert et al. 2007).

Drosophila melanogaster is an ideal model system for investigating odour guided behaviour, its odorant binding proteins (Obps) are expressed in the chemosensory sensilla of the third antennal segment (funiculus), suggesting an important role in olfactory discrimination (Pikielny et al. 1994). Olfactory neurons are activated by different odorants which then generate distinct patterns of neural activity (Anholt et al. 1996). Generally the olfactory receptors (ORs) of insects, although related, are very divergent with very little sequence conservation across species. One exception is the *Drosophila* odorant receptor *Or83b*, which is an unusual OR in that it is co expressed together with all odorant receptors and shares between 67 and 78% of its amino acids with the primary structures of the moth *Heliothis* receptor HR2 and the AgamGPCRor7 receptor. This high degree of conservation presumably reflects the unique role for this odorant receptor in olfaction across species (Vosshall et al. 1999; Krieger et al. 2003).

An individual odorant receptor is not dedicated to the recognition of only a single odour; ORs can be activated by many different odours (Anholt & Mackay 2001). Indications are that olfactory function in response to a broad range of odours in *Drosophila* requires expression of a conventional OR along with *Or83b* in most olfactory neurons (Krieger et al. 2003; Larsson et al. 2004). Data from Larsson et al. (2004) support a model in which *Or83b* acts in concert with other ORs in responding to many different odorants. Larsson et al. (2004) argue against the idea that *Or83b* acts independently to recognise particular odorants or a particular odorant (Larsson et al. 2004). *Or83b* is expressed in many of the cells of the funiculus within the antennae of *Drosophila melanogaster* (Vosshall et al. 1999) and *Or83b* is co-expressed with other ORs in a large proportion of Odorant Sensory Neurons OSNs (Krieger et al. 2003; Vosshall et al. 1999). Larsson et al. (2004) found that in both the antenna and maxillary palp each *Or83b* positive OSN expresses *Or83b* along with at least one conventional OR gene. This also suggests that *Or83b* acts in concert with other ORs to recognise a wide range of odours (Larsson et al. 2004).

The presence of ORs and Obps in the reproductive system could indicate a novel role for odorants in determining fertility. Both pheromones and food odours can act together to attract flies to a feeding site (Bartelt et al. 1985) and as a widely expressed odorant receptor, *Or83b* could be used to determine the presence of food and or pheromones. In this context *Or83b* seemed an ideal choice for exploring the role of internal odorants on rivalry, reproduction and offspring viability. I decided to use males lacking *Or83b* to determine if they have lowered fertility and whether they respond to females and to male rivals in the same way as did wild-type *Drosophila* control males.

4.3] Materials and Method

4.3.1 Does the function of internal odorants in male *Drosophila* affect their behaviour and their fertility?

Control flies used were from the wild-type Dahomey strain, while mutants were *Or83b* knock in mutants (Chapter 2, section 2.1.3). Larvae and fly rearing was carried out as described in Chapter 2. Males and females were collected at eclosion using ice anaesthesia. Females were housed in groups of 10 on a normal yeast diet (100% brewer's yeast per litre of medium) with added live yeast granules. All females used in the experiment were the wild-type Dahomey strain. Males were housed singly on a normal yeast diet as above. The sample size was 40 males per group of each control and *Or83b* mutant treatment and they were kept in these conditions for 2 days.

Males were placed individually into experimental vials supplemented with yeast liquid 24 hours prior to mating, using ice anaesthesia. The SYA medium had 4 grams per litre of charcoal added to enable eggs to be seen and counted (recipe in Appendix 4). At mating a single female was aspirated into each vial. The introduction time of the female and the start and finish times of mating were recorded to the nearest minute. Mating latency and duration times were calculated. Data from flies that didn't mate within 2 hours and any matings that occurred for less than 5 minutes were discarded. Females were left in the vials for 24 hours after mating and the eggs were then counted. The vials were then left for a further 11 days for offspring to emerge, these were also then counted.

4.3.2 Does the function of internal odorants affect mating behaviour, rivalry and reproduction and ultimately offspring viability in *Drosophila melanogaster* males?

Flies for this experiment were from the same stock as the experiment above; rearing and collection was carried out in the same manner. Males were housed with or without a rival; all rivals were wild-type Dahomey strain and were wing clipped to enable identification. The sample size for each group was 40 males of each control and *Or83b* mutants and they were kept with or without rivals for 4 days. Females were placed in mating vials 24 hours before experiment using ice anaesthesia and aspirated into vials with charcoal after mating took place. Experiment ales were placed with females in mating vials while rival males were discarded. Experimental parameters were the same as above, I recorded mating latency and duration, egg and offspring counts.

4.3.3 Statistical Analysis

Statistical analysis was performed using SPSS v 16. Mating latency and mating duration data were tested for normality using Kolmogorov-Smirnov tests and for homogeneity of variance using Levene's tests. All data were appropriate for ANOVA, and Pearson correlations were performed on egg and mating duration data.

4.4] Results

4.4.1 Does the function of internal odorants in male *Drosophila* affect their behaviour and their fertility?

The *Or83b* mutant males took significantly longer to mate than the wild-type controls (ANOVA $F_{1,56}$=8.063, p=0.006, figure 4.6.1). The *Or83b* mutants also mated for significantly longer than the wild-type controls ($F_{1,56}$=14.370, p<0.001, figure 4.6.2). In addition half of the *Or83b* mutants didn't mate within the observation period of 2 hours. Both egg laying and offspring production were not significantly different for the control wild-type and *Or83b* mutant flies, although the percentage of males that had no viable offspring was higher in the mutants than the controls (table 4.7.1). There was no correlation between the number of eggs laid and mating duration (Pearson Correlation r=0.179, N=58, p=0.178), but there was a significant correlation between the number of eggs laid and the number of viable offspring produced (r=0.535, N=58, p<0.001).

4.4.2 Does the function of internal odorants affect mating behaviour, response to rivals and reproduction and ultimately offspring viability in *D. melanogaster* males?

There was a significant difference in mating latency between *Or83b* mutant and control flies ($F_{1,119}$=54.859, p<0.001, figure 4.6.3) with the *Or83b* mutant males taking significantly longer to mate. However, there was no difference in mating latency if *Or83b* lacking males were kept with or without rivals ($F_{1,119}$=0.064, p=0.801). There was a significant difference in mating duration between the control and *Or83b* treatment groups ($F_{1,119}$=75.904, p<0.001, figure 4.6.4) and between the groups kept with and without a rival ($F_{1,119}$=8.565, p=0.004, figure 4). Males kept with rivals mated for longer than those kept without rivals prior to mating, as has been seen previously (Bretman et al. 2010). A slightly higher proportion of the *Or83b* lacking males mated in this experiment than in the experiment described above (28 out of 40 instead of only 21 out of 40). There was no correlation between the number of eggs laid and mating duration (r=0.025, N=123, p=0.780), but there was a significant correlation between the number of eggs laid and the amount of viable offspring produced. (r = 0.790, N=119, p<0.001).

4.5] Discussion

The results showed that the *Or83b* mutant males consistently took significantly longer to mate and then mated for longer than did the wild-type controls. *Or83b* mutants mated for longer when kept with rivals. There was no difference in the number of eggs laid between the mutants that mated and the control groups either with or without rivals. There was a correlation between the amount of eggs laid and the amount of offspring indicating most eggs were viable and that the lack of *Or83b* did not affect fecundity. The results suggest therefore that *Or83b* plays a role in determining a male's mating latency and duration, but not his overall fecundity.

A male's readiness and ability to engage in courtship may be indicative of his general viability. Mating latency has also been suggested as a good indicator of mating propensity (Cook & Cook 1975). It has been suggested that as *Drosophila* has broadly tuned receptors this could compensate for the limited number of odorant receptors it has in comparison to mammals, whilst still enabling flies to detect an equivalent range of odorants (Elmore et al. 2003). *Or83b* acts as a type of odorant co-receptor and is expressed in approximately two thirds of all olfactory receptor neurons in *Drosophila* (Vosshall et al. 1999), other odorant receptors are therefore expressed in far fewer cells (Vosshall et al. 1999; Clyne et al. 1999). Hence *Or83b* functions in concert with other odorant receptors in detecting odorants. Elmore et al. (2003) suggests for example that *Or83b* could work with *Or43b* as an obligate chaperone or that *Or83b* acts as co-receptor (Elmore et al. 2003). The *Or83b* mutant antennae show no odour-evoked responses to a panel of odorants that elicit robust responses from wild-type antenna. Also *Or83b* mutation disrupts *Drosophila* behavioural responses to many odours (Larsson et al. 2004). My results suggest that these altered behavioural responses include mating latency and mating duration. However, my results also suggest that *Or83b* does not affect responses to rivals or a male's overall fecundity.

Many investigations have shown that mutants are on the whole at a disadvantage in competitive mating assays. Often this is attributed to a reduction in fitness of the mutant when compared to the wild-type control (Connolly et al. 1969). Males from the sex-linked *white eye* mutation are considered at a disadvantage, and Connolly also found that the *yellow* bodied mutant males had altered wing vibration patterns, which affected their courtship display. Even this minor reduction in mean vibration length in *yellow* males resulted in their being at a considerable disadvantage (Connolly et al. 1969). The *Or83b* mutants used in these experiments were comparable with the wild-type controls in terms of progeny production, but were slower to mate. It is possible that mating latency differences could indicate the female's unwillingness to mate with the mutant, as well as a courtship deficiency in the *Or83b* males. Another mutant of *Drosophila* called *fruitless* for example, has been shown to have altered courtship steps prior to mating, with the result that many *fruitless* males cannot mate. Of those that can mate the mating latency is up to 4 times longer than the normal average mating time and there is frequent infertility (Lee et al. 2001). There was no evidence in this study that the *Or83b* males suffered lowered fertility.

One caveat to this study is that the *Or83b* mutants have not yet been back crossed into the wild type *Drosophila* population. It will therefore be important to repeat these tests with fully backcrossed stocks to validate these results on mating latency and duration. However, the results serve to show that there are no obvious effects on fertility that result from the lack of *Or83b*, therefore no obvious indication that the lack of this important odorant receptor plays an internal role within the reproductive system in determining a male's fertility.

Many odorants are expressed within the reproductive system; I wanted to test for the global effect of the loss of odorant detection on a male's fertility. Recent studies have indicated that pheromone detection may be linked to genes on the Y chromosome. There are indications that the Y chromosome influences pheromone detection as well as odorant binding proteins suggesting a role for the Y chromosome in mating behaviour (Wang et al. 2009; Jiang et al. 2010). Proteins associated with odorants and pheromone binding have been found expressed in tissue specifically in the male reproductive system (Chapter 3) (Takemori & Yamamoto 2009). The human odorant receptor hOR 17-4 has been found in both the nose and the testes (Vosshall 2004). A study into stallion mating behaviour has shown that vision is more important than olfaction (Anderson et al. 1996), but it is thought to play a more significant role in the sexual behaviour of ducks. Ducks with their sense of smell inhibited copulated less than those with their sense of smell intact, other behaviours for example, aggression were not affected by the loss of olfaction (Balthazart & Taziaux 2009).

4.6] Figures

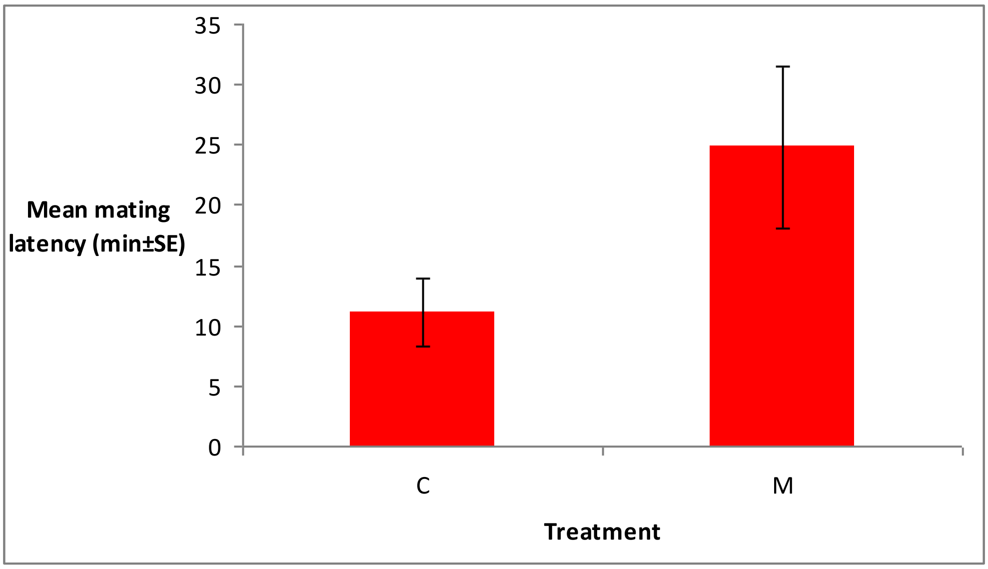

Figure 4.6.1 Mean mating latency (± SE) of wild-type control (C) and *Or83b* mutant (M) male *D. melanogaster*. Mating latency is the time from introduction of the flies until the start of mating.

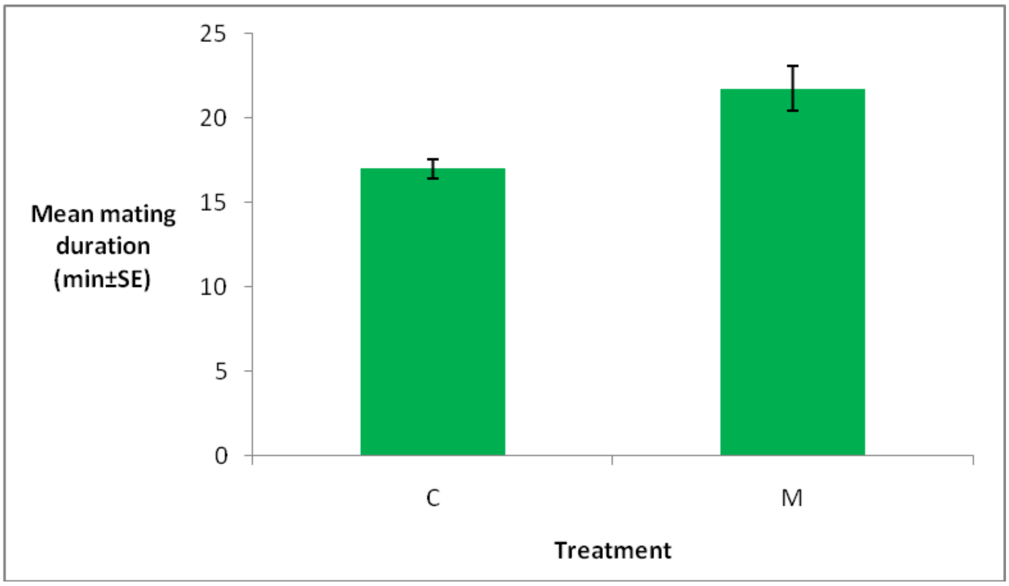

Figure 4.6.2 Mean mating duration (± SE) of wild-type control (C) and *Or83b* mutant (M) male *D. melanogaster*. Mating duration is the time from the start to the finish of mating.

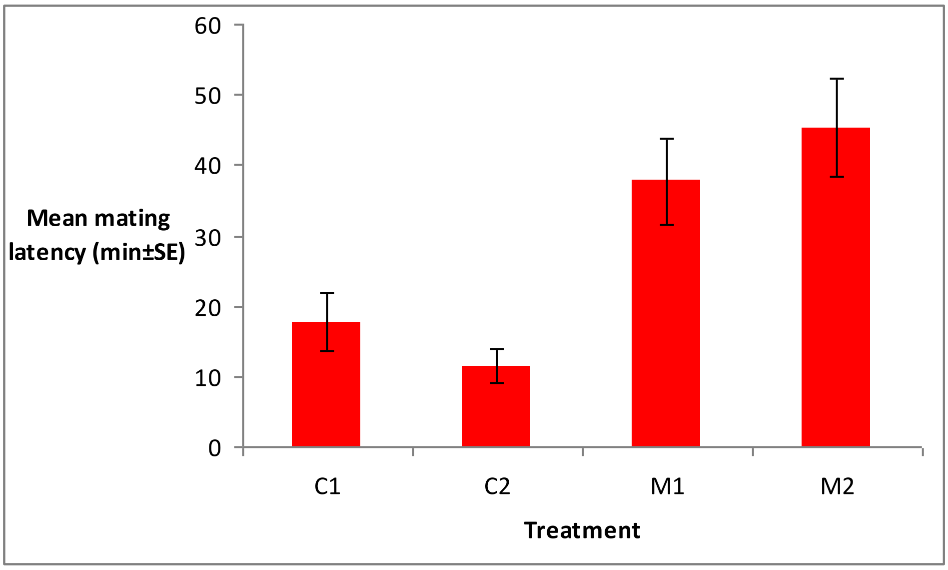

Figure 4.6.3 Mean mating latency (± SE) of wild-type control (C) and *Or83b* mutant (M) male *D. melanogaster* held either with (2) or without (1) rivals prior to mating. Mating latency is the time from introduction of the flies until the start of mating.

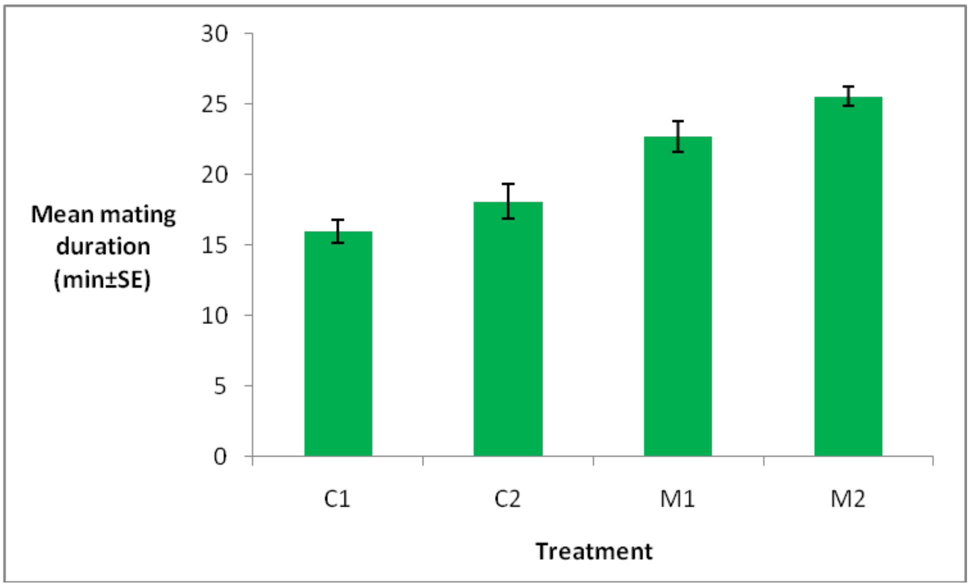

Figure 4.6.4 Mean mating duration (± SE) of wild-type control (C) and *Or83b* mutant (M) male *D. melanogaster* held either with (2) or without (1) rivals prior to mating. Mating duration is the time from the start to the finish of mating.

