Consequences of Plasticity:

How do Individual Conditions Affect Physiology, Survival and Copulatory Behaviours in *Drosophila*?

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Abstract

Drosophila melanogaster are a polygamous species: males and females repeat copulation with multiple mates. When more than one male's sperm have to compete to fertilise the same female's eggs, sperm competition occurs. This has driven individuals to develop physiological and behavioural plastic responses to enable them to outcompete others and increase their chances of siring successful offspring. But what effect do variable environmental conditions have on these responses? Plasticity comes with a cost, often causing reproductive-survival trade-offs to occur. Previous experiments have failed to show how stress impacts reproductive terminal investment, and other plastic responses, so this study aims to contribute knowledge to this field.

The experiments were completed using a combination of stresses to weaken *Drosophila*: a two-day starvation period and either age (three, 14 and 28 days) or varying sperm competition levels (solitary males, pairs, groups of eight and solitary males in the presence of pheromones). Evidence of trade-offs and plastic reproductive terminal investment was investigated by monitoring behavioural responses (courtship behaviours, rejection responses and copulatory durations), physiological responses (spermatogenesis, egg production and fat reserve levels), as well as activity and starvation resistance as a proxy for longevity. Aged and starved flies, and flies that were not placed under sperm competition, were less active and had a shorter lifespan. These weaker males also tended to exert less energy into courtship behaviours; however they also copulated for longer. This suggests that they were investing in reproductive terminal investment. When males were placed with females that had been continuously fed, copulation lasted significantly longer. This could be to enable him to transfer more sperm, or as a method of mate-guarding. In addition to this, older females spent less time rejecting males, suggesting that they were more willing to succumb to copulation. Despite efforts, both males and females produced significantly fewer offspring when placed in stressful environments.

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Author’s Declaration

I hereby declare that all works presented in this thesis were prepared solely by the author, Emily R. Churchill. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

Chapter 1: Introduction

'Fitter' organisms are better adapted to their environment and benefit from higher rates of survival and reproduction than those that are less well adapted. Therefore any heritable characteristics they possess gradually become more common within the population; this is termed natural selection (Darwin 1871; DeBeer 1976; Endler 1986). However there are multiple examples of elaborate traits that have evolved to exist within the population that are detrimental to the survival of individuals. These traits have been selected for by sexual selection (which arises when one sex has a preference for a certain set of characteristics in the other sex) (Bateman 1948; Andersson 1994).

There are two mechanisms of sexual selection: intra-sexual selection and inter-sexual selection. Intra-sexual selection occurs when there is competition between two or more members of the same sex (usually male); they fight for dominance, aiming to kill or deter rivals in order to secure a mate (Bateman 1948). This explains the evolution of male weapons and defensive behaviour. Evidence of this is found in the Japanese horned beetle, *Trypoxylus dichotomus septentrionalis* where males have horns which they use to 'shove' or 'flip' their opponents (Hongo 2003).

Inter-sexual behaviour occurs where members of one sex (usually female) have a preference for a particular characteristic of the opposite sex (Darwin 1871). Thus they pick their mates based on the quality of this trait present on the individual – as a result, the trait or 'ornament' evolves and becomes exaggerated (Andersson 1994; Pomiankowski and Iwasa 1998). This has been observed in stalk-eyed flies, *Cyrtodiopsis dalmanni*; as their name suggests, their eyes are found at the end of long stalks protruding from their head (Chapman et al. 2005). This trait is a handicap to the males, decreasing their chance of survival unless they also develop compensatory traits (Husak et al. 2011). It has been proposed that only healthy males with advantageous genes can afford to produce such a costly trait, thus as females are looking for males in a good condition, they are attracted to those with more exaggerated ornaments – such as larger eye spans in stalk-eyed flies (Panhuis and Wilkinson 1999; Hingle et al. 2001). This concept is known as the Handicap Principle (Zahavi 1975; Iwasa et al. 1991).

1. 1 Competition and Plasticity in Polygamous Species

Environmental conditions are often extremely variable so it is likely that an organism's optimum survival strategy is constantly changing. Phenotypic plasticity is the ability of a genotype to produce different phenotypes depending on the prevailing environmental

conditions (Via et al. 1995; DeWitt et al. 1998). It is an important fitness component as it enables organisms to respond to environmental fluctuations by exhibiting different behavioural and physiological phenotypes (Agrawal 2001). Although it has many benefits, there are constraints that limit its evolution; it is an energetically expensive strategy and relies on a great deal of genetic variation – there must be sufficient underlying genetic diversity to produce these varying phenotypes (DeWitt et al. 1998). Despite these constraints, phenotypic plasticity is widespread, manifesting itself using a variety of methods (Piersma and Drent 2003; Gabriel 2006). It could be one irreversible change; as observed in common frog tadpoles, *Rana temporaria*. Tadpoles become less active at decreasing water levels, and begin metamorphosis earlier (Laurila and Kujasalo 1999). Or it could result in a change that is flexible throughout the individual's lifespan: for example, a Japanese quail, *Cortunix japonica*, changes the size of its organs in response to changes in dietary fibre content (Starck 1999).

Competition also exists between sperm when two or more males compete for the same female's eggs (Parker 1970). Sperm cell morphology, ejaculate components, sexual organs and copulatory behaviours had to adapt to this competition. Males vary sperm allocation according to two possible situations: varying sperm risk (a solitary male compared to one with a single rival) and varying sperm intensity (increasing numbers of rivals) – for example, they transfer a higher quantity of sperm in the presence of more males (Abraham et al. 2015). One particularly famous example of sperm competition is the morphological change in testes size of scarab beetles, *Onthophagus taurus*, in which testes size increases in the presence of sperm competition (Simmons and García-González 2008).

Males also transfer seminal fluid proteins within their ejaculate (Wolfner 2002; Wigby et al. 2009). There are a wide range of proteins, which appear to have evolved to increase male reproductive success in a variety of ways, including: inhibition of female remating, egg laying stimulation, rival sperm eradication, regulation of female attractiveness, more efficient sperm storage and antibacterial proteins (Scott 1986; Clark et al. 1995; Herndon and Wolfner 1995; Tram and Wolfner 1998; Neubaum and Wolfner 1999; Lung et al. 2001)

Sperm competition intensity increases with an increasing male presence. In many species, males release pheromones which can be detected by other individuals of the same or similar species, and alert them of their presence. Sex pheromones are released to attract a member of the opposite sex and to persuade them to acquiesce to copulation (Grillet et al. 2006). Many species of *Drosophila* possess cuticular hydrocarbons that act as courtship

inducing pheromones (Cobb and Jallon 1990). This allows them to assess the species and sex of their potential mate (Antony and Jallon 1982; Cobb and Jallon 1990). The presence of these pheromones could imitate high sperm competition conditions in the absence of flies; enabling the effects of sperm competition to be investigated using secreted pheromones alone.

1. 2 *Drosophila* Courtship and Rejection Responses

Male *Drosophila* perform an elaborate courtship display to attempt to attract a female mate, using a series of visual, olfactory and auditory stimuli (Bennet-Clark and Ewing 1967; Spieth 1974; Markow 1987). Once a male identifies a female, he will orientate himself in her direction, advance towards her and tap her abdomen; following this he will extend and vibrate one of his wings, and if she accepts he will initiate genital licking before finally mounting and beginning copulation (Spieth 1952; Manning 1959; Sokolowski 2001). Females then respond to this ritual either by accepting or refusing, using a range of repulsion behaviours; although initially they flee from any male attempting courtship (Partridge et al. 1987b). Spieth (1952; 1974) lists a number of female rejection responses including fleeing, kicking, fluttering their wings or extruding their ovipositor. The receptiveness of the female determines the length of the display – which can last for just a few seconds, or up to several hours (Bastock and Manning 1955).

Wing extensions are a clear visual cue, so they have been the focus of many sexual behaviour experiments (Brown 1965; Lasbleiz et al. 2006; Yamamoto and Koganezawa 2013). However, for *Drosophila* they are an auditory cue – the extension and vibration of the wing produces a noise referred to as a ‘song’ (Bennet-Clark and Ewing 1970).

Courtship has been shown to require a large amount of energy for a wide range of organisms; including male bullfrogs, *Rana catesbeiana* (Judge and Brooks 2001) and plethodontid salamander, *Desmognathus ochrophaeus* (Bennett and Houck 1983). It is an energetically costly process for *Drosophila* too, even for females which were long thought to play only a passive role; it can be responsible for reductions in longevity (Cordts and Partridge 1996; Clutton-Brock and Langley 1997). It is therefore plausible that courtship effort will decrease when males are placed under stressful conditions, as the expense can no longer be afforded. This indicates that if courtship is an honest signal (Zahavi 1975; Andersson 1994) then the females should acquiesce to copulation in less time with increasing courtship effort.

1. 3 Reproduction in *Drosophila*

Reproduction incurs considerable costs as it requires a large amount of energy, yet it is crucial for all sexual populations to thrive and survive (Hochachka 1992). These reproductive constraints are necessary because without them, survival and fertility will be simultaneously maximised by natural selection (Williams 1966; Harshman and Zera 2007).