4.7] Tables

Table 4.7.1 Percentage of *D. melanogaster* males with no viable offspring in the control (C) wild-type and the *Or83b* mutant (M).

Treatment	Percentage that had no viable offspring
C	2.70
M	14.29

Chapter 5 Olfaction as a cue used by males to detect rival males in *Drosophila melanogaster.*

5.1] Abstract

In any animals or insects olfaction is required in order that chemical information from the external environment can be communicated to the internal environment of the individual. Olfaction is proposed to be important in the detection of conspecifics particularly in the context of reproduction. Antennae are the main external olfactory organs of *Drosophila melanogaster* and house the main odorant receptors. I tested whether removal of the antennal segments containing these receptors affected a male's ability to detect the presence of rivals, as determined by a male's mating duration, (which is extended if rivals are detected). I found that there was no significant difference in the mating duration of males with and without antennae that were exposed or not exposed to rivals prior to mating. Hence there was no evidence that olfaction alone communicates the presence of a male's rivals. Males could have compensated for the loss of olfaction by using visual, tactile, auditory or gustatory cues.

5.2] Introduction

Olfaction is essential for chemical information from the external environment to be transferred to the internal environment. General odorants are important for indicating the presence of food, and pheromones released from an individual can signal rivals or potential mates within a population (Hoffmann 1985; Vosshall 2000; Larsson et al. 2004). Both odorants and pheromones are detected by olfactory sensory neurons (OSNs) in the olfactory system. Mammals detect odorants through the nasal olfactory epithelium via the main olfactory system. Some non-primate species, for example rodents, have a second olfactory system called the vomeronasal pathway, which detects signals via the vomeronasal organ (VNO) (Halpern & Martinez-Marcos 2003). Most insects also have two olfactory organs, these are the antenna and the maxillary palp (Singh & Nayak 1985; Charro & Alcorta 1994). However there is also some evidence to suggest that the labial palp thought to be a taste organ can also sense odours (Kwon et al. 2006). The study of olfaction in insects is an attractive model because a large amount of olfactory driven behaviours are under the control of a nervous system that is somewhat simpler than that of mammals (Benton 2006). The olfactory system of the fruit fly *D. melanogaster* is sensitive to a large variety of odorants and these airborne molecules drive behaviours that include mate attraction and responses to rivals (Carlson 1996).

5.2.1 Mechanism of odorant communication in *Drosophila*

The antenna of *D. melanogaster,* as in other fruit flies, is made up of six segments, each of which appears to have distinct functions. These include audition (hearing) which is signalled by the arista and the second antennal segment (Si Dong et al. 2002). The third antennal segment (the funiculus) along with the maxillary palps are the main olfactory sense organs (Shanbhag et al. 1999). OSNs are linked to the antennal lobe in the central brain by axons and are found in the antennae and the maxillary palp (Hallem & Carlson 2004; Spletter & Luo 2009). The maxillary palp receptors receive olfactory information in a similar manner to the antennae; the maxillary palp axon projects into the brain but to a separate glomerulus in comparison to the antennal axons (Charro & Alcorta 1994). The OSN dendrites which are called sensillae, are present in the sensory organs where they are exposed to the environment (Spletter & Luo 2009). It is via these sensillae distributed over the surfaces of the antennae and the maxillary palp that olfactory recognition occurs (Carlson 1996; Clyne et al. 1997*;* Vosshall et al. 1999). There are about 450 olfactory sensillae found on the third segment of the antenna and about 80 covering the maxillary palp (Carlson 1996), Charro and Alcorta (1994) showed that as an olfactory organ the maxillary palp had a decreased sensitivity to odours by a factor of 1:10 – 1:100 compared to the antennae (Charro & Alcorta 1994). There are three basic types of sensillae: basiconic, coeloconic and trichoid. Each appears to respond to different types of odorants in the environment (Spletter & Luo 2009). It is thought that courtship behaviour in *D. melanogaster* is largely dependent on pheromones (Stocker & Gendre 1989). Consistent with this, the third antennal segment of *Drosophila* is sexually dimorphic with regards to sensillum type, males have about 30% more trichoid sensillae than females, which suggests this plays a role in courtship (Venkatesh & Singh 1984; Stocker & Gendre 1989; Shanbhag et al. 1999).

Both the antennae and maxillary palps carry odorant receptors which are responsible for the detection of odour molecules, i.e. smells. When these receptors are activated a cascade ensues ultimately producing a nerve impulse which is transmitted to the brain. Most odours activate more than one odorant receptor and the whole system is capable of detecting and then distinguishing between an almost infinite number of odorant molecules (Gaillard et al. 2004). Flies with mutations affecting the development of olfactory sensilla have defective odorant receptors or none at all and so have defective olfactory perception (Riesgo-Escovar et al. 1997). Work with a loss of function mutant called *antennaless* by Begg and Hogben confirmed that the lack of antennae can be correlated with a lack of olfactory reception to food odours (Begg & Hogben 1946).

5.2.2 Odorant perception and male responses to rivals

Males that are exposed to other males prior to mating show significantly extended mating duration when they subsequently mate (Bretman et al. 2009). However, the cues that males use to detect rivals have not yet been identified. Given that odorant perception is predicated to be important in the detection of other males, I hypothesised that it might play a role in the detection by males of their rivals. I tested this idea by examining whether the removal of antennal segments that include odorant receptors thought to be important for signalling pheromones would alter the way in which males respond to rivals.

5.3] Material and Methods

5.3.1 The effect of funiculus removal on the behaviour of *Drosophila* males towards rivals and potential mates

Fly rearing was carried out as described in the general method and materials section of Chapter 2. Males and females were collected at eclosion using ice anaesthesia. Males and females were housed in groups of 5 on a normal yeast diet (see Chapter 2 & Appendix 4) with added live yeast granules. The sample size was 40 males per group.

Males were selected randomly and anaesthetized using CO_2, they then either had the third segment of their antenna (funiculus) surgically removed or were placed for the same length of time under anaesthesia without surgical manipulation as a control treatment. Control and funiculus-less males were grouped singly (no rivals) or in groups of two (rivals). Males were kept in these respective conditions for 5 days.

Females were placed individually into experimental vials containing yeast supplement 24 hours prior to mating using ice anaesthesia. Males were then anaesthetized with ice to enable identification of the individuals with no antennae from their rivals, males from the control groups were also anaesthetized for the same length of time to ensure continuity.

At mating a single male was aspirated into each vial. The introduction time of the male and the start and finish times of mating were recorded to the nearest minute. Mating latency and duration times were calculated. Data from flies that didn't mate within 3 hours and any matings that occurred for less than 5 minutes were discarded.

5.3.2 The effect of funiculus and arista removal on the behaviour of male *Drosophila* towards rivals and potential mates

This experiment was the same as above except an extra group of males with their aristae removed were added. Flies were from the same stock as the experiment above; rearing and collection was carried out in the same manner. Males were selected randomly and anaesthetized using CO_2; groups of males had either the funiculi or aristae surgically removed, in another group, males were placed for the same length of time under anaesthesia without surgical manipulation, as a control treatment. Control, funiculus-less and arista-less males were grouped singly (no rivals) or in groups of two (rivals). Rival males used in all groups had their wings clipped under CO_2 anaesthesia to allow identification of the flies to be used in the experiment. Males were kept in their respective conditions for 2 days prior to mating.

For the matings themselves females were placed individually into experimental vials with yeast 24 hours prior to mating using ice anaesthesia and mating was carried out the same as in the first experiment described above. Data from flies that didn't mate within 3 hours and any matings that occurred for less than 5 minutes were discarded.

5.3.3 Statistical analysis

Statistical analysis was performed using SPSS v 16. Mating latency and mating duration data were tested for normality using Kolmogorov-Smirnov tests and for homogeneity of variance using Levene's tests. All data were found to be appropriate for ANOVA analysis.

5.4] Results

5.4.1 The effect of funiculus removal on the behaviour of males towards rivals and potential mates

Mating latency: There were no significant differences in mating latency due to treatment (control versus funiculus removed), (ANOVA $F_{1,39}=0.796$, $p=0.378$). In general, males kept with rivals were significantly slower to mate ($F_{1,39}=4.148$, $p=0.049$) (figure 5.6.1 & table 5.7.1), however there was no significant interaction between the mating latencies of the groups for males held with and without rivals and those with and without a funiculus ($F_{1,39}=1.325$, $p=0.257$).

Mating duration: There were no significant differences in mating duration between males with and without a funiculus ($F_{1,39}$=2.302, p=0.137). Control males kept with rivals mated for longer than those kept singly, as was expected (Bretman et al. 2010) however flies lacking a funiculus mated for less time following exposure to rivals (figure 5.6.2 & table 5.7.2) though neither of these results were statistically significant ($F_{1,39}$=0.179, p=0.674). There was also no significant interaction in mating duration between any of the treatments ($F_{1,39}$=1.994, p=0.166).

5.4.2 The effect of funiculus and arista removal on the behaviour of males towards rivals and potential mates

Mating latency: There was a significant difference in mating latency between flies with intact antennae, those with their aristae removed and those with their funiculus removed ($F_{2,191}$ =3.612, p=0.029). There was also a significant difference in mating latency in flies kept with and without rivals ($F_{1,191}$=13.766, p<0.001). Control males were significantly slower to mate when housed with rivals, as were the flies lacking aristae. Males lacking a funiculus did not appear to respond to rivals (figure 5.6.3 & table 5.7.3). There was a slight difference in mating latency between the two groups of males held with and without rivals and between males with and without antennal structures ($F_{2,191}$=2.366, p=0.097), however, these differences were not significant.

Mating duration: There were no significant differences in mating duration between control, arista-less and funiculus-less males ($F_{2,191}$=0.220, p=0.803). As expected, however, there was a significant difference between males housed with and without rivals ($F_{1,191}$=12.353, p=0.001) males that were kept with rivals mated for significantly longer than those kept singly, for all treatments (figure5.6.4 & table 5.7.4). There was no significant interaction in mating duration between the groups ($F_{2,191}$=0.491, p=0.613).

5.5] Discussion

The main results were that mating latency was affected by the lack of a funiculus but not by the lack of an arista. A significant extension was seen in the mating latencies of males kept with rivals in both wild-type (control) *D. melanogaster* and males lacking an arista; males kept with rivals also had a slower mating latency than males kept singly. However mating latencies were similar for flies that had their funiculus removed whether they were kept in the presence or absence of rivals, indicating the removal of the funiculus had an effect on the ability of males to respond to rivals in terms of mating latency.

Findings were consistent with previous work (Bretman et al. 2010), mating duration for flies kept with rivals prior to mating was significantly longer than those kept on their own, however I found that this was not affected by the presence or absence of the funiculus. Previous data show that exposure to rival males prior to mating increases competitive reproductive success (Bretman et al. 2010). The mating duration results reveal that the flies with the funiculus removed were indistinguishable from wild-type males in this respect. Hence the data suggest that males lacking antennae did perceive and respond appropriately to rivals in terms of mating duration.

In contrast, the males lacking a funiculus did not respond in a normal manner to rivals in terms of mating latency. The length of time taken for the males lacking a funiculus to mate may not be due to a lack of odorant recognition, because fly hearing was also affected by the funiculus removal (Boekhoff-Falk 2005). It has been established that the arista and third segment of antennae are essential for fly hearing; both structures vibrate in response to sound, information is then conducted to the second antennal segment for translation (Todi et al. 2004; Si Dong et al. 2002). It is the rotation of the funiculus within the second segment that is necessary for the transmission of sound from the arista to Johnston's organ (JO) located at the joint between the second and third segments (Si Dong et al. 2002). Therefore the removal of these structures, the arista and the funiculus, could affect hearing as well as the ability to smell. It was observed during experiments that the flies that had their antennae removed were less mobile than those with antennae still intact. The arista and funiculus appear to have a role in gravity sensing, flies detect changes in their orientation relative to gravity through the position of the arista (Kamikouchi et al. 2009; Eatock 2009) which might explain why these males were apparently more sedentary. By removing the arista the ability of the fly to walk upwards is decreased (Kamikouchi et al. 2009). Although both the arista and funiculus are needed to hear it would appear that the removal of the funiculus affects male mobility to a greater extent. I suggest that fly balance may be compromised by the removal of the funiculus and an additional problem for males may be in courting females in addition to being unable to recognize rival males. Smell is not the only sense affected by removal of the arista and funiculus however as these males still responded to rivals as shown by the mating duration data indicating that other cues are used in the detection of rivals.

During the first experiment the males were anaesthetized using ice just before the matings occurred, in order to identify which were the males with the funiculus removed. Experiments have shown that ice anaesthesia affects a male's mating latency for up to 1 hour after treatment (Appendix 1). The antenna experimental flies were anaesthetized twice with ice and were also subjected to CO_2 while having their antennae removed. Control males were also held under CO_2 anaesthetization while experiments were conducted. Exposure to CO_2 appears to have had a detrimental effect on a male's mating and reproductive capacity, and lifespan of the flies used. I found that following CO_2 exposure many flies didn't mate during the 3 hours of allotted time. Interestingly, many flies also had no offspring even when left for 7 days in pairs. Some males across all the groups also died during the experiment causing small numbers in sample sizes. CO_2 has been shown to reduce fecundity, mating success and longevity; it has also been attributed to impaired locomotion and even feeding behaviours in the German cockroach *Blattela germanica* (Nilson et al. 2006).

Overall the tests results suggest that males do not respond to rivals based on smell alone. The fact that males lacking a sense of smell still respond to rivals indicates that they use other senses to compensate the loss of the sensory function. The compensation could occur through other sensory inputs for example gustatory and/or visual. The maxillary palps are also of interest as an olfactory organ. Stocker suggested that basiconic sensilla found on the maxillary palps are good candidates for anti-aphrodisiac receptors (Stocker & Gendre 1989). De Bryune (1999) also found that cis-vaccenyl acetate (cVA) an active pheromone in *Drosophila,* produces a response in one of the two neurons of the maxillary palps, although it is not as strong as the response to some other odours (De Bruyne et al. 1999). The maxillary palps are organs with a reduced sensitivity compared to the antenna but they may play a compensatory role for odorant/pheromone detection if the antennae are damaged. The main taste organ is two labial palps covered in taste bristles (sensilla) found at the end of the proboscis. Because odorant binding proteins (Obps) are expressed in the taste bristles on the labellum, tarsi and wings it is thought they have similar functions to the olfactory sensilla in *Drosophila* (Shanbhag et al. 2001). It is thought that labial palps can also sense odours (Kwon et al. 2006). Several Obps are expressed in taste sensilla and are believed to play a role in moving chemicals from the environment to neurons which express specific gustatory receptors (Shanbhag et al. 2001).

Other possibilities for cues that might be used by males which were not manipulated in this experiment are gustatory, tactile or visual signals, as mentioned above. The gustatory system of *Drosophila* is not restricted to a single taste organ as with mammals. Taste bristles are also located on the legs and the anterior wing margins of the fly. In all there are about 260 taste sensilla on the body while the labial palps contain just 62. This wide distribution of taste cells throughout the fly's body indicates a critical role for chemosensory stimuli to the fly which could affect male rivalry (Amrein & Thorne 2005). Coating of the male legs with a glue or paraffin especially the forelegs which are likely to possess contact chemoreceptors, leads to a decrease in a male flies response to a female through gustatory cues (Stocker & Gendre 1989) and could also affect rivalry between males. A males response to an odorant will depend on the dose of odorant detected (Devaud et al. 2003). Pheromone-binding protein-related protein 2 (PBPRP2) is not generally produced in epidermal cells except in those of the funiculus, maxillary palp and areas in the labellum. In both the second antennal segment and the arista PBPRP2 is absent from the epidermal cells indicating that the expression of PBPRP2 by the epidermis may be related to olfaction in some way (Shanbhag et al. 2001). Surroundings and activity are also important in odour recognition, Devaud et al. found that odour perception is processed differently during exploration flights than it is at feeding sites (Devaud et al. 2003). And although visual cues have not been found to influence courtship of a female (Tompkins et al. 1983) it is possible that seeing another male could affect male rivalry.

I conclude that a *Drosophila* male loses its sense of smell when the funiculus is removed and it may lose its hearing and sense of balance when either the arista on its own or the funiculus and arista are removed. But I have shown that even with these losses males can still detect rivals in some way and respond to them. I believe that males may use their maxillary palps for some odour detection and/or gustatory system or even the visual system to compensate for the loss of olfaction caused by the removal of the funiculus.

5.6] Figures

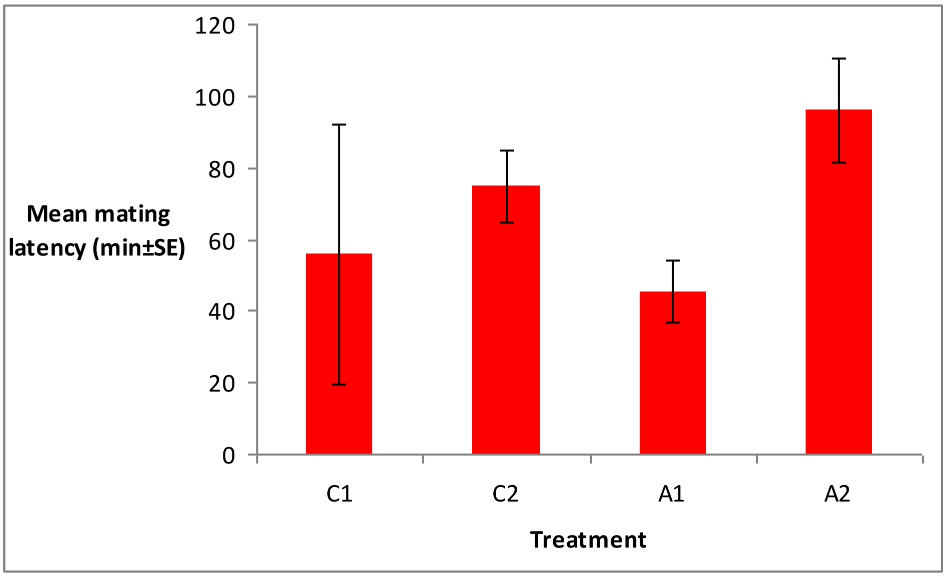

Figure 5.6.1 Mean mating latency (± SE) of male *D. melanogaster,* wild-type control unmanipulated (C) or antenna-less males (A) either in the presence (2 males per vial) or absence (1 male per vial) of rivals, prior to mating. Mating latency is the time from introduction of the flies until the start of mating.

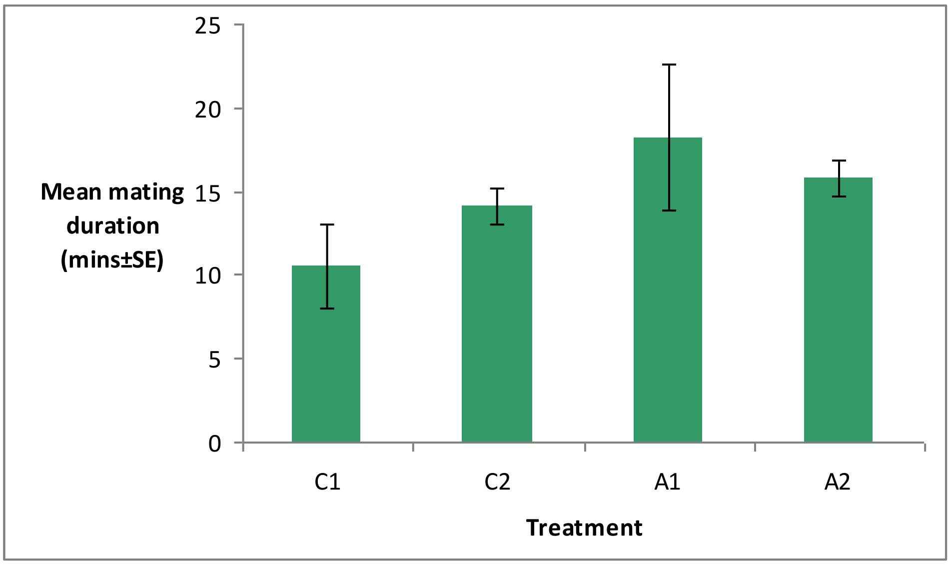

Figure 5.6.2 Mean mating duration (± SE) of male *D. melanogaster,* wild-type control unmanipulated (C) or antenna-less males (A) either in the presence (2 males per vial) or absence (1 male per vial) of rivals, prior to mating. Mating duration is the time from the start to the finish of mating.

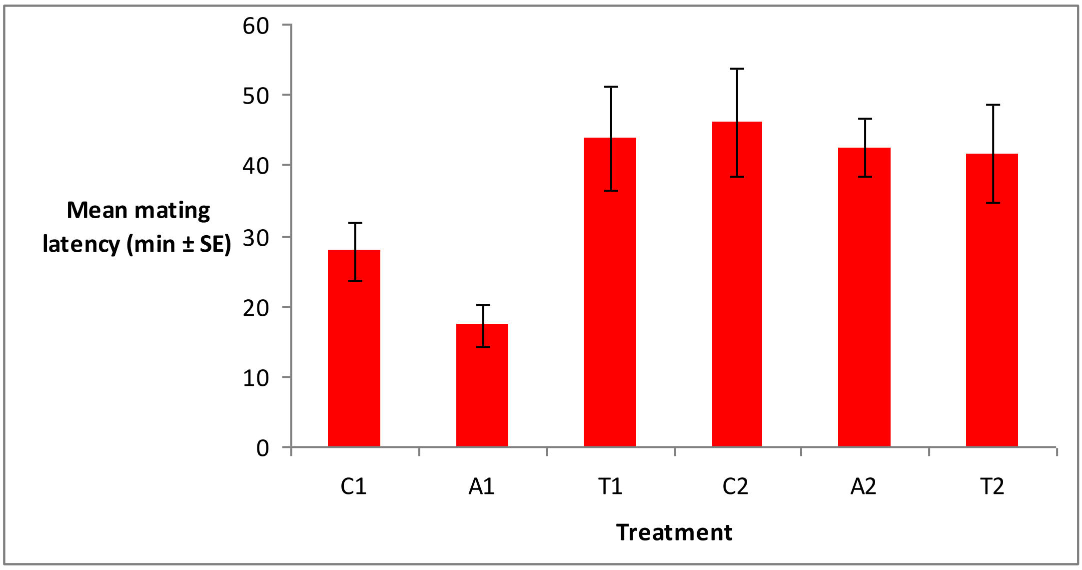

Figure 5.6.3 Mean mating latency (± SE) of male *D. melanogaster*, wild-type control unmanipulated (C), arista-less males (A) or funiculus-less (T) either in the presence (2 males per vial) or absence (1 male per vial) of rivals, prior to mating. Mating latency is the time from introduction of the flies until the start of mating.

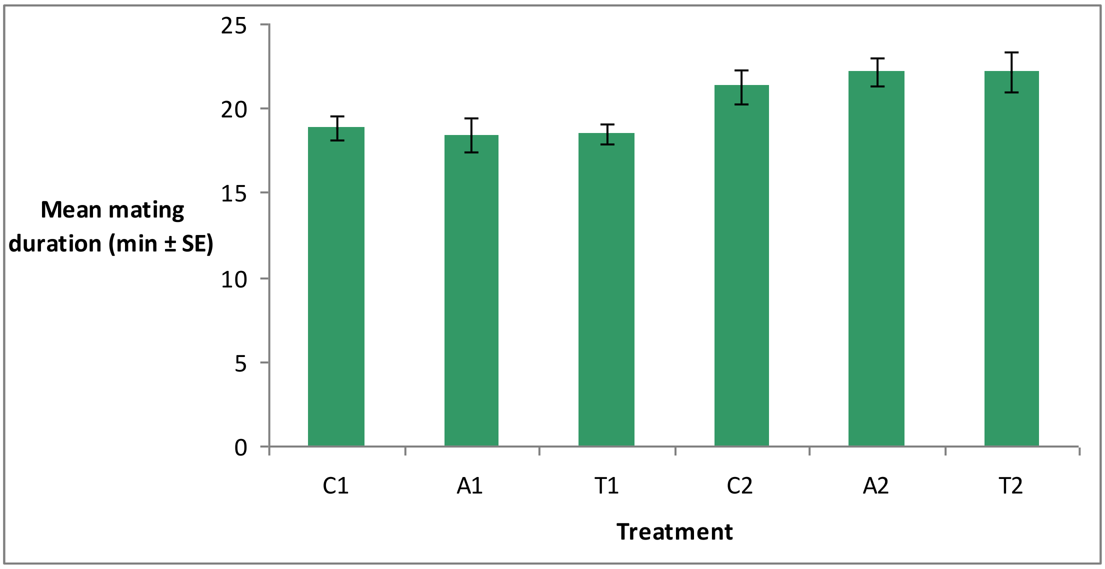

Figure 5.6.4 Mean mating duration (± SE) of male *D. melanogaster*, wild-type control unmanipulated (C), arista-less males (A) or funiculus-less (T) either in the presence (2 males per vial) or absence (1 male per vial) of rivals, prior to mating. Mating duration is the time from the start to the finish of mating.

5.7] Tables

Table 5.7.1 Mean mating latency (± SE) of male *D. melanogaster,* wild-type control unmanipulated (C) or antenna-less males (A) either in the presence (2 males per vial) or absence (1 male per vial) of rivals, prior to mating. Mating latency is the time from introduction of the flies until the start of mating.

Treatment	Mean mating latency	SE
C1	55.75	36.30
C2	74.67	9.91
A1	45.33	8.73
A2	95.87	14.62

Table 5.7.2 Mean mating duration (± SE) of male *D. melanogaster,* wild-type control unmanipulated (C) or antenna-less males (A) either in the presence (2 males per vial) or absence (1 male per vial) of rivals, prior to mating. Mating duration is the time from the start to the finish of mating.

Treatment	Mean mating duration	SE
C1	10.50	2.53
C2	14.07	1.07
A1	18.22	4.37
A2	15.80	1.12

Table 5.7.3 Mean mating latency (± SE) of male *D. melanogaster*, wild-type control unmanipulated (C), arista-less males (A) or funiculus-less (T) either in the presence (2 males per vial) or absence (1 male per vial) of rivals, prior to mating. Mating latency is the time from introduction of the flies until the start of mating.

Treatment	Mean mating latency	SE
C1	27.80	4.15
C2	46.00	7.62
A1	17.21	2.93
A2	42.49	4.06
T1	43.83	7.39
T2	41.63	6.94

Table 5.7.4 Mean mating duration (± SE) of male *D. melanogaster,* wild-type control unmanipulated (C), arista-less males (A) or funiculus-less (T) either in the presence (2 males per vial) or absence (1 male per vial) of rivals, prior to mating. Mating duration is the time from the start to the finish of mating.

Treatment	Mean mating duration	SE
C1	18.86	0.75
C2	21.31	1.02
A1	18.41	0.99
A2	22.16	0.78
T1	18.51	0.58
T2	22.17	1.16

Chapter 6 The effects of adult male nutrition on a male’s reproductive success and ability to respond to rivals.

6.1] Abstract

The level of resources available to adults through diet is expected to determine a male’s overall condition and ability to allocate resources to reproductive activities. I tested these ideas here by altering the amount of protein or sugar available to adult males. I found that a male’s nutritional status significantly altered his mating success but did not affect his responses to rivals. The study shows that male *D. melanogaster* require a high sugar content in their diet to maintain rapid mating latencies and such males mated significantly faster than did males held on a protein only diet or on reduced levels of sugar. Males held on low sugar diets were also found to have lower numbers of offspring compared to other males with normal amounts of sugar in their diet.

6.2] Introduction

The need to gain access to and consume sufficient quantities of food is a fundamental problem that is common to invertebrates and vertebrates. Nutrition can influence an animal’s physiology, behaviour and ultimately their reproductive success (Thompson 1999). *D. melanogaster* are used as a classic insect model for studying many sensory systems, including olfaction and taste. Carbohydrates are a major food source for *D. melanogaster* and many foods that are harmful to humans are also toxic to flies. The detection and uptake of both salts and acids are crucial for electrolyte homeostasis. The fly is extremely sensitive at detecting these chemicals and it is known that *Drosophila* can detect sucrose at levels of 1mM (mini molar) (Amrein & Thorne 2005). The diet of males has been observed to affect male mating success, mating duration and sperm transfer in the Mediterranean fruit fly (Yuval et al. 2002). A pervasive finding is that dietary restriction can lead to an increase in lifespan. This has been consistently seen in work with model organisms such as rodents (Holehan & Merry 1986; Masoro et al. 1991; Masoro 2005) and the fruit fly *D. melanogaster* (Chapman & Partridge 1996), and also occurs across a wide range of different taxa (Carey et al. 2008; Piper et al. 2005; Kirkwood & Rose 1991). In addition many studies have also tested for effects of diet on reproductive behaviour. For example, an adult female’s nutritional status is known to alter her willingness to mate (Chapman & Partridge 1996). There are also some sex differences reported with diet strongly affecting female but not male mating ability (Chippindale et al. 1997). Some effects of diet on adult male reproductive success have been reported. For example, male Mediterranean fruitflies fed low levels of protein mated at a lower frequency than those fed on higher protein diets. Protein deprived males transferred more sperm during mating, but the females to which they mated remated again quickly, resulting in reduced male reproductive success. This indicates that diet is a major factor in determining a male’s reproductive success (Blay & Yuval 1997).

An additional factor crucial for a male’s reproductive success is his ability to respond adaptively to rivals. Males respond to the presence of other males of the same species through mechanisms that can be auditory, visual, olfactory or tactile. A male cricket will increase the amount of sperm transferred to a female when there is sperm competition, in the presence of another male (Gage & Barnard 1996). Males of the Norway rat *Rattus norvegicus* will also ejaculate more sperm in the presence of another male than in the absence of other male rivals (Pound & Gage 2004). Bretman et al. (2009) investigated the responses of males to different numbers of rivals in *D. melanogaster.*

They found that when males were kept with other males prior to mating the duration of their subsequent matings increased significantly (Bretman et al. 2009). Increasing the absolute number of rivals had little effect, exposure to one rival significantly increased mating times, but the addition of more males did not significantly increase or decrease subsequent mating duration (Bretman et al. 2010).

Diet could interact with the ability of males to respond to rivals, by magnifying the effects of trade-offs. There are frequent reports of evolutionary trade-offs between survival and reproduction, for example where restricting the nutrients available gives rise to an increased life expectancy but a lowered reproduction rate, as shown in *D. melanogaster* (Chippindale et al. 1993). Such effects are also seen in other taxa, for example Holehan and Merry (2005) showed reducing the calorie intake of rodents increased life expectancy but resulted in a loss of reproduction (Holehan & Merry 1985). This shift in resources from reproductive activity to the survival in adults under dietary restriction could alter the degree to which a male can respond to rivals.

Adult male nutrition could also have a significant impact on a male's ability to allocate resources towards reproductive processes. In the context of ruminant livestock breeding, the benefits of nutrients on the viability of sperm have been well documented (Robinson et al. 2006; Kendall et al. 2000) as have the effects of dietary restriction on female fertility in species such as *D. melanogaster* (Chapman & Partridge 1996). In *D. melanogaster* female egg production increases linearly with increased dietary yeast (Bass et al. 2007). At higher food levels a female will mate more, produce more eggs and have a shortened lifespan (Chapman & Partridge 1996; Chippindale et al. 1997; Chippindale et al. 1993). There has been less work on the effect of diet in males; however males are reported to increase reproductive output with increasing diet quality (Carey et al. 2008). As with females, males also experience a cost to reproduction (Carey et al. 2008; Yuval et al. 1998). Males thus have a significantly shorter lifespan when housed with females than when kept as virgins (Chippindale et al. 1997). Fricke et al. (2008) provided evidence that adult male nutrition impacted on the male's ability to gain matings with non-virgin females and that there was an optimum level of nutrition required for male reproductive success (Fricke et al. 2008).

Here I tested for the effects of adult nutrition on male reproductive success and the ability of males to respond to rivals. I predicted that nutrient shortage would lead males to have decreased reproductive success and a decreased capacity to respond adaptively to rivals. I then tested the effects of dietary components (yeast versus sugar) and the effects that were observed.

6.3] Material and Methods

6.3.1 <u>The effects of nutrition on male reproductive success and rivalry in male *Drosophila*</u>

In this section I conducted three experiments to determine male reproductive success and ability to respond to rivals for males when held in differing nutritional conditions:

1. Groups of males were exposed to different amounts of yeast in their diets for 7 days and recorded their response to rivals (section 6.3.1.1).
2. The experiment was then repeated but for an extended period of 14 days (section 6.3.1.2).
3. The amounts of (a) yeast and (b) sugar in a male's diet were varied over 7 days, and included males held under starvation conditions (section 6.3.1.3).

6.3.1.1 Experiment 1. Effects of dietary yeast on male reproductive success and responses to rivals - 7 days of exposure to different diets and the presence or absence of rivals

Fly rearing was carried out as set out in the general materials and methods (Chapter 2). Males and females were collected at eclosion using ice anaesthesia. Females were housed in groups of 5 on a normal 100% yeast diet (100g brewer's yeast per litre medium) with added live yeast granules. Males were collected and kept in groups of 5 for 1 day (Appendix 3) and then randomly allocated to the different food groups containing either low or standard quantities of yeast, namely 20% (20g brewer's yeast per litre medium) or 100% yeast diets (no live yeast granules added). Males on each diet were housed either singly (1 per vial) or with rivals (4 per vial). The sample size was 40 per group of males and they were held in their respective conditions for 7 days before mating with virgin females that had all been reared on normal food.

Females were placed singly into experimental vials containing agar 24 hours prior to mating, using ice anaesthesia. Females were housed on just agar (no food) to remove any confounding effect of diet during the matings themselves. The effect of this procedure itself was tested in later experiments to make sure that the food used in the mating vials had no biasing effect on the outcome of the experiment (Appendix 2). At mating a single male was aspirated into each vial. The introduction time and start and finish times of mating were recorded to the nearest minute. Flies that didn't mate within 3 hours and any matings under 10 minutes were discarded.

6.3.1.2 Experiment 2. Effects of dietary yeast on male reproductive success and responses to rivals - 14 days of exposure to different diets in the presence or absence of rivals

This experiment was conducted to determine whether any differences in the ability of males to respond to rivals was increased when males suffered severe nutritional stress by exposing them to different diets for an extended period of 14 days compared to 7 days as above. Fly rearing was performed as before except that males were held in their respective conditions for 14 days before mating with females that were reared on normal food. Both males and females were given fresh food of the respective type after 7 days.

6.3.1.3 Experiment 3. Effects of (a) dietary yeast and (b) dietary sugar on male reproductive success and responses to rivals – 7 days of exposure to different diets in the presence or absence of rivals

I varied the amount of (a) yeast and (b)sugar during the period of exposure to rival males to test which dietary components might affects a male's reproductive success and ability to respond to rival males. Fly rearing was conducted as described above. The nutrition varied for each group and males were placed in 1 of the 4 food groups as above either singly or with rivals. The food groups were: control (100% yeast & 100% sugar), low yeast (20% yeast & 100% sugar), no yeast (0% yeast, 100% sugar) and plain agar (0% yeast & 0% sugar). The diets used were as defined in Appendix 4.

6.3.2 The effects of nutrition differences in yeast and sugar on male reproductive success, responses to rivals and fecundity

I conducted two further experiments to determine if there were any fitness differences apparent in males held on different diets of yeast and sugar, in addition to the ability to respond to rivals. I also measured whether these differing nutritional conditions affected offspring viability as well as mating frequency (section 6.3.2.1).