Drosophila are polygamous: both males and females repeat courtship and copulation on multiple occasions (Dobzhansky and Pavlovsky 1967). Polyandry evolves whenever a female benefits from having multiple mates (Arnqvist and Nilsson 2000). Mating with multiple males enables female *Drosophila* to improve upon her mate choice; if the latter sires are more attractive and better quality mates there is an increased chance they will provide her with more grand-offspring (Weatherhead and Robertson 1979) – and it compensates for any infertility previous mates may have. However the cost of this strategy is high, as copulation often causes harm to both males and females (Fowler and Partridge 1989; Kuijper et al. 2006).

The good gene theory suggests that females should choose a high quality mate so her offspring and grand-offspring inherits genetic advantages (Yasui 1997). Females also benefit from mating with an attractive mate, as then her offspring are likely to be more attractive to their potential mates, so they will have a higher mating success. This is known as the 'sexy sons' hypothesis (Weatherhead and Robertson 1979).

In order to compete against other potential mates, male *Drosophila* have been forced to evolve behavioural and physiological adaptations. Like many insects, they practice mate-guarding and manipulation of female-remating (Alcock 1994). To aid mate-guarding, males have small sex combs on their front legs that enable them to hold onto females during copulation (Kopp 2011). However, the cost is a limiting factor of these responses as they are energetically expensive.

Often an increase in reproduction rate causes a decline in survival or fertility (Williams 1966; Partridge and Andrews 1985; Harshman and Zera 2007; Lee et al. 2008). Both males' and females' lifespans are affected by increased sexual activity, caused by the cost of the necessary muscular movements in male courtship efforts, the female's struggle to flee and in copulation itself (Partridge and Farquhar 1981; Fowler and Partridge 1989). It has also been shown that sexual activity elevates metabolic rate in female *Drosophila simulans* (Giesel et al. 1989) which in turn reduces lifespan (Miquel et al. 1976).

Production of sperm and seminal fluids also comes at a high physiological cost to males (Dewsbury 1982; Galvani and Johnstone 1998) which is why insemination rate declines with age (Snoke and Promislow 2003). If courtship is unsuccessful, this is time and energy wasted for the fruit fly.

If an organism has been placed under stressful conditions and is close to death, it will need to make a trade-off in order to maximise its reproductive potential. It may exert all of their remaining energy into mating for a final time, prioritising passing on their genes, rather than attempting to conserve enough energy to survive. This theory is referred to as terminal investment (Clutton-Brock 1984; Andrade and Kasumovic 2005). If an individual remains in poor conditions long-term, morphological changes have been shown to occur across generations; as observed in the scarab beetles. Although their testes size increased under high sperm competition, it decreased when the individuals were maintained in nutrient-deficient conditions (Simmons and García-González 2008).

1. 4 Physiological Effects of Environmental Conditions

Larger *Drosophila* are of a higher quality and are better able to cope with a change to poorer conditions as they have more energy and fat reserves to aid them; they take longer to starve as 'the per weight energy expenditure of larger animals is lower than that of smaller ones' (Partridge and Farquhar 1983), which makes them more attractive to the opposite sex. This is reflected in their lengthened lifespan and greater lifetime mating success (Partridge et al. 1987b; Partridge and Farquhar 1983).

Females benefit from mating with larger males, because according to the 'sexy sons' hypothesis, this means their offspring will also be larger and more attractive – and will therefore have a greater mating success (Weatherhead and Robertson 1979; Møller and Alatalo 1999). Larger females are also considered to be more attractive, and males prefer to mate with them (Long et al. 2009); despite them requiring more energy to court as they are more reluctant to mate than smaller individuals and continue to flee for a longer period of time (Turiegano et al. 2012). As larger females are more attractive, they are able to be more selective about which males they allow to mate with them. They prefer to mate with larger males despite increasing size causing them more harm and causing a greater reduction to their lifespan – possibly due to courtship or ejaculate toxicity (Partridge et al. 1987a; Partridge and Fowler 1990; Chapman 1992; Lung et al. 2002; Pitnick and García-González 2002).

As well as altering their behaviour, some organisms are capable of modifying their physiology too – or behave in a way that causes an alteration to their appearance. For example, when organisms find themselves in a nutrient deficient environment, they will resort to using stored fats for energy, which will reduce their body mass (Djawdan et al. 1998; Arrese and Soulages 2010). Additionally, many males alter the composition of their ejaculate and the quantity of sperm they transfer in varying conditions (Wigby et al. 2009; Bretman et al. 2011).

Oviposition and ovulation incur some physical costs too (Fowler and Partridge 1989; Wang et al. 2001; Wigby and Chapman 2005). Larger females are better able to cope with this and have a higher fecundity (Lefranc and Bundgaard 2000). Therefore, there is a positive correlation between the nutrition available in the environment and the number of eggs produced (Terashima and Bownes 2004); and when given the option the female will consume more food than sterile females (Barnes et al. 2008). However, longevity trades-off against reproduction: the more eggs a female fruit fly lays, the shorter her lifespan is (Alpatov 1932; Partridge et al. 2005; Lee et al. 2008). Diet further influences this trade-off, as 'the nutritional intake that maximises lifespan results in poor reproductive performance and vice versa' (Maklakov et al. 2008).

Similarly, in males insemination rate declines with age, as production of sperm and seminal fluids are energetically expensive processes (Pitnick et al. 1995; Snoke and Promislow 2003). Courtship intensity and fecundity also decline with decreasing male size (Partridge et al. 1987a; Partridge et al. 1987b). A negative correlation is again observed between reproductive success and longevity (Partridge and Andrews 1985; Cordts and Partridge 1996). It is therefore logical that males adjust their ejaculate quality and quantity based on environmental conditions and the female's mating status (Dewsbury 1982; Friberg 2006; Cameron et al. 2007; Lüpold et al. 2011).

Many studies have investigated the effects of male characteristics on copulatory behaviour, yet despite females often determining when (if ever) a pair will mate, there has been considerably less research into the importance of female characteristics – or the combined impacts caused by both sexes. Additionally, little is known about the consequences of a variable environment and how it affects *Drosophila melanogaster* copulatory behaviour and physiology. This study attempts to address these omissions by investigating the effects of individuals' size, and alterations to living conditions and sperm competition intensity on various responses; including male courtship behaviours, female

rejection responses, latency to court, courtship duration, copulation duration, numbers of sperm and eggs produced, levels of fat reserves, activity and longevity.

Chapter 2: The Effect of Age and Starvation on Courtship, Copulation and Egg Survival

2. 1 Introduction

2. 1. 1 Courtship Behaviours

Male *Drosophila melanogaster* have to perform an elaborate and energetically expensive courtship ritual before a female becomes willing to mate with them (Bennet-Clark and Ewing 1967; Spieth 1974; Markow 1987). Courtship behaviours include wing extensions, chasing, abdomen tapping and genital licking (Spieth 1952; Manning 1959; Sokolowski 2001). This costly reproductive behaviour has an adverse effect on male lifespan (Partridge and Farquhar 1981; Dewsbury 1982; Cordts and Partridge 1996; Lee et al. 2008) so any trait or condition that has previously weakened flies will have a further detrimental effect.

Nutrient deficiency causes individuals to become weaker, and forces a courtship-longevity trade-off (Cordts and Partridge 1996; Droney 1998; Fricke et al. 2008; Fricke et al. 2015). Individuals in a better quality environment, with a body in good condition, court more than those which are weak (Droney 1998; Wagner JR and Hoback 1999; Kotiaho 2002; Engqvist 2009). Younger and larger males are of a higher quality, so they are able to invest more into their lifetime reproductive success (Partridge et al. 1987a; Partridge et al. 1987b; Jones and Elgar 2004; Dhole and Pfennig 2014). They are better able to cope with variations within the environment, and suffer comparatively less in nutrient-deficient environments (Partridge and Farquhar 1983).

2. 1. 2 Rejection Responses

Females also play an active role in courtship. They flee from males and refuse their advances through a series of rejection responses: kicking, fluttering their wings and extruding their ovipositor (Partridge et al. 1987a; Spieth 1952; Spieth 1974). This activity has similar effect on female longevity: lifespan decreases with increasing rejection responses (Clutton-Brock and Langley 1997; Travers et al. 2015).

Plastic responses (responses to a changing environment as a result of phenotypic plasticity) in females behaviour were observed under stressful environmental conditions (Terashima and Bownes 2004; Barnes et al. 2008; Maklakov et al. 2008).

2. 1. 3 Copulation

Females often dictate if and when copulation occurs (Bastock and Manning 1955). If the male is comparatively 'fitter' (that is larger, younger or healthier) or comparatively more attractive than the female, then it is likely that the female will acquiesce to copulation quicker (Lefranc and Bundgaard 2000; Fricke et al. 2008). Equally males adjust their energy spent according to female attractiveness (Lüpold et al. 2011). If a male is of a poor quality and is less attractive, the female may never accept him as a mate.