4a I exposed groups of males to different amounts of yeast in their diets for 7 days and recorded their response to rivals (section 6.3.2.1).
4b I recorded mating frequency of the males from the different yeast diets.
4c I measured whether different amounts of yeast affected offspring production.

5a I exposed groups of males to different amounts of sugar in their diets for 7 days and recorded their response to rivals (section 6.3.2.).
5b I recorded mating frequency of the males from the different sugar diets.

Fly rearing was carried out as set out in the general materials and methods (Chapter 2). Males and females were collected at eclosion using ice anaesthesia. Females were housed in groups of 5 on a normal yeast diet seeded with live yeast granules until use in the experiment. Males were collected and placed immediately into their food groups. This was done to prevent the potentially confounding effect of a male building up food resources by feeding before allocation to the respective food treatments (Appendix 3). Males were randomly assigned to one of 4 different food groups and were placed either with (4 per group) or without (singly) rivals until mating.

6.3.2.1 Experiments 4. Effect of variations in the dietary yeast intake on male reproductive output and response to rivals

4a. Variation in dietary yeast levels : Males were kept in 4 different nutritional environments, control (100% yeast & 100% sugar), low yeast, normal sugar (20% yeast & 100% sugar), no yeast, normal sugar (0% yeast & 100% sugar), and plain agar (0% yeast & 0% sugar). No live yeast granules were added at any time to these diets. Males were placed in 1 of the 4 food groups above either singly or with rivals.

Females were placed singly into experimental vials with only agar 1 hour prior to mating using ice anaesthesia. At mating a single male was aspirated into each vial. The introduction time and start and finish times of mating were recorded to the nearest minute. Flies that didn't mate within 2 hours and any matings under 5 minutes were discarded.

4b. Mating frequency: Males mating frequencies in each group with varying nutritional conditions in yeast were recorded.

4c. Offspring counts: After matings were finished females were removed from their vials and placed in vials with normal food and live yeast granules for 24 hours and then discarded. Vials were left for 12 days for offspring to emerge and so determine reproductive success of each of the males in their different diet groups.

6.3.2.2 Experiments 5. Effect of variations in the dietary sugar intake on male reproductive output and response to rivals

5a. Variation in dietary sugar levels: Males were kept as above, control (100% yeast & 100% sugar), normal sugar, no yeast (100% sugar & 0% yeast), low sugar, no yeast (20% sugar & 0% yeast) and plain agar (0% sugar & 0% yeast). Males were placed in 1 of the 4 food groups as above either singly or with rivals.

Females were placed singly into experimental vials with only agar 1 hour prior to mating using ice anaesthesia. At mating a single male was aspirated into each vial. The introduction time and start and finish times of mating were recorded to the nearest minute. Flies that didn't mate within 2 hours and any matings under 5 minutes were discarded.

5b. Mating frequency: Males mating frequencies in each group with varying nutritional conditions in sugar were recorded.

6.3.2.3 Statistical analysis

Statistical analysis was performed using SPSS v 16. Mating latency and mating duration data were tested for normality using Kolmogorov-Smirnov tests and for homogeneity of variance using Levene's tests. If data distributions were not normal, the data were logged to improve normality. Data from mating duration and mating latency were then subjected to univariate ANOVA. Chi-square tests were conducted to determine if there was a difference in the number of males in each treatment that did and did not mate. I tested for a correlation between mating duration and the time to mating using the Pearson's correlation test. Pearson correlations were also performed to determine whether there were any associations between mating duration and the number of offspring produced.

6.4] Results

6.4.1 The effects of nutrition on male reproductive success and rivalry in male *Drosophila*

6.4.1.1 Experiment 1. Effects of dietary yeast on male reproductive success and responses to rivals - 7 days of exposure to different diets in the presence or absence of rivals

Mating latency: The mating latency data were not normally distributed, so all data were first logged, which corrected this problem. There were no significant differences between males kept on different diets in terms of mating latency (ANOVA $F_{1,141}$=1.732, p=0.190). Males kept with rivals prior to mating had longer mating latencies than males kept singly, although rival versus non rival mating latencies were not significant ($F_{1,141}$ =5.895, p=0.16, figure 6.6.1). There was no significant interaction between the mating latency of males kept on different food groups and whether they were with or without rivals prior to mating ($F_{1,141}$=0.027, p=0.870). The results show that the time it takes to mate is not dependent on having yeast in the diet, and that the presence or absence of rivals was also not affected by these 7 day diet treatments.

Mating duration: Male diet had no significant effect on mating duration ($F_{1,141}=0.148$, $p=0.702$). As expected, there was a significant difference in the mating duration of males kept with and without rivals, with males exposed to rivals mating for significantly longer ($F_{1,141}=33.481$, $p<0.001$, figure 6.6.2). There was no interaction for mating duration between males kept on different food groups and whether they were kept with or without rivals prior to mating ($F_{1,141}=0.130$, $p=0.718$). This suggests that differences in the level of yeast in the diet over the 7 day period tested do not affect a male's ability to detect rivals.

6.4.1.2 Experiment 2. Effects of dietary yeast on male reproductive success and responses to rivals - 14 days of exposure to different diets in the presence or absence of rivals

Mating latency: There were no significant differences in mating latency between males kept on different diets ($F_{1,103}=2.147$, $p=0.146$) or between males kept with and without rivals prior to mating ($F_{1,103}=0.114$, $p=0.737$). However I did find a significant interaction between nutrition and the presence or absence of rivals ($F_{1,103}=8.791$, $p<0.05$, figure 6.6.3). The males exposed to the lower nutrition took longer to mate when kept alone, while males exposed to higher nutrition took longer to mate when housed with rivals. This result was not seen in the 7 day experiment above which may indicate that longer exposures to the different diets may enhance the effects of exposure to rivals.

Mating duration: There was no significant difference in mating duration between the males kept on the different diets ($F_{1,103}=0.793$, $p=0.375$). There was however, as expected, a significant extension of mating duration for males exposed to rivals prior to mating ($F_{1,103}=8.034$, $p<0.05$, figure 6.6.4). As above (section 6.4.1.1) there was no interaction for mating duration between males kept on the different food groups and whether they were kept with or without rivals prior to mating ($F_{1,103}=0.008$, $p=0.927$). These results suggest that diet has no effect on a male's ability to respond to rivals.

6.4.1.3 Experiment 3. Effects of dietary yeast and dietary sugar on male reproductive success and responses to rivals – 7 days of exposure to different diets in the presence or absence of rivals

Mating latency: Here there was a significant difference in mating latency between the males kept on different diets ($F_{3,243}=18.408$, $p<0.001$, figure 6.6.5). Males in the 'no food' group (i.e. agar only) took significantly longer to mate than males on any of the other diets. There was also, as before, a significant difference in latency between the males kept with and without rivals ($F_{1,243}=5.758$, $p=0.017$, figure 6.6.5). The males held on the no food (agar only) diet did not differ in latency according to whether they were or were not housed with rivals prior to mating. However, males held on all other diets had longer latencies following previous exposure to rivals. There was no significant interaction between the different nutritional condition of the males and whether they were kept with or without rivals ($F_{3,243}=0.601$, $p=0.615$). Therefore starved males did not respond normally to rivals, and took significantly longer to mate compared to males from other food groups.

Mating duration: The mating duration data were log transformed to improve normality. No significant differences were found in the mating duration of males held on different diets ($F_{3.232}=1.341$, $p=0.262$), but significant differences were found in mating duration of males kept with and without rivals ($F_{1,232}=7.009$, $p=0.009$, figure 6.6.6) as before. There was no significant interaction between the mating duration of males held with or without rivals ($F_{3,232}$=0.419, p=0.740). This shows again that a male's ability to respond to rivals was not affected by the diets tested here.

6.4.2 The effects of nutrition differences in yeast and sugar on male reproductive success, responses to rivals and offspring number and viability

Below are the results of the experiments to determine if altering the different major nutritional components of the male diets affected mating and/or offspring viability.

6.4.2.1 Experiment 4a. Effects of variations in dietary yeast on male mating latency and duration times

Mating latency: A significant difference in mating latency was seen between males kept in different nutritional conditions ($F_{3,227}$=4.489, p=0.004, figure 6.6.7). The males held on normal yeast levels but no added sugar took longer to mate. No significant differences were seen in males kept with or without rivals ($F_{1,227}$=0.097, p=0.755), although those without rivals were slower to mate in the two groups held on diets containing yeast but no sugar, in comparison to those on both yeast and sugar or no yeast or sugar. There was no interaction between the different diet treatments and the presence or absence of rivals ($F_{3,227}$=0.086, p=0.968). The results suggest that sugar may be an important energy resource for male flies and needed to sustain high levels of courtship.

Mating duration: There was no significant difference in mating duration of those kept on different nutritional diets ($F_{1,227}$=0.898, p=0.443). However, there was a significant difference in mating duration between those kept with and without rivals ($F_{1,227}$=8.418, p=0.004, figure 6.6.8). Those exposed to rivals prior to mating took longer to mate than those without rivals, except for the group with no food (agar only), which did not make the adjustment in response to rivals. Diet therefore appears, across the range tested, to have minimal effects on male responses to their rivals.

6.4.2.2 Experiment 4b. Nutritional effects of varied dietary yeast on mating frequency

There was a significant difference in mating frequency between males held on different diets (Pearson Chi-Square χ^2 =96.252, df=7, p<0.001). Over 80% of each group mated except for those on no food, where 50% and 22% of the males with and without rivals, respectively mated (table 6.7.1). Hence in the absence of yeast and sugar a significantly lower proportion of males mated.

6.4.2.3 Experiment 4c. Nutritional effects of varied dietary yeast on offspring number and offspring viability

Of those males that mated (see above) all diets achieved >70% viable offspring regardless of the diet on which they were held. In addition, all males kept without rivals prior to mating had more offspring than those kept with rivals, except in the group with 100% yeast and no sugar, in this group those kept with rivals had almost 14% more offspring. There was also evidence that the number of viable offspring produced was dependent on diet ($F_{3,223}$=2.994, p=0.032, figure 6.6.9). Males in the control groups and those kept on no food (agar only) produced more offspring than those with differing amounts of yeast but no sugar. This could indicate that yeast on its own has a detrimental effect on fecundity or that sugar is required for optimum levels of offspring production.

There was no correlation between the mating duration and number of offspring in any of the different dietary groups (Pearson Correlation *r*=0.027, *N*=230, *p*=0.688).

6.4.2.4 Experiment 5a. Effects of variations in dietary sugar on male mating latency and duration times

Mating latency: There was no effect of varying sugar on mating latency ($F_{3,164}$=1.249, *p*=0.294), although the males with no food tended to take less time to mate when held with or without rivals prior to mating. The control group took longer to mate when kept with rivals but the results were not significant ($F_{1,164}$=0.161, *p*=0.689, figure 6.6.10).

Mating duration: There was no overall effect of variation in sugar in the diet on mating duration ($F_{3,164}$=0.874, *p*=0.456). Mating duration was affected by the presence of a rival in all the groups; males kept with rivals mated for longer in every group ($F_{1,164}$=20.571, *p*<0.001, figure 6.6.11). There was no interaction between the two groups, diet and with or without rivals ($F_{3,164}$=2.236, *p*=0.086).

6.4.2.5 Experiment 5b. Nutritional effects of varied dietary sugar on mating frequency

There was a significant difference in mating frequency between the males held on different diets (χ^2=35.664, *df*=7, *p*=0.001). Those that mated on normal food and a high sugar diet had higher mating frequencies (< 78%) than those on low sugar or no food diets (>54), (table 6.7.2).

6.5] Discussion

Taken together my results show that the ability of a male to respond to rivals as shown by extensions to mating duration was not affected by any of the different diets tested. However, the amount of sugar in the diet has a significant effect on mating latency, males lacking sugar were slower to mate and mated less frequently than did males that were housed on medium containing yeast. Also in one experiment the data suggested that males housed on medium containing no sugar fathered fewer offspring (section 6.4.2.3.)

6.5.1 The effects of nutrition on rivalry of male *Drosophila*

When males had been held on different diets for 14 days an interaction was found between the food type and whether they had been kept with and without rivals. Males on normal food took longer to mate when kept with a rival prior to mating, while males on the low yeast food took longer to mate when housed singly. Males in the no food (agar only) group also took a lot longer to mate than males held on other diets and did not show a response to the presence of rivals. In all the experiments male mating duration was significantly longer when males were kept with rivals. This was expected based on previous studies (Bretman et al. 2009).

It has been previously observed that a male’s nutritional status can alter his mating success. For example, Blay and Yuval (1997) discovered that male Mediterranean fruit flies fed on low levels of protein copulated less than those fed on higher protein diets. In my experiments any differences in the mating latencies of flies kept under differing nutritional conditions were inconsistent, except for the males that had no food for 7 days. Under those conditions male mating latencies were not affected by the presence or absence of rivals prior to mating. The time taken to mate was also significantly longer than any of the other groups by approximately 50 minutes. The effects of adult diet on male mating activity have been documented in various insect species (Blay & Yuval 1997). A nutritionally poor diet in the male mormon cricket, *Anabrus simplex* has been observed to decrease the number of sexually active males (Gwynne 1993). An inadequate protein diet has been known to lead to a partial or complete cessation of mating activity by the male tephritid fly, *Anastrepha oblique* (Perez-Staples et al. 2008). In my experiments male mating activity did slow down in males held on the no food diet, as shown by the mating latency results.

Males subjected to severe nutritional stress (held on agar only diets) took longer to mate, but once they did mate they retained the ability to respond to rivals. Mating duration was found to depend on the presence or absence of rivals, but was not linked to the nutritional status of the male in any case. In all three experiments males consistently mated for longer when they had previously been kept with rivals. Previous studies have shown that female flies prefer larger males (Partridge & Farquhar 1983; Partridge et al. 1987) and males that have been deprived of nutrition during development may be smaller and so less attractive (Friberg & Arnqvist 2003). Studies of male size in crickets have shown that when females are paired with smaller males they take longer to mate and may interrupt sperm transfer or reject matings altogether (Bateman et al. 2001).

My results show that nutrition makes little difference to the ability of males to respond to rivals in respect to mating duration. Whether males are well fed or literally starving they still respond to a rival in the same way by mating for longer than males that are not exposed to rivals prior to mating. No differences were seen in mating latencies across the diet treatments in the first two experiments. However, in the third experiment the males held on the no food (agar only) diets took significantly longer to mate and did not adjust mating duration in response to the presence of rivals. This indicates starved males under some parameters may not respond to rivals in the expected fashion.

6.5.2 The effects of nutrition on rivalry and reproductive success in male *Drosophila*

In the second set of experiments I tested whether differing nutritional diets affected mating and/or offspring viability. I also tested the effect of placing males onto their respective diets immediately following eclosion (Appendix 3). I found that groups of males on yeast diets had higher frequencies of mating than those on no food (agar only) and that males on high sugar diets had higher levels of mating than those on low or no sugar. I discovered no effect of diet on the presence or absence of rivals in mating frequency.

The males kept with rivals prior to mating mated for longer than those without. Bass et al. (2007) found that the addition of sugar to the culture medium was detrimental to egg laying. They found that flies moderate their feeding in response to added sugar levels, so the lowered egg laying on a high sucrose diet is unlikely to be an effect from reduced feeding and more likely to be an adverse physiological effect due to the presence of these unnaturally high levels of sucrose. It is also reported that the type of yeast can alter lifetime fecundity (Bass et al. 2007). We calculated the amount of surviving offspring from each treatment to determine if nutrition can cause differences in egg to adult survival. In all groups both with variable yeast and sugar levels 70% of flies produced viable offspring. Variation in yeast levels revealed that the number of viable offspring was dependent on the nutritional status of the male, with those held on no food having more offspring.

Variation in the concentration of sugar provided no evidence for differences in egg viability. Previous research has shown that an absence of nutritional components imposes a limit on the egg laying capacity in females, due to a depletion of parental reserves. Flies with less reserves have less energy to invest in offspring production so egg laying is curtailed (Bass et al. 2007). The male Mediterranean fruitfly fed low levels of protein led to a lower frequency of copulation. But although protein deprived males transferred more sperm to their mates than those on the higher protein diets the females they copulated with quickly mated again. This indicates that diet is a major factor in determining a male's reproductive success (Blay & Yuval 1997).

I was unable to reliably count the offspring from the experiment that tested the effect of variation in dietary sugar because the samples were not stored correctly before counting (results section 6.4.2.6), so further work is needed to fully clarify offspring effects found. In the last two experiments (section 6.3.2.1) agar only medium was used in the mating vials so as not to confound the results with effects of a full diet in those mating vials. A later experiment (Appendix 2) indicated that there is a significant effect on mating duration dependent on the medium in the mating vials (male rivalry was not tested). However, this effect showed no interaction across the different diets hence there is no evidence for a biasing effect of the food medium used in the mating vials on my results.

In many mate choice studies females are presented with just one male and so this may often not represent the natural situation because there is a potential absence of male-male competition and female choice. The laboratory conditions in this experiment required only one male per vial so there was no mate choice for the female. Under natural conditions nutrition may be poor and some individuals may not be able to secure a mate. Although research has shown that poorly fed adults may accumulate enough energy resources to match well-fed competitors if given enough days to feed (Aluja et al. 2001). The males from the first part of this experiment were transferred to their respective diets one day after eclosion while the second set of experiments were placed immediately on their respective diets following eclosion. However there was no evidence that the males left for one day on normal food built up a reserve of nutrients that affected the outcome of the experiments (Appendix 3). I did find however, that many males that survived on poor diets died immediately following copulation; this could be because of the detrimental effect that copulation has on starvation resistance (Zwaan et al. 1991).

In some circumstances prey deprivation or an inadequate diet can lead to a partial or complete cessation of male mating activity (Anderson & Franks 2001). Adult tephritid fruit flies need to ingest carbohydrates and water constantly to survive and protein is required to attain sexual maturity (Aluja et al. 2001). The effect of diet on an adult male's mating activity has been documented in various insect species. Food intake has been found to have a positive effect on testis size and to influence mating duration in yellow dung flies, *Scatophaga stercoraria* (Ward & Simmons 1991). Research has shown that a poor diet can affect mating behaviours. For example, protein deprived black blow fly (*Phormia regina*) males mated less and inseminated fewer females than those on a higher protein diet (Stoffolano et al. 1995). Males that have been fed higher protein diets court females more and copulate more frequently than the same species fed on sugar (Yuval et al. 2002). The males of the calliphorid fly (*Phormia regina*) have a high reproductive success rate when fed high levels of protein compared to protein deprived males (Stoffolano et al. 1995). Also females that mate with a protein deprived male are significantly more likely to re-mate again quickly. A poor diet results in a decrease in the number of sexually active male mormon crickets *Anabrus simplex* and alters sexual selection dynamics (Gwynne 1993). Also, fewer mating attempts were made by males of the bushcricket (*Requena verticalis*) when fed on a low protein diet (Schatral 1993). Blay and Yuval (1997) concluded that diet is an important factor determining a male's reproductive success in the Mediterranean fruit fly (*Ceratitis capitata*). Shelly et al. (2002) suggests that diet can even affect the attractiveness of male Mediterranean fruit flies by altering their pheromone signalling (Shelly et al. 2002).

Nutritional studies have been performed on *D. melanogaster* using both yeast and sucrose. Yeast extract has been found to be detrimental to fecundity and lifespan in high concentrations (Bass et al. 2007). Dietary restriction has been shown to give rise to increased longevity but also reduced fecundity. Diet has also been found to have strong effects on female reproductive traits (Chippindale et al. 1997). My results here show that males are also sensitive to the effects of diet and that the sugar component of the diet is particularly important in determining a male's reproductive success.

In future work it would be interesting to determine if the number of eggs laid by the low or no sugar males was the same as the number of offspring hatched, to determine if many eggs laid are not viable or if fewer eggs are laid. In the scorpionfly (*Panorpa cognate*) higher nutrient availability for males can increase copulation frequency, and cause females to become reproductively active at a younger age (Engqvist & Sauer 2003). Further studies need to be conducted to investigate the effects of different diets and dietary components across the entire life cycle. It would also be useful to determine if, over generations, there are any evolutionary changes in behaviour to compensate for the prevailing diets experienced.

6.6] Figures

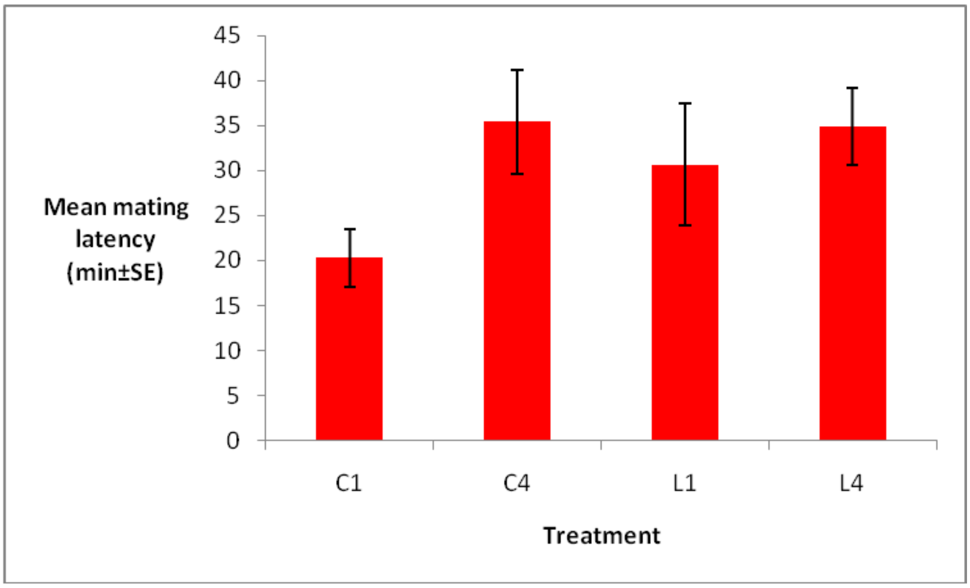

Figure 6.6.1 Mating latency data from experiment 1. Mean mating latency (±SE) of wild-type *D. melanogaster* males kept for 7 days on either normal (C) or low (L) protein food either in the presence (4 males per vial) or absence (1 male per vial) of rivals, prior to mating. Mating latency is the time from introduction of the flies until the start of mating.

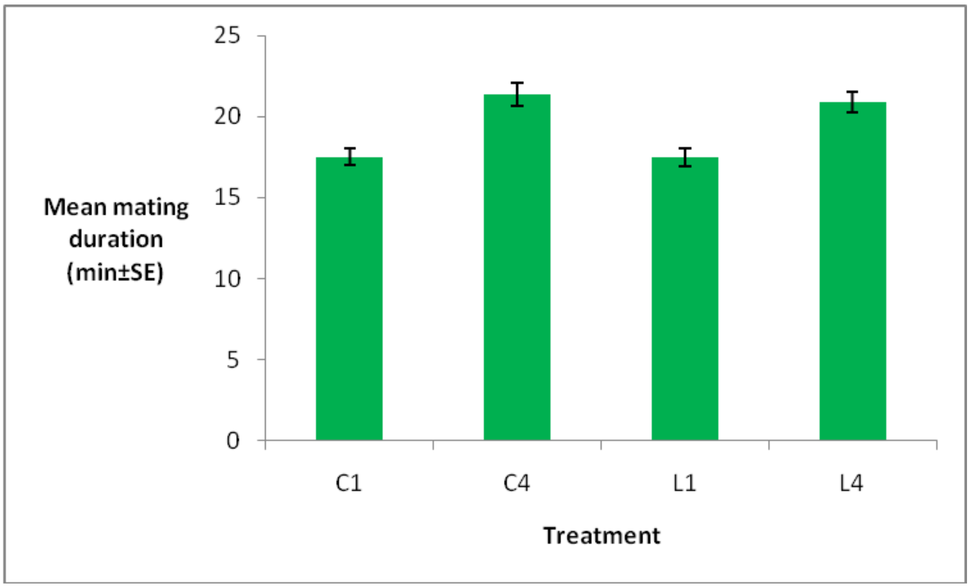

Figure 6.6.2 Mating duration data from experiment 1. Mean mating duration (±SE) for wild-type *D. melanogaster* males kept for 7 days on either normal (C) or low (L) protein food either in the presence (4 males per vial) or absence (1 male per vial) of rivals, prior to mating. Mating duration is the time from the start to the finish of mating.

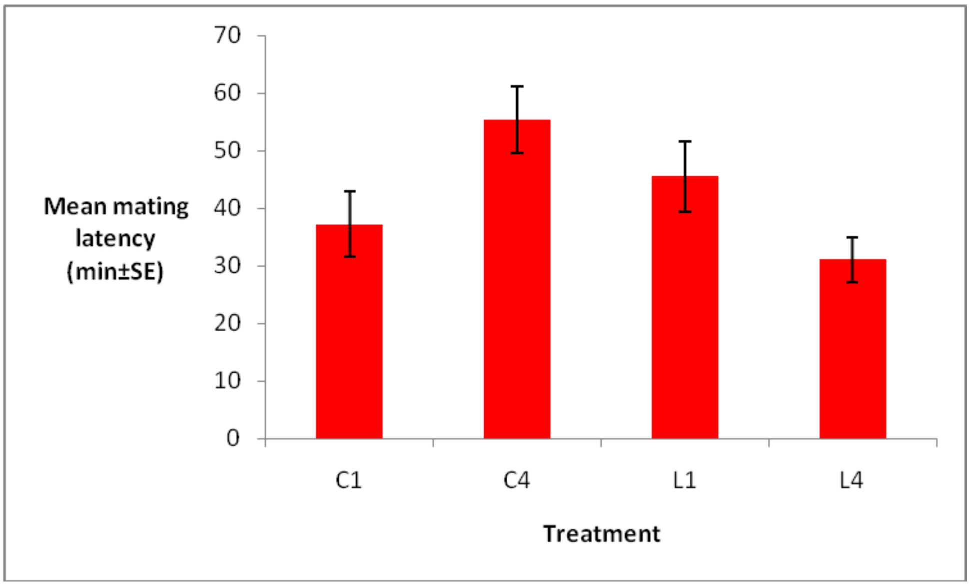

Figure 6.6.3 Mating latency data from experiment 2. Mean mating latency (±SE) for wild-type *D. melanogaster* males kept for 14 days on either normal (C) or low (L) protein food either in the presence (4 males per vial) or absence (1 male per vial) of rivals, prior to mating. Mating latency is the time from introduction of the flies until the start of mating.

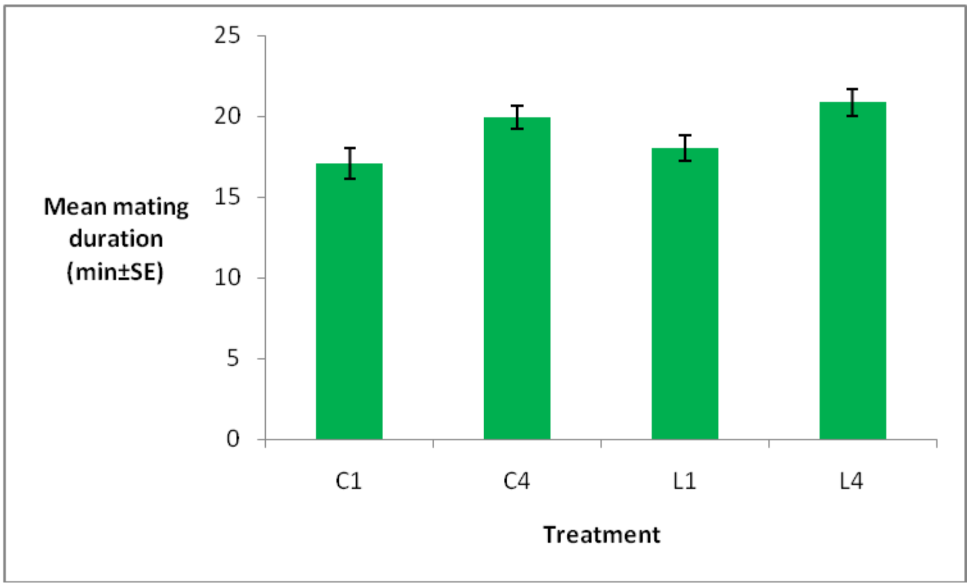

Figure 6.6.4 Mating duration data from experiment 2. Mean mating duration (±SE) for wild-type *D. melanogaster* males kept for 14 days on either normal (C) or low (L) protein food either in the presence (4 males per vial) or absence (1 male per vial) of rivals, prior to mating. Mating duration is the time from the start to the finish of mating.

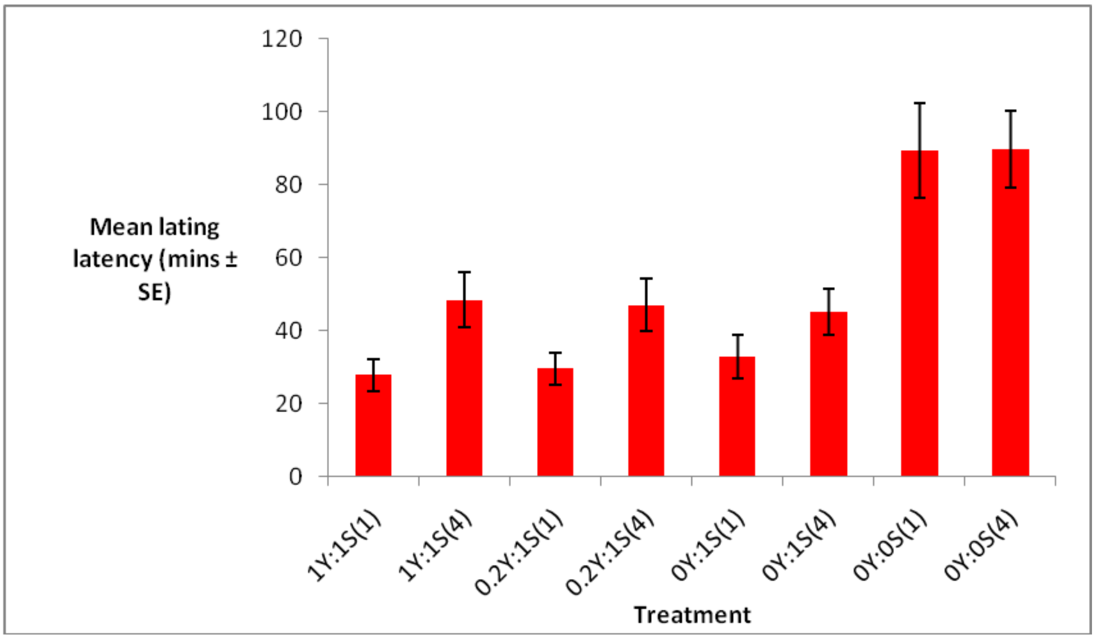

Figure 6.6.5 Mating latency data from experiment 3. Mean mating latency (±SE) of wild type *D. melanogaster* male flies kept for 7 days on different diets 100% yeast: 100% sugar (1Y:1S), 20% yeast: 100% sugar (0.2Y:1S), 0% yeast: 100% sugar (0Y:1S) and 0% yeast: 0% sugar (0Y:0S). For each food treatment males were kept either in the presence (4 males per vial) or absence (1 male per vial) of rivals prior to mating. Mating latency is the time from introduction of the flies until the start of mating.

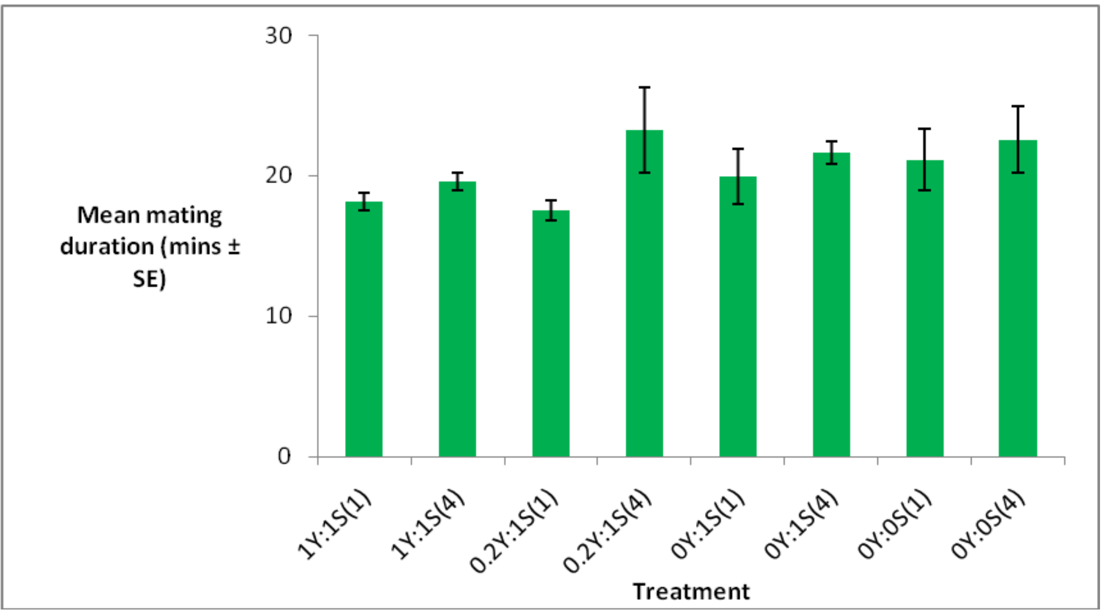

Figure 6.6.6 Mating duration data from experiment 3. Mean mating duration (±SE) of wild type *D. melanogaster* male flies kept for 7 days on different diets 100% yeast: 100% sugar (1Y:1S), 20% yeast: 100% sugar (0.2Y:1S), 0% yeast: 100% sugar (0Y:1S) and 0% yeast: 0% sugar (0Y:0S). Males held on each diet were kept either in the presence (4 males per vial) or absence (1 male per vial) of rivals, prior to mating. Mating duration is the time from the start to the finish of mating.

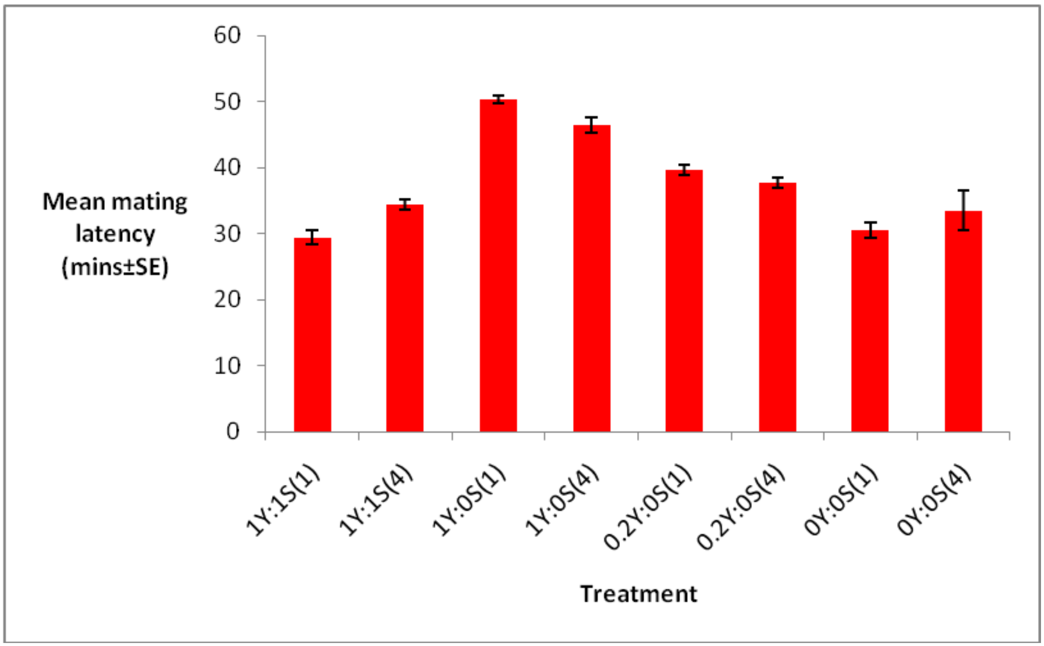

Figure 6.6.7 Mating latency data from experiment 4a. Mean mating latency (±SE) of wild type *D. melanogaster* male flies kept for 7 days on different diets 100% yeast: 100% sugar (1Y:1S), 100% yeast: 0% sugar (1Y:0S), 20% yeast: 0% sugar (0.2Y:0S) and 0% yeast: 0% sugar (0Y:0S). Males on each diet were kept either in the presence (4 males per vial) or absence (1 male per vial) of rivals prior to mating. Mating latency is the time from introduction of the flies until the start of mating.