When the environment is so poor that it is a threat to the pair's survival, reproductive terminal investment may be observed (Clutton-Brock 1984; Krams et al. 2015). If the pair perceives it to be their last chance to mate and pass on their genetic information, they may exert all of their remaining energy into copulation, and consequently oviposition (Clutton-Brock 1984; Creighton et al. 2009; Krams et al. 2015). Reproductive behaviour is further affected by size. Both males and females are considered to be more attractive when they are bigger and younger (Partridge et al. 1987a; Cook and Cook 1975; Lefranc and Bundgaard 2000; Turiegano et al. 2012; Dhole and Pfennig 2014; Travers et al. 2015). Latency to copulate is shorter, and copulation duration is longer, when the mate is more attractive (Ewing 1961; Cook and Cook 1975; Lefranc and Bundgaard 2000).

2. 1. 4 Hypotheses

The effects of male characteristics on copulatory behaviours have been widely studied (Partridge et al. 1987a; Partridge et al. 1987b; Jones and Elgar 2004; Fricke et al. 2008), but comparatively fewer investigations have been completed on the impacts of females – despite some studies suggesting female characteristics are of more importance than male's (Lefranc and Bundgaard 2000).

The research in this chapter aims to provide more information on the interaction between male and female *Drosophila* conditions, investigating how size and nutrition availability affects courtship behaviours, repulsions responses, copulation and reproductive success. This will create a clearer picture on how different organisms will react to the changing environment; such as loss of food resource as caused by a changing global climate (McMichael et al. 2007). It could also provide a greater understanding of how food distributions will help protect populations of endangered species, or how and when food should be distributed when human aid is required.

The hypotheses are:

- Older males that experience a period of starvation will have a longer courtship latency as they will be less willing to exert energy into expensive processes
- Older and starved males will also have a shorter courtship duration as they will be weaker and will not be able to afford the expense
- Males that are starved and males that are older will copulate for longer than high quality males if they invest in reproductive terminal investment
- Latency to court will be shorter when females are older and fed – they will be less attractive so males will be less willing to court or copulate with them
- For the same reasons, courtship duration will be longer where females are older and have experienced a starvation period
- Copulation will last longer when females are fed and younger – they will be more attractive so males will aim to transfer more sperm and guard the females from rival males
- Courtship efforts will decrease with increasing age and starvation in males as they will not be able to afford the expense
- Courtship efforts will also increase when females are young and not exposed to a period of starvation – these females will be more attractive and are therefore worth investing more energy in
- Fewer rejections will be observed when male mates are younger and not starved because females will find them more attractive
- More rejections will occur with younger, fed females because they will be more discriminatory
- Males that are older and experience a starvation period will produce fewer offspring because they will be weaker and have less remaining energy
- Older and starved females will also produce fewer offspring, and fewer eggs in total for the same reasons

2. 2 Methods

2. 2. 1 *Drosophila* Maintenance

F1 virgin male and female *Drosophila* were collected from crosses of Oregon-R and Canton-S. Both strains were maintained in 40ml vials on a standard agar-based medium of 25g of yeast per litre (Bass et al. 2007) at a constant temperature of 25ºC (Imasheva et al. 1998) in a 12 hour light:dark cycle beginning at eight o'clock. Between 20 and 30 *Drosophila* were housed in each vial; to minimise inbreeding all vials were mixed every three to four days. To ensure behavioural normality F1 crosses were used in this experiment. Four virgin Oregon-R females were placed in a 40ml vial with four Canton-S males and allowed to breed; after three days they were removed to minimise overcrowding and reduce the likelihood of the vials having a significant effect on the flies' behaviours and responses. To ensure their virginity, flies were removed from the vials less than six hours after eclosion (Moatt et al. 2013).

The F1 virgins were anaesthetised using carbon dioxide so they could be sexed and were then placed in 40ml vials in solitude, to avoid any effect of competition. Following this, they were moved to 24 hour light at 25ºC, where they remained until they reached the appropriate age for testing.

2. 2. 2 Treatment Protocol

There were 16 different treatments in total, covering both sexes, three ages and three different starvation patterns: males and females at three, 14 and 28 days old that were either not starved, starved at days two and three, or starved during the final two days before testing (days 13 and 14 for 14 day olds, and 27 and 28 for 28 day olds) (as described in figure 1); see table 1 for the number of *Drosophila used*.

Table 1. The number of replicates used in each treatment.

NS=Not starved SE=Starved in final two days S3=Starved at 2-3 days old

SE and S3 are the same treatment in three day old flies.

3=Three days old 14=14 days old 28=28 days old

Males				**Females**			
	NS	**SE**	**S3**		**NS**	**SE**	**S3**
3	16	11	-	**3**	19	15	-
14	16	17	17	**14**	14	15	15
28	15	15	14	**28**	15	12	14

Flies that were exposed to the starvation treatment were placed in similar 40ml vials; however these contained 7ml of starvation agar (appendix 1.ii). Previous unpublished research from within this laboratory found that the mean date at which a fly died in starvation was after three days ($\bar{x}$=3.68, SD=7.92, n=37); so a two day starvation period was used in this experiment to handicap the *Drosophila* without causing their death. The flies need to be stressed in order to observe terminal investment, but still need to be able to participate in courtship and copulation rituals. To ensure that the effects observed were caused by the effects of starvation rather than the stress from translocation to new vial, each fly experienced a change in vial even if they were not being placed in a starvation treatment. The flies were transferred to a new vial at a similar time each day where possible, to keep starvation periods precise.

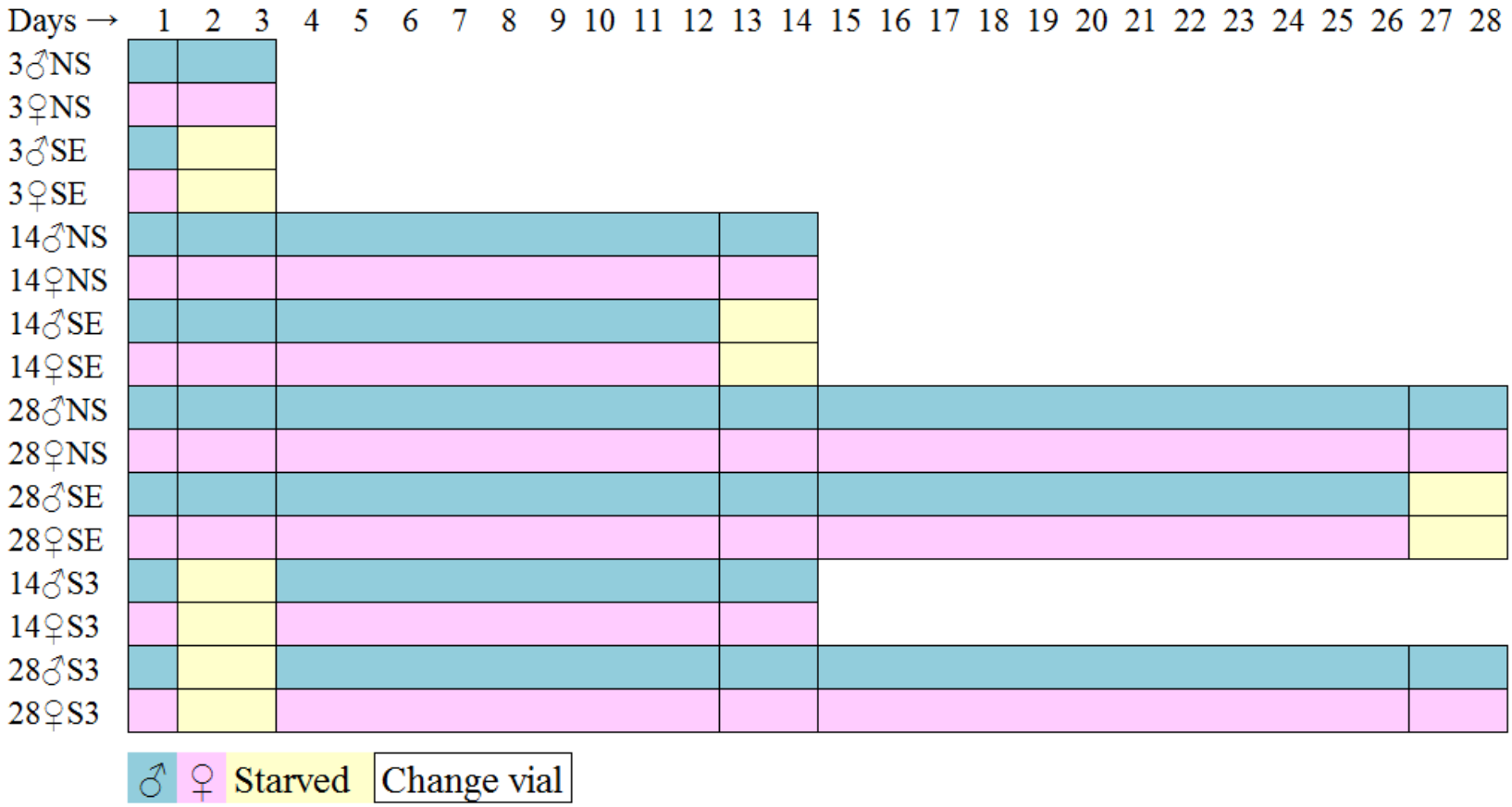


Figure 1. The sixteen treatments used in this experiment; showing the duration of starvation periods and when translocation occurred.

NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing

3=3 days old 14=14 days old 28=28 days old

2. 2. 3 Experimental Technique

Wing extensions have been extensively studied as they give a clear visual cue, and there is no ambiguity in the male's intentions (Brown 1965; Lasbleiz et al. 2006; Yamamoto and Koganezawa 2013). This behaviour is easy to observe even when collecting data from video footage. Many investigations have been completed by analysing data collected from video footage (Full and Tu 1991; Schenk and Bacher 2002; Manoukis et al. 2014). However, if the footage is not of a high quality it can be difficult to identify the different behaviours exhibited. The benefit of using video footage is that it can be paused and rewound to enable behaviour sequences to be re-evaluated to check for discrepancies, and it often enables the viewer to observe more total replicates. In order to attempt to clarify whether video footage or observation is the most effective method, both have been used in this investigation. The hypothesis was that data collected from the video footage will have fewer observations but should not be significantly different from that collected using direct observations.

2. 2. 4 Copulation Observations

When the fruit flies reached the required age they were aspirated into the mating arena with a standard mating partner – a seven day old male or female *Drosophila*, that had been fed throughout life and was maintained in a solitary vial at 25ºC in constant light. Each cylindrical mating arena slot was approximately 6200mm^3 and contained a small amount of cotton wool (2000mm^3) to minimise space and increase the encounter rate; 0.01g of active yeast granules were also added to promote egg laying and therefore encourage copulation. To further increase encounter rate, the activity of the flies was increased by heating the mating arena to 27ºC (Imasheva et al. 1998). A digital video (Logitech HD Pro Webcam C910) recorded the flies' behaviour when present in the mating arena, and the first five minutes of courtship were also directly observed. Courtship was recorded as having begun after the first wing extension was completed by the male; this definition was used as it is a clear visible and non-ambiguous movement. This five minute period was chosen because previous unpublished work from the laboratory has shown that the behaviours initiated in the first five minutes of courtship are representative of behaviours exhibited over the entire courtship period ($p<0.001$, d.f.=42, $r=0.612$). Courtship and copulation do not always occur, so time limits were set: if courtship was not initiated within 30 minutes, and copulation within 75 minutes, then the pair was recorded as not courting or copulating respectively. When courtship did not occur, courtship latency equals 30 minutes (1800s); and when copulation did not occur, copulation latency equals 75 minutes (4500s) (Eastwood and Burnet 1977; Nandy et al. 2012). To assess the impact

of time windows on measures of courtship and copulation, all statistical analyses were completed again with larger limits (3600s when courtship did not occur and 6300s when copulation did not occur). However when watching the video footage, if the pair were observed mating within 75 minutes despite taking longer than 30 minutes to court, the courtship data was still included in the analysis.

During direct observation, the number of wing extensions the male initiated was counted, as well as tapping, licking and mounting (appendix 2.i); these were summed to calculate 'total courtship events'. The female rejection responses, fleeing, kicking and fluttering of wings (appendix 2.ii), were also observed within this five minute period – 'total rejection responses' were also calculated by summing these three responses. Connolly and Cook (1973) interpreted extrusion as a way of 'physically preventing copulation', rather than inhibiting courtship behaviours. It is also a subtle behaviour that would often not be captured by video, so it was not included in this study. These behaviours were watched at the time of completion as they are more subtle and require smaller movements which were difficult to observe from the video footage. Larger movements, including males' chasing and females' fleeing were counted afterwards from the footage. Courtship latency, courtship duration, copulation latency and copulation duration were also calculated from the video footage.

The video was re-analysed using a time-event recording programme: this enabled the duration of each courtship or rejection behaviour, and total courtship or rejection response duration (appendix 2.iii) to be calculated. If the male did not court, a value of zero for courtship behaviours was given. However, female rejections were left blank as they were not given the opportunity to reject. The data collected by eye was compared to that collected with use of the video using a paired t-test, to determine whether using video footage is an effective method, and could be used to count the subtle behaviours completed by the fruit flies.

After copulation, the males were aspirated into small tubes (1200mm^3) which were then put into a DAM (*Drosophila* Activity Monitor) (DAM, Trikinetics, Waltham, MA, USA). The data collected from this stage of the method will be analysed and discussed in Chapter 5.

2. 2. 5 Video Footage as an Analytical Method

The number of wing extensions recorded by direct observation of mating pairs were broadly similar to that observed from watching the video footage ($t(202)=0.188$, $p=0.852$).

However, there were significantly more abdomen taps, mounting events and kicks observed from the video footage (abdomen taps: $t(204)=-12.286$, $p=2.20e^{-16}$; mounts: $t(184)=17.008$, $p=2.20e^{-16}$; kicks: $t(200)=20.727$, $p=2.20e^{-16}$). The number of wing flutters observed using each method were different, but this was not significant ($t(202)=0.200$, $p=0.842$).

Pearson product-moment correlations were used to identify significant positive correlations in almost all behaviours measured using video footage and direct observations (wing extensions: $r=0.891$, d.f.=201, $p=2.20e^{-16}$; abdominal taps: $r=0.560$, d.f.=203, $p=2.20e^{-16}$; mounting events: $r=0.782$, d.f.=184, $p=2.20e^{-16}$; kicks: $r=0.826$, d.f.=200, $p=2.20e^{-16}$). However, wing flutters from video footage and direct observations were not significantly correlated ($r=0.0141$, d.f.=202, $p=0.842$).

Based on these results, data collected from observing the video footage was analysed in this report. This is because more data can be collected using video footage as it can be re-watched multiple times. This also enables mistakes to be corrected if they occur.

2. 2. 6 Females' Reproductive Investments

The females were aspirated into an empty 40ml vial which was upturned onto a circle of red agar-based medium (appendix 1.iii), coloured with natural food dye to enable better vision of eggs. The vials were placed in damp sand so the agar-based medium retained moisture; see figure 2. The females remained in this vial for one day and were then placed in a second identical vial for one further day. One day after the fruit fly had been removed, the total number of eggs was counted, and then a day later total number of unhatched eggs were counted, enabling total eggs and total hatched eggs to be calculated when combined with the second vial – see table 2 for further clarification.

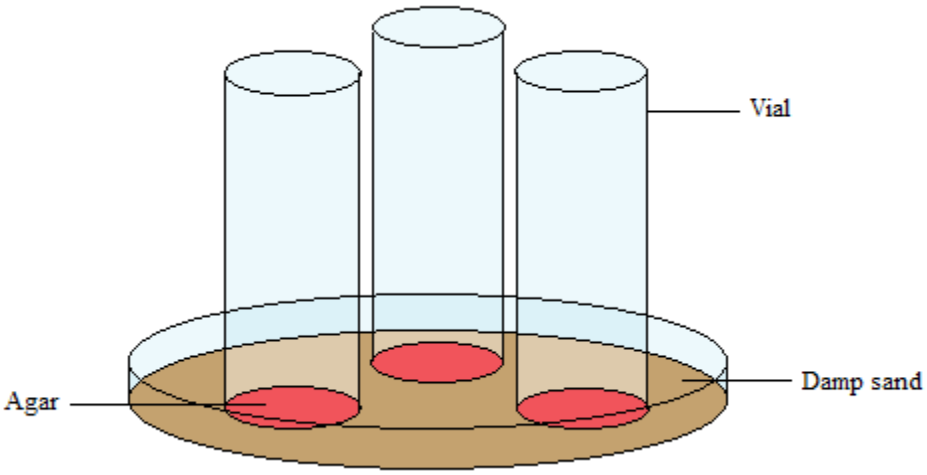


Figure 2. The set up for counting female *Drosophila* eggs; red agar placed on damp sand covered by a 40ml vial.

Table 2. A timeline describing how the females' eggs were counted.

Day One	**Day Two**	**Day Three**	**Day Four**
Add fly to first vial	Count total eggs	Count unhatched eggs	
	Move fly to second vial	Count total eggs	Count unhatched eggs

2. 2. 7 Body Size Measurements

Once the flies had died, their left wings were removed and measured to allow an investigation into whether there was an effect of body size. The lengths were measured to the nearest 0.005mm under a binocular microscope (Nikon Eclipse E200 equipped with a graticule) at four times magnification. The measurements were taken from the intersection of the anterior cross vein and the longitudinal vein, to the intersection of L3 with the distal wing margin (Partridge et al. 1987a); see figure 3. The difference in size was calculated by subtracting the male's wing length from the female's wing length. Further unpublished research has shown that the size of the left wing and right wing of *Drosophila melanogaster* is significantly similar, so where the left wing was absent or damaged, the right wing was used.