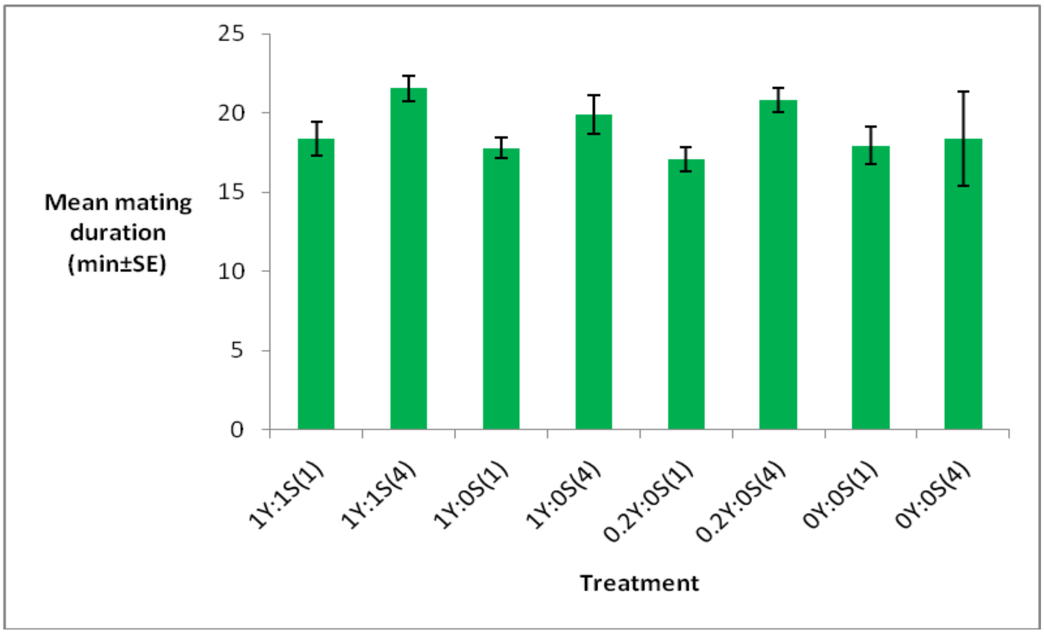

Figure 6.6.8 Mating duration data from experiment 4a. Mean mating duration (±SE) of wild type *D. melanogaster* male flies kept for 7 days on different diets 100% yeast: 100% sugar (1Y:1S), 100% yeast: 0% sugar (1Y:0S), 20% yeast: 0% sugar (0.2Y:0S) and 0% yeast: 0% sugar (0Y:0S). Males on each diet were kept either in the presence (4 males per vial) or absence (1 male per vial) of rivals prior to mating. Mating duration is the time from the start to the finish of mating.

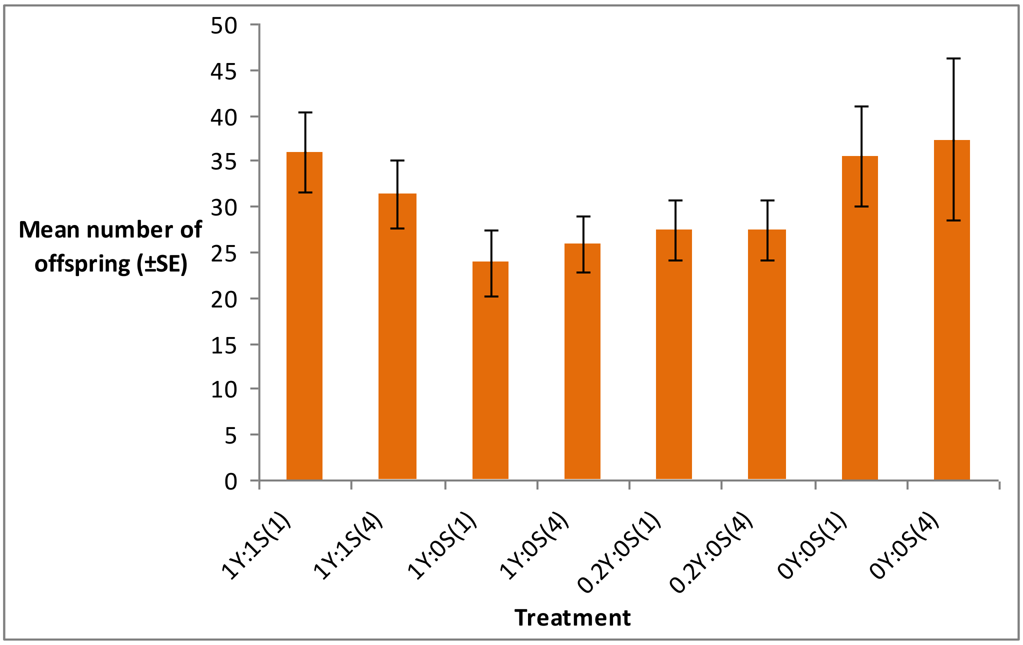

Figure 6.6.9 Offspring number data from experiment 4c. Mean number of offspring (±SE) of wild type *D. melanogaster* male flies kept for 7 days on different diets 100% yeast: 100% sugar (1Y:1S), 100% yeast: 0% sugar (1Y:0S), 20% yeast: 0% sugar (0.2Y:0S) and 0% yeast: 0% sugar (0Y:0S). Males on each diet were kept either in the presence (4 males per vial) or absence (1 male per vial) of rivals prior to mating.

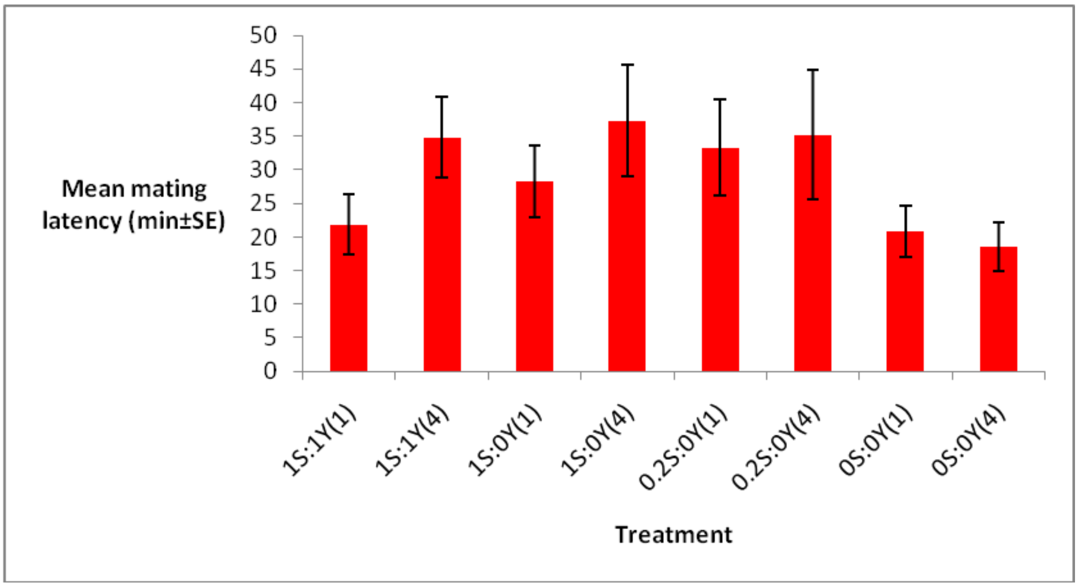

Figure 6.6.10 Mating latency data from experiment 5a. Mean mating latency (±SE) of wild type *D. melanogaster* male flies kept for 7 days on different diets 100% sugar: 100% yeast (1S:1Y), 100% sugar: 0% yeast (1S:0Y), 20% sugar: 0% yeast (0.2S:0Y) and 0% sugar: 0% yeast (0S:0Y). Males on each diet were kept either in the presence (4 males per vial) or absence (1 male per vial) of rivals prior to mating. Mating latency is the time from introduction of the flies until the start of mating.

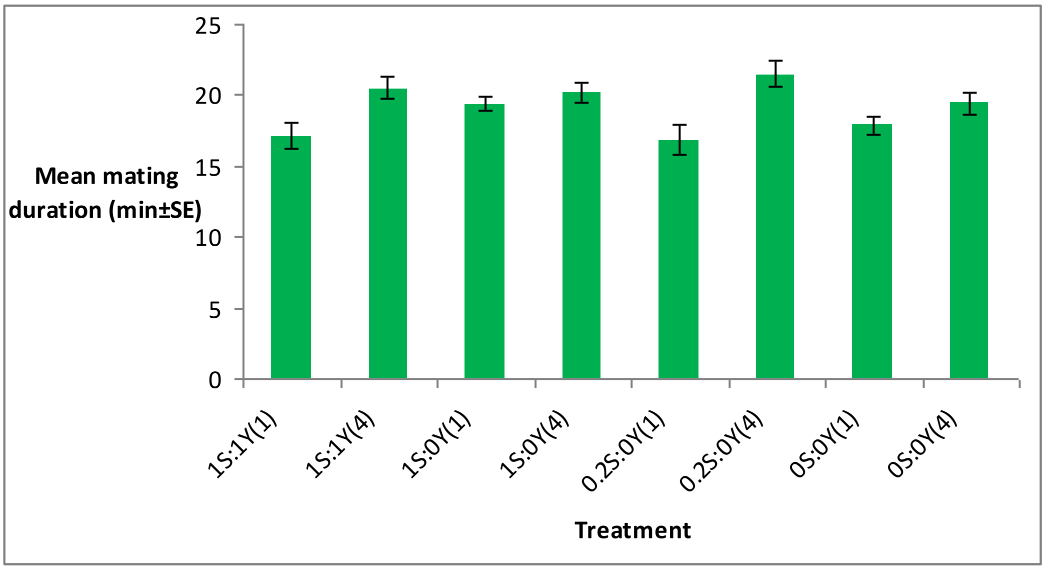

Figure 6.6.11 Mating duration data from experiment 5a. Mean mating duration (±SE) of wild type *D. melanogaster* male flies kept for 7 days on different diets 100% sugar: 100% yeast (1S:1Y), 100% sugar: 0% yeast (1S:0Y), 20% sugar: 0% yeast (0.2S:0Y) and 0% sugar: 0% yeast (0S:0Y). Males on each diet were kept either in the presence (4 males per vial) or absence (1 male per vial) of rivals prior to mating. Mating duration is the time from the start to the finish of mating.

6.7] Tables

Table 6.7.1 Mating frequency data from experiment 4b. The percentage of male *D. melanogaster* on different diets that mated, (100% yeast: 100% sugar (1Y:1S), 100% yeast: 0% sugar (1Y:0S), 20% yeast: 0% sugar (0.2Y:0S) and 0% yeast: 0% sugar (0Y:0S) and in the presence (4) and absence (1) of rivals).

Treatment	Males mated	Total in group	Percentage mated
1Y:1S(1)	36	40	90
1Y:1S(4)	36	39	92.31
1Y:0S(1)	34	39	87.18
1Y:0S(4)	37	38	97.37
0.2Y:0S(1)	33	40	82.50
0.2Y:0S(4)	33	39	84.62
0Y:0S(1)	18	36	50.00
0Y:0S(4)	8	37	21.62

Table 6.7.2 Mating frequency data from experiment 5b. The percentage of male *D. melanogaster* on different diets that mated, (100% sugar: 100% yeast (1S:1Y), 100% sugar: 0% yeast (1S:0Y), 20% sugar: 0% yeast (0.2S:0Y) and 0% sugar: 0% yeast (0S:0Y) and in the presence (4) and absence (1) of rivals).

Treatment	Males mated	Total in group	Percentage mated
1S:1Y(1)	25	32	78.13
1S:1Y(4)	26	31	83.87
1S:0Y(1)	24	30	80.00
1S:0Y(4)	26	30	86.67
0.2S:0Y(1)	16	32	50.00
0.2S:0Y(4)	17	32	53.13
0S:0Y(1)	16	41	39.02
0S:0Y(4)	20	38	52.63

Chapter 7 Conclusion

The main themes of this thesis were to establish the functions of odorant receptor (OR) and odorant binding protein (Obp) genes expressed in the head and reproductive organs of *D. melanogaster*. It examined whether there was evidence within clusters of odorant genes of new functions as indicated by changes in expression patterns between different cluster members, coupled with evidence of higher rates of evolutionary change in odorant genes enriched for expression in the reproductive system. This thesis determined whether males with a loss of function mutation for an odorant receptor (*Or83b*) with widespread expression suffered lowered fertility and whether they retained the ability to detect the presence of rivals. It then went on to ascertain whether the loss of olfactory reception obtained by removing the male antennae affected a male's ability to detect rivals and if this would also affect his general fertility levels. Lastly this thesis discovered the effects of different diets on reproduction and fecundity and determined whether diet affected a male's ability to detect and respond to rivals.

7.1] New functions and rapid evolution of odorant genes expressed in the reproductive system of the fruitfly

I found that OR and Obp genes can be expressed in the reproductive tissues as well as in the olfactory system, and some odorant genes were expressed only in the reproductive system. Many odorant genes occur in clusters and the change I observed in tissue specific expression within gene clusters suggests there may have been changes in function following gene duplication. There was some evidence that OR and Obps found in the head were more highly conserved across species than those found in the male accessory glands.

7.2] Silencing internal odorants affects mating latency but not rivalry or offspring viability

In Chapter 4 I showed that *Or83b* mutant males took considerably longer to mate than wild-type *Drosophila* and that they mated for a longer duration suggesting this mutation could affect courtship by the male through altered recognition and processing of odours. There was no evidence that fecundity was affected in *Or83b* lacking males.

7.3] Olfaction as a cue used by males to detect rival males in *Drosophila melanogaster*

My results from Chapter 5 provided evidence that a male compensated for the loss of its funiculus when exposed to rivals. However, a male's latency to mate was altered by the loss of the ability to hear (lack of arista) or detect odorants (lack of funiculus).

7.4] The effects of adult male nutrition on a male's reproductive success and ability to respond to rivals

Finally, I discovered that a male's ability to respond to rivals is not dependent on his nutritional condition. However, males were slower to mate when there was reduced sugar in their diet, and reduced sugar also resulted in the production of fewer offspring. However, males needed sugar to enable rapid matings and males with low levels of sugar or no food mated in lower numbers than those with yeast in their diet.

7.5] Overall conclusions

Overall I have found that OR and Obp genes are duplicated in the reproductive organs of *Drosophila* and that the genes in these clusters may be evolving new functions. Also there is some evidence to suggest that genes expressed in the male accessory glands are evolving faster than those expressed in the head. Odorants although important are not the only way *D. melanogaster* interact with the environment. Other sensory factors are also involved in the ability to recognise rivals and potential mates. Although how males perceive the presence of rivals is not yet known, it is likely to involve at least in part the detection of male specific volatile pheromones (Bretman et al. 2010). Research suggests nutrition is important to a female's mating ability (Bass et al. 2007) but I have shown that males also require particular nutrients in their diet for optimal reproduction and offspring viability.

7.6] Wider context

Research into chemotaxis between sperm and egg could result in fertility treatments in the future. Understanding the functions of ORs and Obps in the reproductive system is important to determine how chemotaxis might occur. Understanding how internal and external cues are used concerning the reproductive environment will also help research in this area. The amount of resources available to a male for allocation towards reproduction is dependent on his nutritional state. Studying how the diet affects mating behaviour and reproductive success is therefore important as it determines how likely is a male able to express his reproductive potential.

7.7] Future work

More sophisticated analysis is required on the sequence changes in OR and Obp genes expressed in the reproductive organs, but especially those expressed in the male accessory glands, to confirm the patterns of evolutionary change and also identify which amino acid residues are changing. Some of the odorant genes found in clusters appear to have new functions and some expressed in the male accessory glands are conserved across only a few species.

Work on the role of internal odorants is important, as shown in this thesis, mating latency can be affected by a broadly expressed receptor. Mutation of the *Or83b* receptor results in severe olfactory defects that alter adult metabolism, enhance stress resistance and extend lifespan in *Drosophila* (Libert et al. 2007). Research into other receptors that are found only in the reproductive organs of *Drosophila* would be ideal to determine if where a receptor is expressed makes a difference to male rivalry, reproduction and fecundity. The fact that the human odorant receptor hOR 17-4 has been found to be expressed in the nose and sperm suggests it may play a similar role in both these organs (Vosshall 2004). More research is needed to determine if some receptors help the communication between sperm and egg in some way. This could pave the way for new fertility treatments in the future.

We now know that although a male cannot compensate for the loss of its antennae in regards to mating latency it can still recognise rivals, indicating odorants are not the only way in which *D. melanogaster* interact with their environment and detect cues. More study on other senses for example hearing, sight and touch would help to further understand how *D. melanogaster* males experience their social and sexual environment.

A male's ability to respond to rivals is not affected by his nutritional condition. But I have shown that adult male nutrition is important in determining male reproductive success and that differences in a male's dietary components can have effects on different aspects of his reproductive success. More work is definitely needed in this area to investigate further the links between specific diet components and specific reproductive traits and in particular whether such differences are seen in both sexes. These results are important for understanding how reproductive strategies evolve in males and females under varying resource levels.

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Appendix I Effect of ice anaesthesia on mating latency.

A1.1] Introduction

Drosophila are routinely anaesthetized to facilitate handling, the main two methods being carbon dioxide (CO_2) and chilling, where flies are placed over ice for a short time. Flies may be damaged just by, or show altered responses following, different handling procedures. For example in one experiment (Chapter 5) I found that the use of anaesthesia resulted in increased death rates in both control and experimental groups. The flies were anaesthetized using ice at eclosion then anaesthetized using CO_2 for antennal removal and then anaesthetized again using ice prior to the mating assays. Mating latency was lengthened and mating duration reduced as a result of these procedures. Hence in behavioural studies, great care should be taken to minimise exposure to such anaesthetics.

Previous studies have assayed the effects of CO_2 exposure on behaviour. For example CO_2 exposure causes a reduction in fecundity, mating success and longevity in the German cockroach *Blattela germanica*. Elevated CO_2 levels have also been shown to increase development time and impair locomotion and feeding behaviour (Tanaka 1982). CO_2 has a marked effect on longevity, fecundity and mating success in individuals exposed at very young ages (Perron et al. 1972; Barron 2000; Barron 2000). Nilson et al. (2006) discovered that the required time for recovery from anaesthesia is proportional to the time spent exposed (Nilson et al. 2006). For anaesthesia to be used in behavioural experiments the effects on behaviour must be minimized. Here I tested whether there was a standard time required for males to recover from ice anaesthesia. I wanted to determine whether the males used in Chapter 5 had sufficient time to recover from their ice anaesthesia and test whether their mating behaviour was affected.

A1.2] Materials and Method

Larvae and flies were reared at standard densities as in Chapter 2, collected at eclosion, and males and females placed directly into vials containing yeast in groups of 5 and maintained for 4 days. Females were placed in mating vials with yeast 24 hours prior to the experiment using ice anaesthesia. Control males (C) were pooted directly into the mating vials on the day of the experiment. The other two groups were anaesthetized using ice, one group was placed directly into the mating vials following anaesthesia (I) and the other group was left for 1 hour before they too were pooted into mating vials (H). This experiment therefore tested the effect of recent anaesthetization using ice on male mating behaviour.

Time of entry into the vial was recorded along with start and finish times of mating, to the nearest minute. Mating latency and duration times were calculated. Data from flies that didn't mate in under 3 hours and any matings lasting less than 5 minutes were discarded.

A1.2.1 Statistical analysis

Statistical analysis was performed using SPSS v 16. Mating latency and mating duration data were tested for normality using Kolmogorov-Smirnov tests and for homogeneity of variance using Levene's tests. Data from mating duration and mating latency data were then subjected to ANOVA.

A1.3] Results

Effect of ice anaesthesia on male mating latency: There was a significant difference in mating latency between anaesthetized and non-anaesthetized males (ANOVA $F_{2,24}$=5.297, p =0.012). Males from the control group, those that were mated with no anaesthetic, and those that were left to recuperate for an hour after ice anaesthesia had almost the same mating latency, while the males that were placed in the vial directly following ice anaesthesia took much longer to mate (figure A1.5.1).

Effect of ice anaesthesia on mating duration: There were no significant differences in mating duration between the different groups of males ($F_{2,24}$=0.505, p=0.610, figure A1.5.2). The control group, mated without ice anaesthesia, the group mated straight after ice anaesthesia and the group mated one hour following ice anaesthesia all had similar mating durations.

A1.4] Discussion

There was a clear effect of ice anaesthesia on male mating latency, with males that were anaesthetized directly prior to mating with a female taking almost twice as long to mate in comparison to males that had 1 hour to recover.

Flies in the antennal experiment (Chapter 5) were chilled to enable the sorting of males from females as is the normal procedure. These were then anaesthetized using CO_2 to remove antennae (controls were also anaesthetized) and then chilled again after 5 days to sort flies from multiple groups into *antennaless* males and to discard vials. The high death rate before and during the experiment across all groups was the motivation for this test here of the effect of ice anaesthesia on male mating latency.

It might have been useful to test whether males still responded to rivals as expected under the conditions tested here. It would be useful in the future to investigate more explicitly the effects of different exposure times under anaesthesia.

I conclude that, unless unavoidable, flies should not be anaesthetized on the day of the experiment. If flies must be anaesthetized they should be left for at least one hour after to allow for a full recovery. Also it is indicated from experiments in Chapters 5 and 6 that using multiple anaesthesia should be avoided if possible as this may have a detrimental effect on fly mortality.

A1.5] Figures

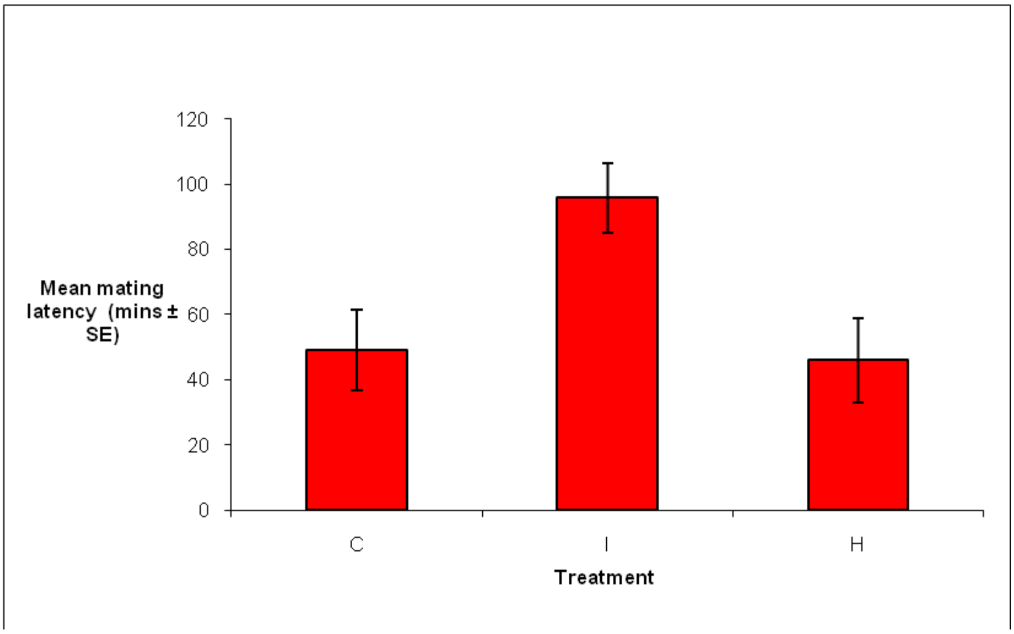

Figure A1.5.1 Mean mating latency (± SE) of wild-type *D. melanogster* males. Control males (C) were placed directly into mating vials. The other two groups were anaesthetized using ice, one group was placed directly into the mating vials (I) and the other was left for 1 hour before also being placed into their mating vials (H).

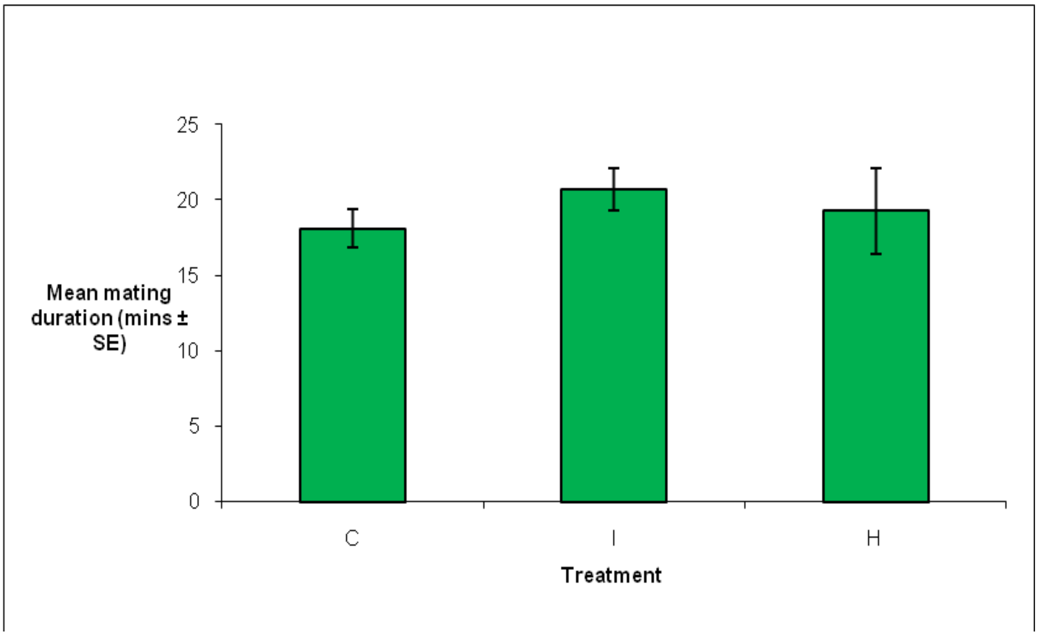

Figure A1.5.2 Mean mating duration (± SE) of wild-type *D. melanogaster* males. Control males (C) were placed directly into mating vials and the other two groups were anaesthetized using ice, one group was placed directly into the mating vials (I) and the other was left for 1 hour before also being placed into their mating vials (H).

Appendix II. Effect of the medium used in mating vials on the mating success and fertility of adult males subjected to differing diets from eclosion.

A2.1] Introduction

In the experiments in Chapter 6, I tested the effects of nutrition on male mating behaviour and fecundity. However, in those experiments it was not clear whether the optimal way to conduct the experiment was to hold females for the matings on normal food or on agar only food. The aim was to avoid confounding the effects of the male's diet with those encountered during the matings themselves. I therefore conducted extra experiments here to test the effect of the food medium on which pairs were held for matings, on mating latency, duration and subsequent fertility.

There are many environmental factors which can influence and eventually lead to a number of consequences for an animal's morphology and/or behaviour. Factors such as larval density, temperature of the environment and nutritional stress, all these can potentially affect body size, secondary sex traits and even mating performance in some insect taxa (He & Tsubaki 1992; Gage 1995; Hosken et al. 2000).

Nutrition and the specific amount of food components available can have a considerable influence on an individual's development and viability (Hosken et al. 2000). It has been observed in mature sheep and goats that changes in nutrition lead to a profound response in the testicular size of the males which in turn affects the rate of production of spermatozoa (Martin & Walkden-Brown 1995). The nutritional status of an adult can have effects on reproductive investment. Fricke et al. (2008) found evidence that an adult male's ability to gain a mating with a female was affected by his diet (Fricke et al. 2008). This may be related to the growth and development of the reproductive organs and such energy requirements may also differ between the sexes (House 1962).

I tested here for an effect of the food medium used in mating vials and whether this would have an effect on the outcome of experiments that tested different diets on male reproductive success.

A2.2] Materials and Method

All flies were raised at standard density on standard food medium as stated in Chapter 2. Males were collected at eclosion and placed immediately in individual vials for 7 days on one of 4 different diets, normal (N: 100% yeast, 100% sugar), yeast only (Y: 100% yeast, 0% sugar), sugar only (S: 0% yeast, 100% yeast) and agar only (X: 0% yeast, 0% sugar).

Females were kept in groups of 10 from eclosion on standard medium until 1 hour before mating when they were transferred to vials containing either normal SYA food (N: 100% yeast, 100% sugar) or just agar (X: 0% yeast, 0% sugar)

After mating females were removed from mating vials and all were placed on normal SYA food medium in vials supplemented with added yeast to encourage egg laying. Females were removed after 24 hours and discarded and the vials were left for 12 days when offspring were counted to determine offspring viability for each treatment.

A2.2.1 Statistical analysis

Statistical analysis was performed using SPSS v 16. Mating latency and mating duration data were tested for normality using Kolmogorov-Smirnov tests and for homogeneity of variance using Levene's tests. Data from mating duration and mating latency were then subjected to ANOVA. Pearson correlation tests were used to test for associations between mating duration and the number of offspring.

A2.3] Results

As expected, the diet of the adult males had a significant effect on mating latency (ANOVA $F_{3,241}=8.158$, $p<0.001$). Males on normal SYA food or just yeast mated significantly more quickly than those on just sugar or no food, which is consistent with the results in Chapter 6 and Appendix III. However, there was no significant difference in mating latency between groups held on the two different types of media in the mating vials ($F_{1,241}=2.839$, $p=0.093$, figure A2.5.1).

Adult male diet also had a significant effect on mating duration ($F_{3,241}=2.675$, $p=0.048$). This time the food used in the mating vials did have a significant effect ($F_{1,241}=6.333$, $p=0.013$). Males generally mated for significantly longer when mating in vials containing agar alone, with the exception of the yeast only treatment males where there were no differences in duration on either type of mating vial food (figure A2.5.2).

Adult male diet had a significant effect on the percentage of mating that occurred. There were significantly fewer (<50%) matings by males that were held on agar only medium prior to mating (Pearson Chi-Square $\chi^2=38.340$, $df=7$, $p<0.001$, table A2.6.1). There was no significant effect of nutrition on the production of offspring by the males ($F=0.762$, df=7, $p=0.620$, figure A2.5.3). There were also no significant correlations between mating duration and the number of offspring produced (Pearsons correlation $r=0.009$, $N=247$, $p=0.893$).

A2.4] Discussion

The nutritional status of the male had an impact on mating latency and mating duration. To mate takes energy and those males who were starved took longer to mate, possibly due to reduced courtship, and also mated for longer. Interestingly the type of media in the mating vial did have a significant effect on mating duration, with males mating, when held on agar only in the mating vials, for longer than those on normal SYA medium in mating vials.

Only 50% of the males kept on agar only food and held in agar only mating vials mated in comparison to 76% of the males kept on agar only food and held in normal SYA mating vials. Males may therefore be able to feed up quickly when presented with food medium following a period of starvation. The lack of resources could also affect a male's ability to court. Blache et al. (2002) studied the reproductive endocrine responses to changes in diet of mature male sheep. They concluded that glucose does not appear to be involved directly in reproduction, but that fatty acids can using dependant pathways initiate changes in testicular function (Blache et al. 2002).

In my study offspring viability did not appear to be affected by the male's nutritional status. Males of the tiger swallowtail butterfly *Papilio glaucus L.* (*Lepidoptera: Papilionidae*) receiving an enhanced diet of electrolyte and amino acids produced more viable offspring than control males (Lederhouse et al. 1990). Fricke et al. (2008) found that adult male nutrition had no significant impact on paternity share but that males kept on an intermediate diet (yeast levels at 100g/l) fathered more offspring than those on low or even high nutritional diets (Fricke et al. 2008).

I determined that the nutritional status of the male affected mating latency and this is consistent with the results in Chapter 6 and Appendix III. Mating duration in this experiment was also affected by the diet of the male. Offspring viability was not affected.

Mating duration, but not mating latency was affected by the type of food used in the mating vials. However, there were no interactions between these effects and the different male diets. Therefore there is no reason to believe that the use of either diet in the mating vials was problematic or had a biasing effect in terms of the effects seen in the main data chapters.

A2.5] Figures

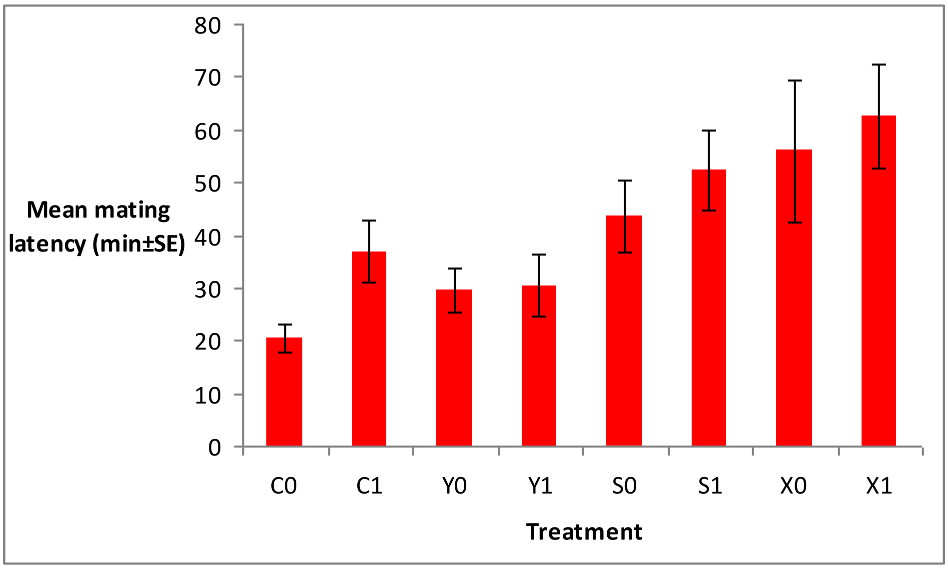

Figure A2.5.1 Mean mating latency (± SE) of wild-type *D. melanogaster* males placed immediately into their respective groups and kept for 7 days on different diets; standard SYA, 100% yeast: 100% sugar for control (C), 0% yeast: 100% sugar (S), 100% yeast: 0% sugar (Y) and agar only, 0% yeast: 0% sugar (X). Each group was placed in a mating vial with a female on either just agar (0) or on normal SYA food (1).

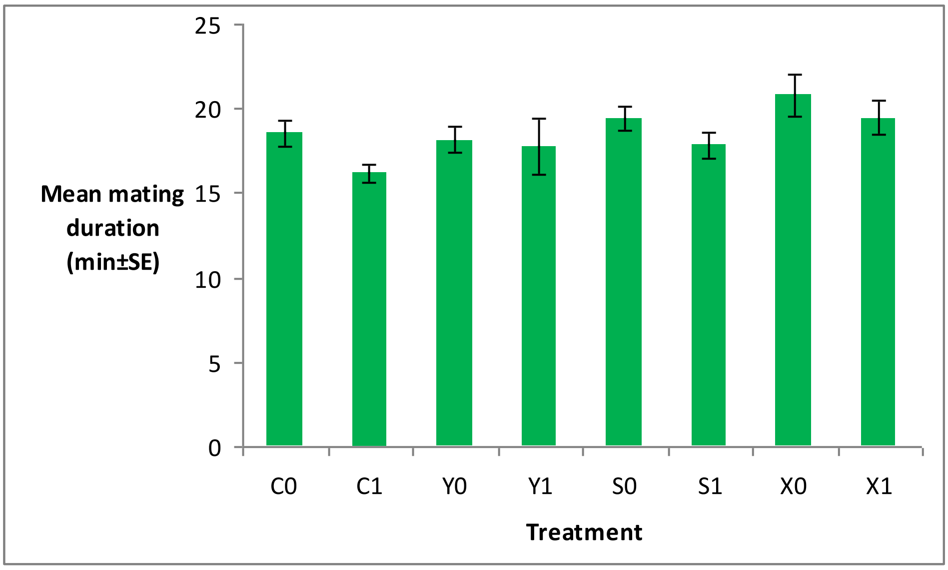

Figure A2.5.2 Mean mating duration (± SE) of wild-type *D. melanogaster* males placed immediately into their respective groups and kept for 7 days on different diets; standard SYA, 100% yeast: 100% sugar for control (C), 0% yeast: 100% sugar (S), 100% yeast: 0% sugar (Y) and agar only, 0% yeast: 0% sugar (X). Each group was placed in a mating vial with a female on either just agar (0) or on normal SYA food (1).