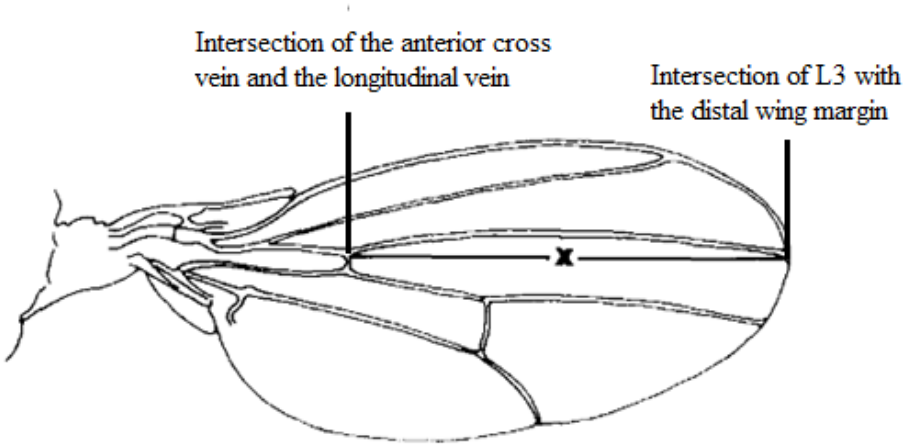


Figure 3. 'X' describing how the wing size measurement was taken, in between the two landmarks – adapted from Partridge, Hoffmann, and Jones 1987a.

2. 2. 8 Statistical Analysis

Statistical analyses were performed using the statistical software package R version 3.0.2 (R Core Team 2013). The data was broken down into several subsets: male and female data were analysed separately, further analysis was completed dependent on whether the pairs mated or courted. Binomial general linear models (GLMs) were used to assess whether courtship or copulation occurrence was affected by treatment. One-way and two-way ANOVAs and ANCOVAs were used to analyse the impacts of age and starvation on courtship behaviours (and rejection responses), including courtship latency, courtship duration (copulation latency) and copulation duration. Post-hoc Tukey HSD tests were then used where significant effects occurred. Additionally Pearson product-moment

correlations were completed on all courtship behaviours and rejection responses to establish whether any patterns arose.

The time and date of testing, the parent vial the fly originated from (parent vial one was the vial in which the test fly originated, and parent vial two is the vial that the mating pair eclosed from), and which mating arena each fly occupied, were recorded so these could be included in the above mentioned tests to assess whether any of these had a significant effect on the results found. The number of replicates and levels measured in each variable is described in table 3. It was often the case that the explanatory variables had small sample sizes and would cause many spurious significances. When this was true, they were removed from the model; which variables were included is stated with the presentation of results.

Table 3. The levels in each explanatory variable, and the total number of replicates measured.

NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing

3=3 days old 14=14 days old 28=28 days old

♂=male ♀=female

Explanatory Variable	3♂NS		3♀NS		3♂SE		3♀SE		14♂NS		14♀NS		14♂S3		14♀S3	
	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates
Date	11	16	11	19	8	11	9	15	12	16	12	14	11	17	10	15
Time	15	16	17	19	10	11	12	15	14	16	10	14	11	17	11	15
Parent vial one	12	16	15	18	8	11	10	15	10	12	9	14	12	14	9	13
Parent vial two	14	16	15	19	10	11	13	15	12	16	12	14	15	17	14	15
Mating arena	6	16	6	19	8	11	7	15	7	16	7	14	7	17	7	15
Male size	6	16	7	17	6	11	8	13	7	15	5	9	5	14	8	12
Female size	6	10	6	13	7	8	6	9	6	10	7	10	6	11	6	9
Size difference	6	10	7	11	7	7	5	8	8	9	5	6	6	9	7	9
TOTAL	-	16	-	19	-	11	-	15	-	16	-	14	-	17	-	15

14♂SE		14♀SE		28♂NS		28♀NS		28♂S3		28♀S3		28♂SE		28♀SE		TOTAL	
Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates
11	17	11	15	11	15	8	15	7	14	9	14	8	15	9	12	26	240
12	17	12	15	10	15	11	15	10	14	12	14	11	15	10	12	48	240
12	12	12	13	5	5	3	4	1	2	3	4	2	4	3	3	61	158
15	17	13	14	12	15	8	14	10	14	11	14	11	15	9	12	79	238
6	17	6	15	7	15	7	15	6	14	8	14	8	15	7	12	8	240
5	14	7	12	7	13	6	12	6	14	6	10	6	14	7	11	19	205
7	12	6	9	7	4	5	7	4	9	4	7	7	13	2	4	16	148
7	9	8	8	6	7	5	6	6	9	3	5	11	12	4	4	20	129
-	17	-	15	-	15	-	15	-	14	-	14	-	15	-	12	-	240

To determine the efficiency of video analysis, paired t-tests and Pearson product-moment correlations were completed on the data observed through video footage and direct observation.

2. 3 Results

This experiment investigates the combined effects of age, starvation and body size on *Drosophila* courtship latency, courtship duration (also equal to latency to mate) and copulation duration.

One hundred and twenty one males and 119 females were used in this experiment across eight treatments (for each sex): ages three, 14 and 28 days old and starved at days two and three, two days prior to testing or not starved; see figure 1. Of these 240 flies, 198 (82.5%) engaged in courtship, and 160 of these (66.67% of the total flies) went on to copulate.

Not all flies courted and mated, so the data was analysed three times using the complete set, and using subsets of just those that courted and just those that mated. Limits were set to determine the maximum possible courtship latency (1800s) and duration (4500s). To ensure that these limits had no significant effects on the results, the data analysis was completed a second time using a different set of limits – 3600s for courtship latency and 6300s for courtship duration. No difference was found between these two analyses, indicating that there was no significant effect of the limited observation window.

2. 3. 1 Poor Quality Males Begin Courtship Later

Male condition (caused by the treatment effects of age and starvation) did not have a significant effect on whether the pair engaged in courtship or not (starvation: t=-0.066, d.f.=71, p=0.947; age: t=1.922, d.f.=71, p=0.0546); neither did the male or female size, the size difference between the pair or the position within the mating arena (table 4).

Table 4. *Drosophila* response to age and starvation; none of the measured treatments had a significant impact on the likelihood of the male to initiate courtship.

	Estimate	Standard Error	Z Score	P Value
(Intercept)	1.848	25.245	0.073	0.942
Male Starvation	-0.009	0.138	-0.066	0.947
Male Age	0.109	0.241	-0.454	0.650
Size Difference	-0.110	0.241	-0.454	0.650
Male Size	-0.010	0.440	-0.023	0.981
Mating Arena Slot	0.013	0.135	0.096	0.924
Male Starvation:Male Age	-0.002	0.005	-0.453	0.651

Degrees of Freedom = 71

Male treatments also had no effect on whether the courting pairs went on to copulate (starvation: t=-0.526, d.f.=71, p=0.599; age: t=1.364, d.f.=71, p=0.173); male and female size, size difference between the pair and mating arena slot also had no effect (table 5).

Table 5. None of the treatments had a significant impact on the likelihood of the successful copulation.

	Estimate	Standard Error	Z Score	P Value
(Intercept)	-7.264	19.940	-0.364	0.716
Male Starvation	-0.060	0.114	-0.526	0.599
Male Age	0.053	0.039	1.364	0.173
Size Difference	0.016	0.185	0.085	0.932
Male Size	0.144	0.347	0.416	0.678
Mating Arena Slot	0.008	0.110	-0.073	0.942
Male Starvation:Male Age	0.000	0.004	-0.020	0.984

Degrees of Freedom = 71

Latency of the male to court was unaffected by starvation period, but was significantly longer in older males (age: $F_{2, 100}$=4.200, p=0.0172; starvation: $F_{2, 100}$=1.945; p=0.148); see figure 4. Post-hoc TukeyHSD tests showed that 28 day old flies took significantly longer to court than 14 day old flies (p=0.0165).

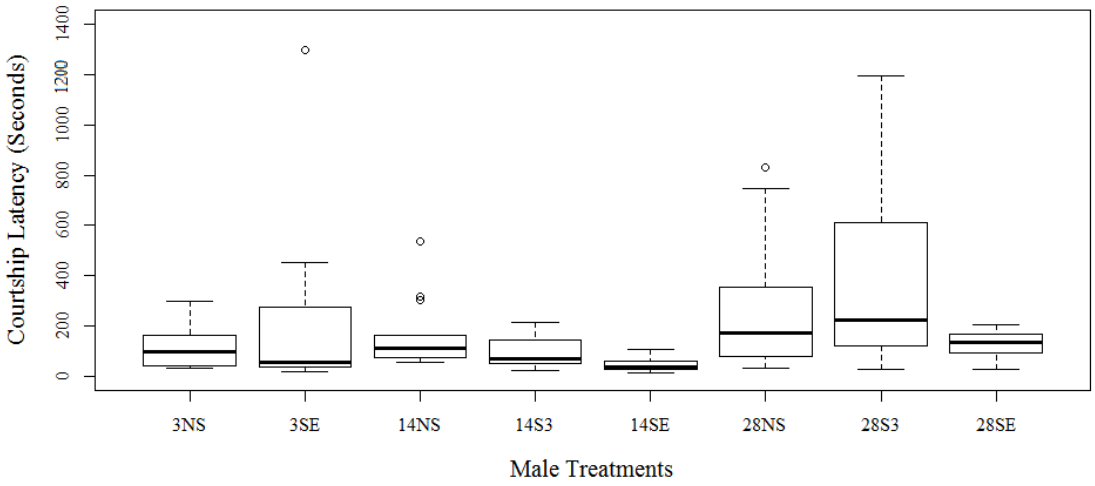


Figure 4. Courtship latency is longer in older flies ($F_{2, 100}$=4.200, p=0.0172).

NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing

3=3 days old 14=14 days old 28=28 days old

Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

Starvation had no significant effect on copulation latency of those males that did initiate courtship, however increasing age caused an increase in latency to court (age: $F_{2, 93}$=3.381, p=0.0383; starvation: $F_{2, 93}$=0.378, p=0.686); refer to figure 5. Post-hoc TukeyHSD tests identified that 28 day old males took significantly longer to initiate copulation than 14 day old males (p=0.0423).

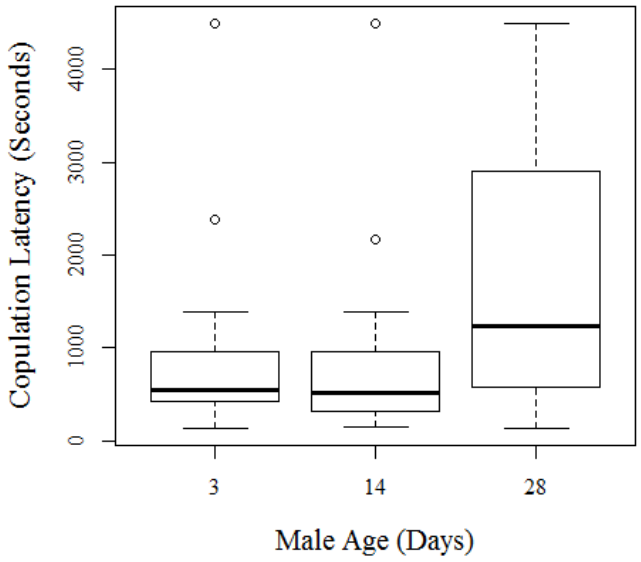


Figure 5. Copulation latency is longer in 28 day old flies than 14 day old males (p=0.0423).. Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

Neither age nor starvation had a significant effect on the duration of copulation in those that went on to mate (age: $F_{2, 79}$=0.623, p=0.538; starvation: $F_{2, 79}$=0.883, p=0.417).

2. 3. 2 High Quality Females Copulate for Longer

The treatment that the females experienced, had no significant effect on whether their male mating partners initiated courtship (starvation: t=-1.333, d.f.=56, p=0.182; age: t=1.654, d.f.=56, p=0.0981); neither did male and female size, size difference between the mating pair and position occupied in the mating arena (table 6).

Table 6. None of the measured treatments had a significant impact on the likelihood of the male to initiate courtship.

	Estimate	Standard Error	Z Score	P Value
(Intercept)	-34.559	22.921	-1.508	0.132
Female Starvation	-0.256	0.192	-1.333	0.182
Female Age	0.097	0.059	1.654	0.098
Size Difference	0.309	0.274	1.128	0.259
Male Size	0.611	0.398	1.537	0.124
Mating Arena Slot	0.004	0.176	0.025	0.980
Female Starvation:Female Age	0.011	0.012	0.939	0.348

Degrees of Freedom = 56

Males were less likely to invest in copulation when the females were starved immediately before testing; but no effect of age was observed (starvation: t=-2.268, d.f.=56, p=0.0233; age: t=1.953, d.f.=56, p=0.0508). No effect of male or female size, size difference between the pair or position within the mating arena was identified either (table 7).

Table 7. *Drosophila* response to age and starvation; none of the measured treatments had a significant impact on the likelihood of successful copulation.

	Estimate	Standard Error	Z Score	P Value	
(Intercept)	-24.553	17.158	-1.431	0.152	
Female Starvation	-0.316	0.139	-2.268	0.023	*
Female Age	0.086	0.044	1.953	0.051	
Size Difference	0.180	0.232	0.777	0.437	
Male Size	0.435	0.297	1.468	0.142	
Mating Arena Slot	-0.047	0.149	-0.319	0.749	
Female Starvation:Female Age	0.010	0.005	1.818	0.069	

Degrees of Freedom = 56

Latency to court was not significantly affected by the condition of the male's mating partner (age: $F_{2, 103}$=0.098, p=0.906; starvation: $F_{2, 103}$=0.159, p=0.854). However female condition did have a significant effect on copulation latency; when the female had experienced a period of starvation, latency to copulate was longer, although female age had no effect (age: $F_{2, 89}$=0.990, p=0.375; starvation: $F_{2, 89}$=9.900, p=1.31e^{-4}). Post-hoc TukeyHSD tests were used to show that courtship duration (or copulation latency) lasts significantly longer in females that were starved two days before being placed in the mating arena (p=1.15e^{-4}), and were starved early in life (p=0.0389), compared to when the females were not starved; see figure 6.

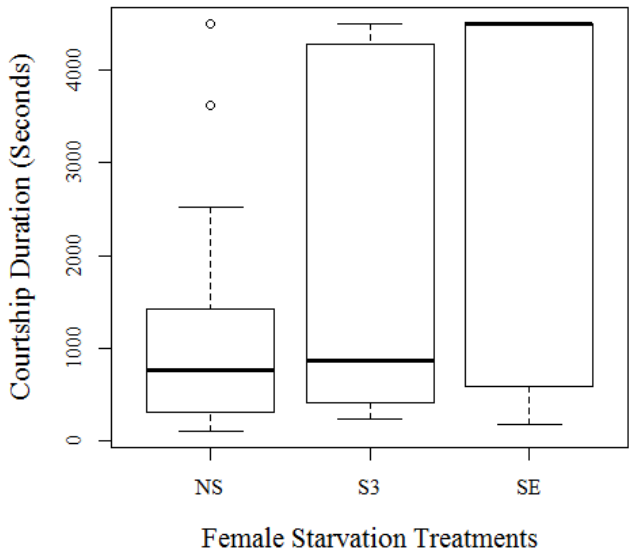


Figure 6. Female starvation periods caused a decrease in copulation latency ($F_{2, 89}$=9.900, p=1.31e^{-4}).
NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing
Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

Female age had no effect on the duration of copulation, but starvation periods significantly reduced copulation duration (age: $F_{2, 87}$=1.028, p=0.362; starvation: $F_{2, 87}$=15.577, p=1.65e^{-6}). Post-hoc TukeyHSD tests identified that fed females mated for longer than

those that were starved at age two-three days old (p=0.00796) and those starved two days prior to testing ($p=1.30e^{-6}$); see figure 7.

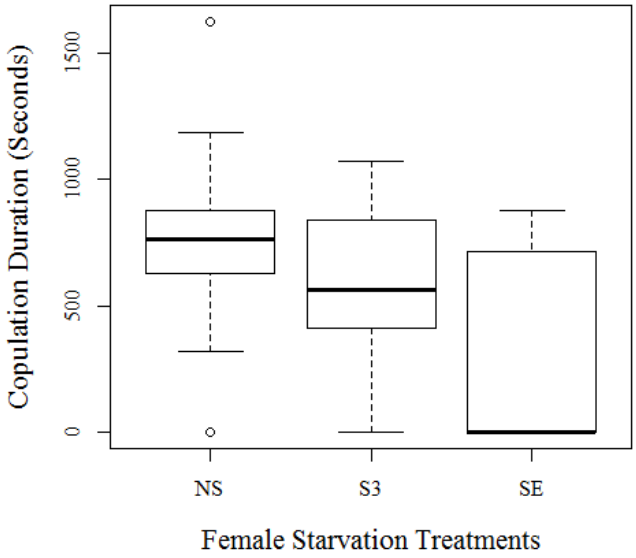


Figure 7. Female starvation reduces copulation duration ($F_{2, 87}=15.577$, $p=1.65e^{-6}$).
NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing
Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

2. 3. 3 Twenty Eight Day Old Males Initiate Less Courtship Behaviours

Pearson product-moment correlations were completed and all courtship behaviours were highly positively correlated to the total number of courtship events in both male and female treatments (wing extensions: r=0.974, d.f.=209, $p=2.20e^{-16}$; chasing events: r=0.546; d.f.=209, $p=2.20e^{-16}$; abdomen taps: r=0.665, d.f.=209, $p=2.20e^{-16}$; genital licks: r=0.473, d.f.=209, $p=3.87e^{-13}$; mounting events: r=0.296, d.f.=209, $p=1.26e^{-5}$).

The age of the male, but not starvation treatment, significantly affected both the total number of courtship events (age: $F_{2, 89}=13.520$, $p=7.46e^{-6}$; starvation: $F_{2, 89}=1.198$, p=0.307), and the total time the male spent courting (age: $F_{2, 89}=15.588$, $p=1.57e^{-6}$; starvation: $F_{2, 89}=0.0169$, p=0.983). TukeyHSD tests showed that total courtship events declined in 28 day old males compared to three (p=0.0256) and 14 day olds ($p=4.80e^{-6}$)

(see figure 8). Time spent courting also declined in 28 day old males compared to three ($p=0.0422$) and 14 day olds ($p=8.00e^{-7}$) (see figure 8).