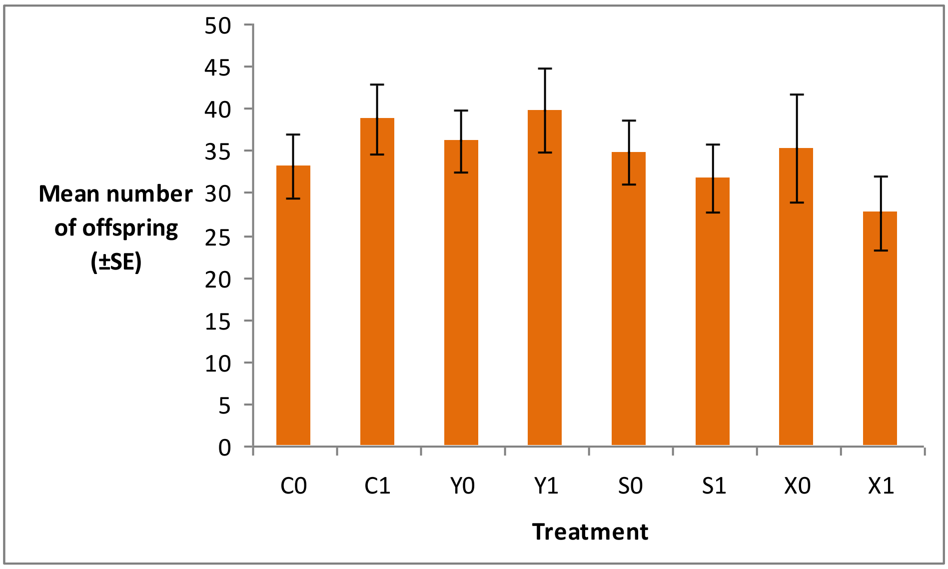

Figure A2.5.3 Mean offspring amounts (± SE) of wild-type *D. melanogaster* males placed immediately into their respective groups and kept for 7 days on different diets; standard SYA, 100% yeast: 100% sugar for control (C), 0% yeast: 100% sugar (S), 100% yeast: 0% sugar (Y) and agar only, 0% yeast: 0% sugar (X). Each group was placed in a mating vial with a female on either just agar (0) or on normal SYA food (1).

A2.6] Tables

Table A2.6.1 Percentage of wild-type *D. melanogaster* males that mated from each different nutritional group after 7 days; standard SYA, 100% yeast: 100% sugar for control (C), 0% yeast: 100% sugar (S), 100% yeast: 0% sugar (Y) and agar only, 0% yeast: 0% sugar (X). Each group was placed in a mating vial with a female on either just agar (0) or on normal SYA food (1).

Treatment	Percentage mated
C0	87.50
C1	95.00
Y0	87.50
Y1	92.50
S0	87.18
S1	82.50
X0	44.44
X1	76.67

Appendix III. Experiment to test whether access to food 24 hours from eclosion before assigning to differing nutritional diets affects a male's subsequent reproductive success

A3.1] Introduction

In the experiments in Chapter 6 I investigated the effects of nutrition on a male's reproductive success. It was not clear however whether the best way to test this was to allow all flies to eclose and develop fully over 24 hours before assigning them to their nutritional treatments, or whether to put them on their respective diets immediately following eclosion. I therefore conducted the experiment described here to determine whether an individual male's reproductive success is affected by feeding in the first 24 hours of life.

One condition critical for the survival of many organisms is their ability to adapt to different food in their diets. The main requirements for most animals diets are carbohydrates, fats and proteins (Dagon et al. 2005; Zinke et al. 2002). The intake of nutrients has a profound effect on development, fertility and lifespan. It is thought there is a trade off between sustaining development and fertility versus lifespan of the individual (Kirkwood 2002).

The nutritional requirements for larvae may be relatively uniform, unlike those needed for adults which may vary more widely. Research has shown that nutrition is very important for the development of a healthy foetus and so for viable offspring, but this is often more geared towards the female (House 1962). Breeders of ruminant livestock recommend that the male must receive adequate nutrition for 2 months prior to breeding (Robinson et al. 2006). This is because there are beneficial effects on sperm motility and the percentage of live sperm correlated with proper nutrition (Robinson et al. 2006; Kendall et al. 2000). Restricting levels of available nutrients can reduce a males fecundity (Chippindale et al. 1993) indicating a males general health can affect his reproductive viability.

The aim was to test whether a male given 24 hours to stock up on food would be in better condition than males placed directly into their food groups, and ultimately whether this would affect their reproductive success.

A3.2] Materials and Method

All males and females were raised on standard medium at a standard density (Chapter 2). Single males were collected at eclosion and kept on one of 4 diets (40 per group), normal (N: 100% yeast, 100% sugar), yeast only (Y: 100% yeast, 0% sugar), sugar only (S: 0% yeast, 100% sugar) and agar only (X: 0% yeast, 0% sugar) for 7 days immediately following eclosion. Another 160 males from the same cohort were placed on the normal (N) diet for 24 hours following eclosion before being randomly assigned (40 per group)to the diets above (N,Y,S & X) for 6 days. Males were transferred between vials without using anaesthesia (Appendix 1).

Females were kept in groups of 10 with added yeast granules and were placed in mating vials 24 hours before mating experiments began, with added liquid yeast to enable egg laying. Females were removed after 24 hours and the vials left for 12 days when offspring were counted.

A3.2.1 Statistical analysis

Statistical analysis was performed using SPSS v 16. Mating latency and mating duration data were tested for normality using Kolmogorov-Smirnov tests and for homogeneity of variance using Levene's tests. Data from mating duration and mating latency data were then subjected to ANOVA. Pearson Correlation tests were used to determine links between mating duration and the number of offspring produced.

A3.3] Results

Consistent with the previous results, mating latency was altered by adult male nutritional regime (ANOVA $F_{3,279}$=9.650, p<0.001). Mating latency was slower when males were kept on the agar only or sugar only diets in comparison to the yeast and normal diets (figure A3.5.1). Here there were no significant differences across any diet treatments in terms of mating duration ($F_{3,280}$=0.835, p=0.476, figure A3.5.2). The main purpose of this experiment was to test whether there was any effect of the diet the males experienced in the first 24 hours following eclosion. Here there were no significant differences between groups placed immediately onto their diets or those that had 24 hours on normal food prior to being placed on different diets in either mating latency, ($F_{1,279}$=0.093, p=0.297) or mating duration ($F_{1,280}$=0.621, p=0.431).

There were no significant differences in the percentage of males that mated from each group, i.e. from those that were placed directly onto the different diets versus those that had 24 hours on normal food prior to being placed onto the different diets. There was also no significant difference in offspring production and male nutrition in any of the groups ($F_{3,278}$=2.176, p=0.091, figure A3.5.3). But there was a significant correlation between mating duration and the number of offspring produced, (Pearson correlation r=0.177, N=286, p=0.003). All groups except those on a sugar diet had more offspring when placed on their diet after being allowed to build up their reserves for 24 hours.

A3.4] Discussion

The main findings were that mating latency was dependent on male nutritional treatment, and that males fed on normal or yeast diets mated quicker than those on no food or just sugar (as in Appendix II & Chapter 6). There was no difference in mating latency according to whether males were placed immediately on their diet or 24 hours later after presumably building up some food reserves. Although no significant differences were found in mating duration there was a significant correlation between the mating duration and the number of viable offspring produced. It would appear that yeast is important for mating to take place quickly, those flies with no yeast took longer to mate but diet didn't affect mating duration (Geer 1967). More offspring were produced across all the groups except the sugar only diet when males were placed on the diets following 24 hours on the full diet.

The reserve of food stores from larval feeding can prove a problem for feeding studies of adult nutritional requirements (Geer 1967). Zinke et al. (2002) showed that sugar on it own is not sufficient to quench a larva's hunger and although sugar fed larvae live longer than starved larvae they are smaller as adults compared to those larvae fed on yeast (Zinke et al. 2002). There are data to suggest that poorly fed adults may accumulate enough energetic resources to match well-fed competitors, if given enough days to feed (Aluja et al. 2001). The flies from this experiment were put on their diets directly following eclosion but they still behaved in the same way as the controls, showing they had sufficient reserves from their larval stage to enable them to perform in a comparable manner to males that were allowed 24 hours of full feeding before being allocated to the diet treatments.

The reason for undertaking this experiment was to test whether differences in the treatment of adult males directly following eclosion would obscure the later effects of the diets imposed. I found no indication that this was the case. There is therefore no evidence that this variation to the experimental protocols has any biasing effect on the outcomes of the experiments descibed in the main chapters.

A3.5] Figures

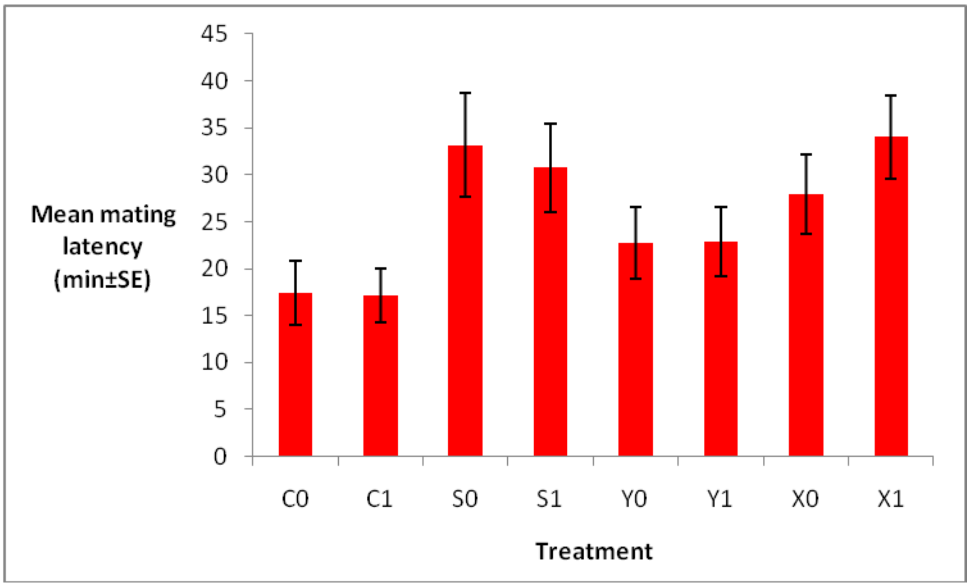

Figure A3.5.1 Mean mating latency (± SE) of wild-type *D. melanogaster* males kept for 7 days on different diets; standard SYA, 100% yeast: 100% sugar for control (C), 0% yeast: 100% sugar (S), 100% yeast: 0% sugar (Y) and agar only, 0% yeast: 0% sugar (X). Each group was placed immediately (0) in to their respective groups or given 1 day to feed up on normal SYA food (1) and then transferred to their respective diets for 6 days.

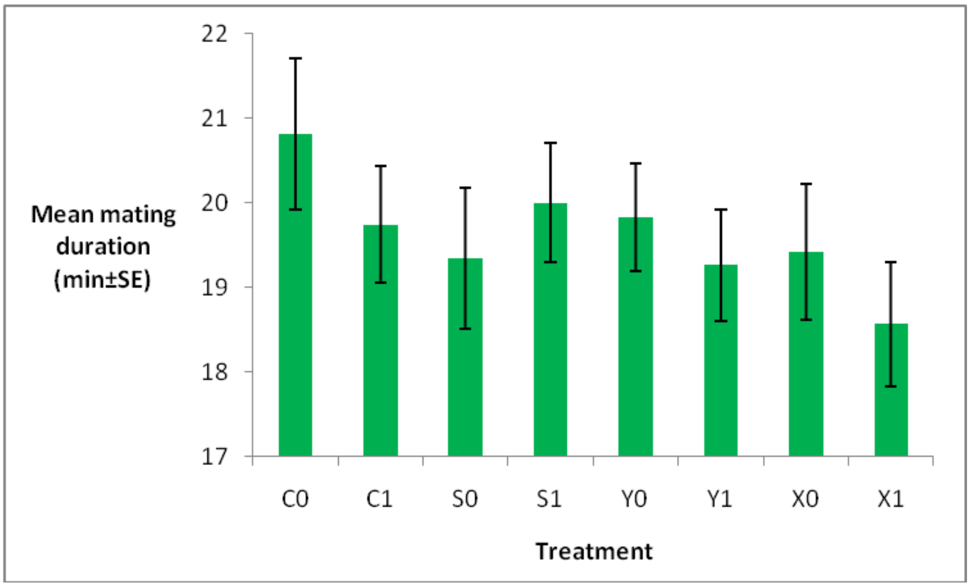

Figure A3.5.2 Mean mating duration (± SE) of wild-type *D. melanogaster* males kept for 7 days on different diets; standard SYA, 100% yeast: 100% sugar for control (C), 0% yeast: 100% sugar (S), 100% yeast: 0% sugar (Y) and agar only, 0% yeast: 0% sugar (X). Each group was placed immediately (0) in to their respective groups or given 1 day to feed up on normal SYA food (1) and then transferred to their respective diets for 6 days.

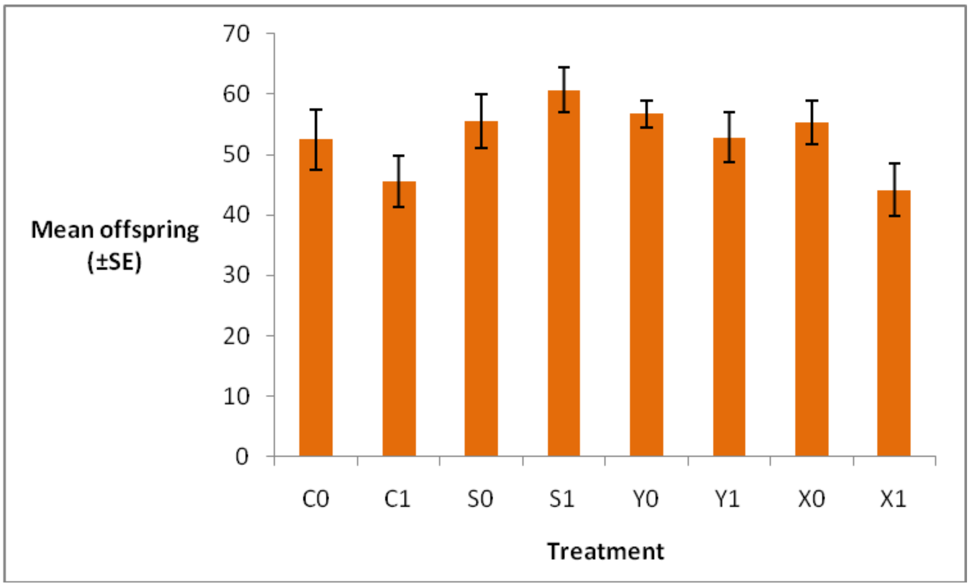

Figure A3.5.3 Mean number of viable offspring (± SE) of wild-type *D. melanogaster* males kept for 7 days on different diets; standard SYA, 100% yeast: 100% sugar for control (C), 0% yeast: 100% sugar (S), 100% yeast: 0% sugar (Y) and agar only, 0% yeast: 0% sugar (X). Each group was placed immediately (0) in to their respective groups or given 1 day to feed up on normal SYA food (1) and then transferred to their respective diets for 6 days.

Appendix IV. Food recipes and methods

A4.1] ASG medium for keeping flies

The brewer's yeast was obtained from DCL yeast Ltd (Scotland, UK), Nipagin powder from Clariant UK Ltd (Pontypridd, UK), Proprionic acid from Sigma (Dorset, UK) and the grape juice from Solvino Ltd (London, UK).

Standard ASG medium per litre of water

1L distilled water
10g agar
85g sugar
20g brewer's yeast
60g maize
25ml Nipagin solution

Preparation method: Mix agar and water in a saucepan and bring to the boil. Take off the heat and stir in all dry ingredients thoroughly. Cool to approximately 60°C before adding the Nipagin solution, stir and dispense.

A4.2] SYA medium for experiments

Standard SYA medium per litre of food medium

970ml distilled water
15g agar
75g sugar
100g brewer's yeast
30ml Nipagin solution
3ml Propionic acid

Preparation method: Prepare as in ASG recipe above but add both the Nipagin and Propionic acid after cooling and dispense.

A4.3] Nutrition experimental media values used in Chapter 6

SYA media with restricted yeast and sugar (per litre of food medium)

100% yeast: 100% sugar as above (standard)
100% yeast: 20% sugar = 100g yeast: 13g sugar (w/w)
20% yeast: 100% sugar = 20g yeast: 75g sugar (w/w)

A4.4] Nipagin solution (used in all food recipes as an antifungal agent)

10% Nipagin solution

1900ml 100% ethanol
100ml distilled water
200g Nipagin powder

A4.5] Charcoal SYA medium

Charcoal was added to the SYA medium in some cases to facilitate egg counting, 4g of charcoal powder was added per litre of standard SYA food medium.

A4.6] Live yeast paste

The live yeast used was delivered in 500g tins from DCL yeast limited.

Live yeast paste was used to supplement the grape juice medium used for egg laying (section 2.1.3). A small amount of yeast granules were mixed with a few drops of water to form a paste for use with the grape juice medium. Also a standardised volume of liquid yeast was added to vials containing larvae to encourage growth, (section 2.2.1).

A4.7] Grape juice medium

The grape juice medium was used to collect fly eggs.

Recipe for approximately 18 Petri dishes standard size (8.5 diameter by 1.5 deep)

550ml distilled water
25g agar
300ml concentrated red grape juice
21ml Nipagin solution

Preparation method: Mix 500ml of the water and agar as they are bought to the boil. Add the red grape juice and bring to the boil again, simmer for a few minutes. Add the remaining 50ml of water and allow mixture to cool to about 60°C before adding the Nipagin solution. Immediately dispense into petri dishes and leave to cool at room temperature. These were then kept in the freezer until required.