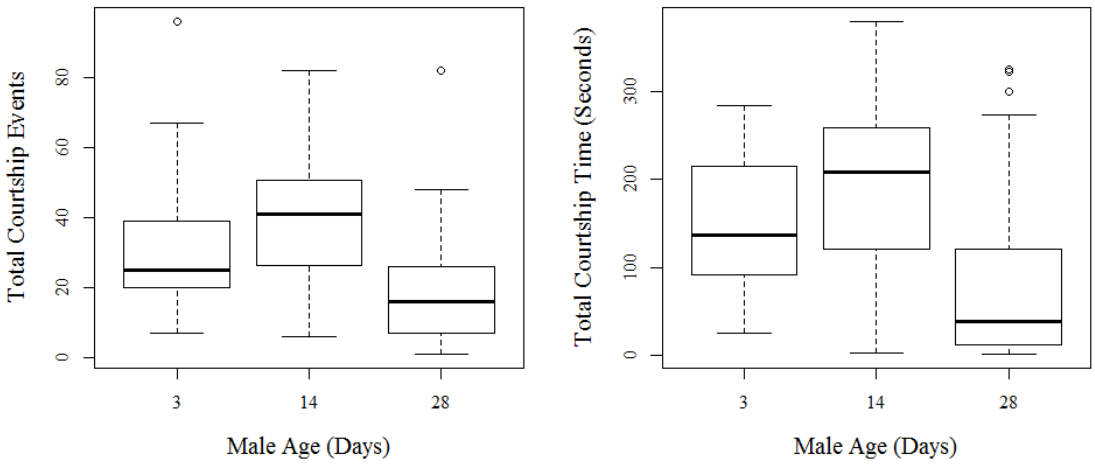


Figure 8. Male condition affects the amount invested in courtship – both in number of events and total time spent courting. Both decline with an increasing age (number of events: $F_{1, 72}=22.578$; time spent courting: $F_{1, 64}=18.604$, $p<0.001$).
Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

Further information on how male condition affects the number of wing extensions, abdomen taps, genital licks and chasing events is provided in appendix 5.i.

2. 3. 4 Courtship Behaviours are Affected by Female Condition

The condition of the female had no significant effect on the total number of courting events the male engaged in (age: $F_{2, 82}=0.160$, $p=0.853$; starvation: $F_{2, 82}=1.085$, $p=0.343$), or the total time spent courting (age: $F_{2, 82}=0.589$, $p=0.557$; starvation: $F_{2, 82}=0.503$, $p=0.607$).

Further information on how the condition of the female affects the number of wing extensions, abdomen taps, genital licks and chasing events is reported in appendix 5.ii.

2. 3. 5 Male Condition Alters Rejection Responses of the Female

The number of fleeing events and the number of kicks were highly positively correlated (using Pearson product-moment correlations) with the total number of rejection responses observed in both male and female treatments (fleeing events: $r=0.885$, d.f.=208,

$p=2.20e^{-16}$; kicks: r=0.327, d.f.=208, $p=1.21e^{-6}$). However the number of times a female fluttered her wings to reject a male was not significantly correlated to the total number of rejection responses she completed (r=0.117, d.f.=208, p=0.0896); because of this, analysis of wing flutters has been completed separately, in addition to the total number of rejection responses.

Male age (but not exposure to starvation periods) had a significant effect on the total number of rejection responses completed by the female (age: $F_{2, 91}=3.533$, p=0.0333; starvation: $F_{2, 91}=1.675$, p=0.193) and time spent rejecting the male (age: $F_{2, 91}=19.356$, $p=9.90e^{-8}$; starvation: $F_{2, 91}=0.658$, p=0.520). Female rejections peak at an intermediate male age (see figure 8). Post-hoc TukeyHSD tests showed that females rejected males advances for significantly less time when the males were 28 days old compared to three day olds ($p=6.53e^{-4}$) and 14 day olds ($p=1.00e^{-7}$); and significantly less often when they were 28 days old compared to 14 (p=0.0353); see figure 9.

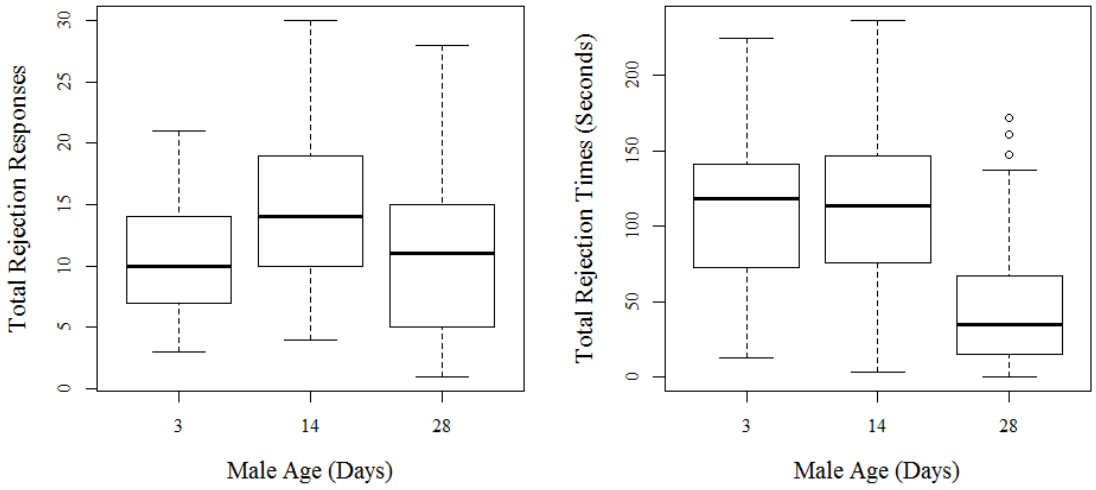


Figure 9. Females rejected males advances for significantly less time when they were 28 days old compared to three day olds ($p=6.53e^{-4}$) and 14 day olds ($p=1.00e^{-7}$); and significantly less often when they were 28 days old compared to 14 (p=0.0353).
Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

There was no significant effect of male age or starvation on the number of wing flutters completed by his female mate (age: $F_{2,\ 91}=0.261$, p=0.771; starvation: $F_{2,\ 91}=1.392$, p=0.254).

Further information on the effect of male condition on the number of fleeing events and kicks the female initiated is provided in appendix 6.i.

2. 3. 6 Older Females Spend Less Time Rejecting Males

The female's condition did not have a significant effect on the number of rejection responses she initiated (age: $F_{2,\ 84}=0.751$, p=0.475; starvation: $F_{2,\ 84}=0.383$, p=0.683); however, older females spent less time rejecting the males' advance than younger females (age: $F_{2,\ 84}=4.055$, p=0.0208; starvation: $F_{2,\ 84}=0.223$, p=0.801) (see figure 10). Post-hoc TukeyHSD tests showed that 28 day old females rejected for significantly less time than 14 day olds (p=0.0477).

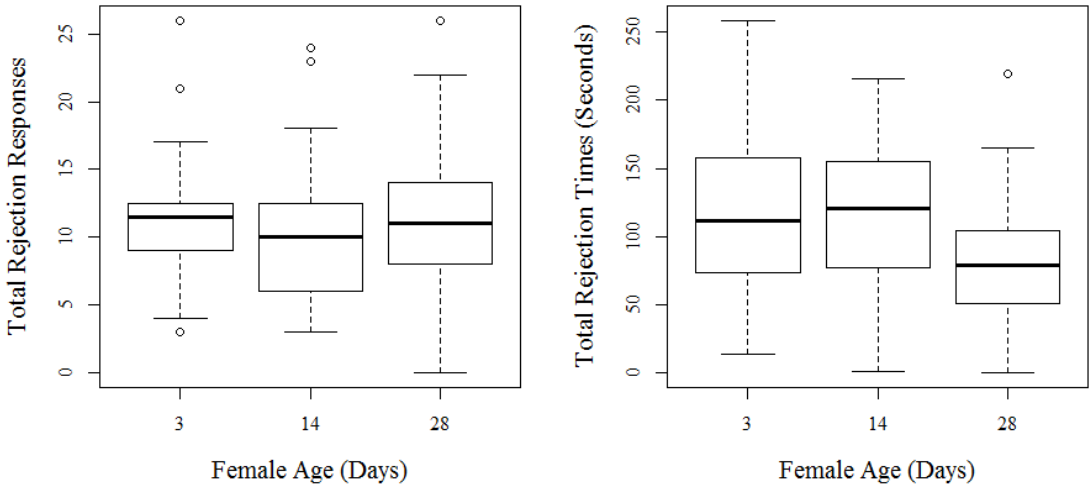


Figure 10. The effect of female age on the total number of rejection responses completed and the time spent completing them.

NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing

3=3 days old 14=14 days old 28=28 days old

Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

There was no significant effect of female age or starvation on the number of wing flutters the female completed to deter the males (age: $F_{2, 84}$=1.788, p=0.174; starvation: $F_{2, 84}$=2.215, p=0.115).

Additional information on the effect of male condition on the number of fleeing events and kicks the female initiated is reported in appendix 6.ii.

2. 3. 7 Older Males Produce Fewer Offspring

Male treatment had no significant effect on the total number of eggs that the female laid (age: $F_{2, 66}$=2.668, p=0.0769; starvation: $F_{2, 66}$=1.754, p=0.181). It did however, have an effect on the number of larvae that hatched. There was no effect of starvation but older males produced significantly fewer offspring than younger males (figure 11) (age $F_{2, 68}$=3.744, p=0.0287; starvation: $F_{2, 68}$=1.368, p=0.262): 28 day old males sired fewer successfully hatched larvae than three day old males (TukeyHSD: p=0.0213).

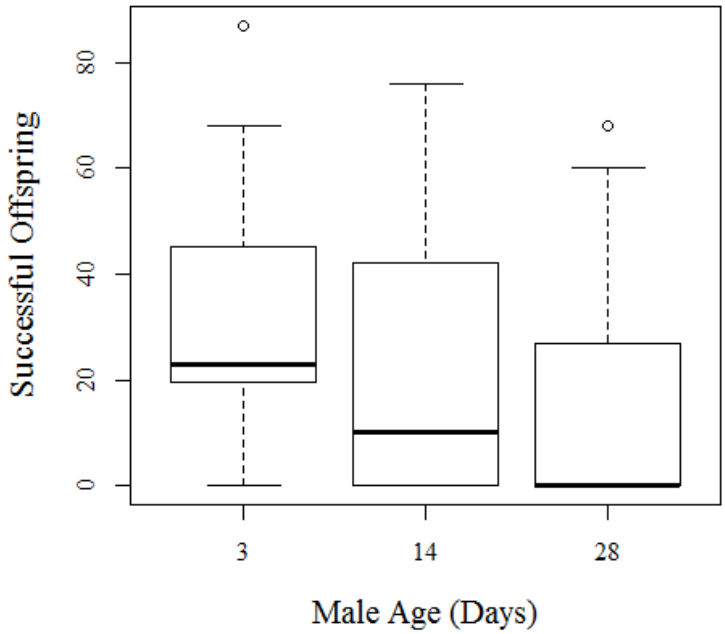


Figure 11. Older males produced significantly fewer offspring than younger males (age $F_{2, 68}$=3.744, p=0.0287; starvation: $F_{2, 68}$=1.368, p=0.262).

NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing

3=3 days old 14=14 days old 28=28 days old

Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

2. 3. 8 High Quality Females Produce More Offspring

Both age and starvation period of the female *Drosophila* had a significant effect on the total number of eggs she was able to lay (see figure 12) (age: $F_{2, 69}$=4.899, p=0.0102; starvation: $F_{2, 69}$=6.094, p=0.00365). Post-hoc TukeyHSD tests showed that females that were fed produced significantly more eggs than those starved two days prior to testing (p=0.00563). Three day old females laid significantly more eggs than 28 day old females (TukeyHSD: p=0.00753).

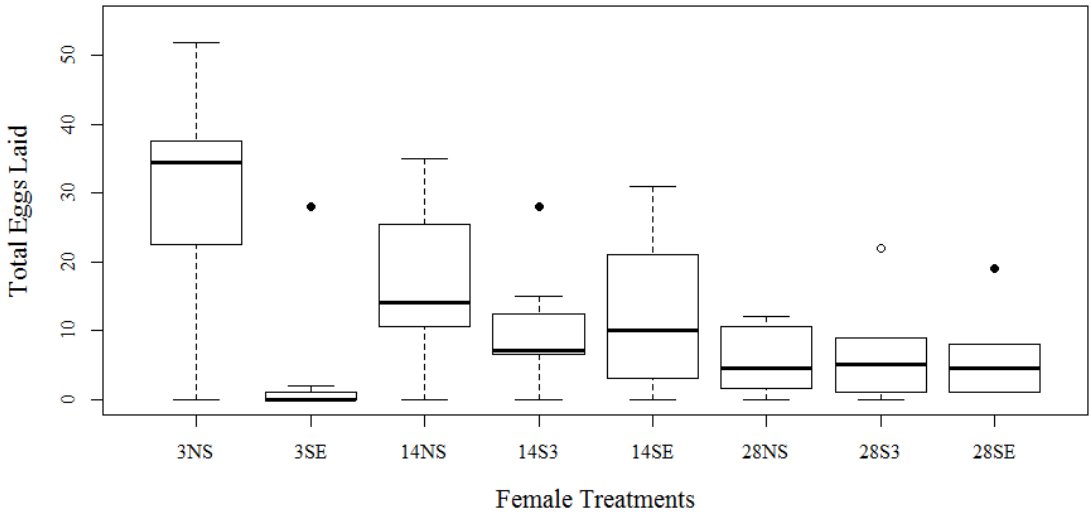


Figure 12. Increasing age and starvation of the females significantly reduced the total number of eggs she was able to lay (age: $F_{2, 69}$=4.899, p=0.0102; starvation: $F_{2, 69}$=6.094, p=0.00365).
NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing
3=3 days old 14=14 days old 28=28 days old
Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

Female condition also had a significant effect on the number of successful offspring she produced (figure 13) (age: $F_{2, 70}$=7.044, p0.00163; starvation: $F_{2, 70}$=5.768, p=0.00480). Post-hoc TukeyHSD tests revealed that three day old females produced significantly more successful offspring than either 14 day olds (p=0.00360) or 28 day olds (p=0.00522). Post-hoc tests also showed that females that were not exposed to any period of starvation produced significantly more successful offspring than those that experienced starvation late in life (p=0.00679).

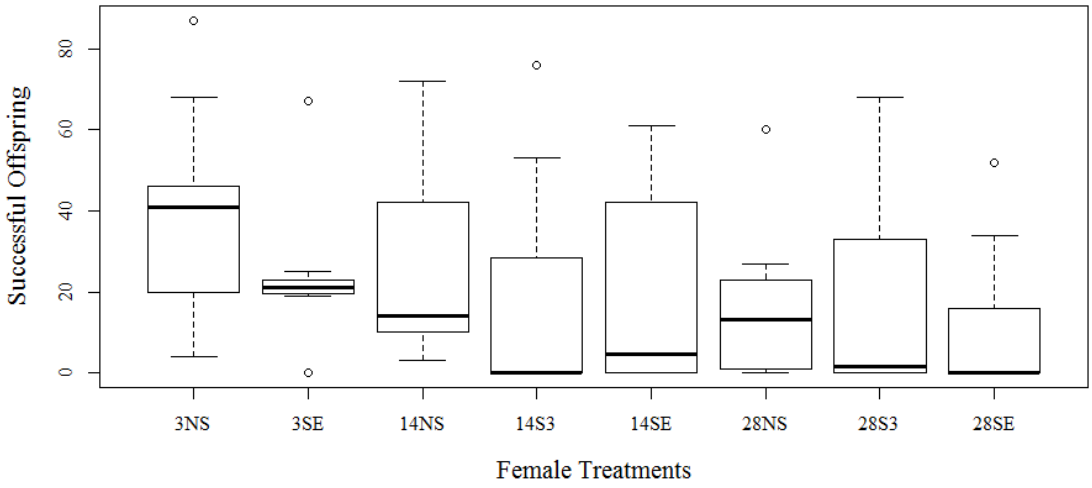


Figure 13. Increasing age and starvation of the females significantly reduced the number successful offspring (age: $F_{2, 70}$=7.044, p0.00163; starvation: $F_{2, 70}$=5.768, p=0.00480).

NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing

3=3 days old 14=14 days old 28=28 days old

Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

2. 4 Discussion

The experiment investigated the effect of age and starvation treatments on both male and female *Drosophila*. Various courtship behaviours and rejection responses were analysed to gain an understanding of male and female receptiveness. Courtship and copulation durations, as well as number of eggs laid, were analysed to attempt to understand the effect these stresses had on *Drosophila* reproductive success.

Courtship displays differ in duration, due to the variation in the effort that the males invest and the receptiveness of the female (Bastock and Manning 1955). When females are less keen to copulate, latency to mate (or courtship duration) is longer. It therefore follows that when females are more attractive (robust, larger and younger), or males less attractive (smaller or older), the latency to mate increases – as more attractive *Drosophila* are can afford to be more discriminating (Bonduriansky 2001; Dukas and Baxter 2014; Baxter et al. 2015).

When flies experience a period of starvation it lowers their fitness and therefore their attractiveness. The extent to which starvation affects them however, depends on their initial fitness level. Larger individuals have more resources and are better able to cope with starvation, so it reduces their fitness less (Miller and Thomas 1958). The impact of starvation varies depending on the amount of food that the fly consumed before it was placed in the starvation treatment (so some flies were pushed closer to death than others); this could not be controlled, but all flies were fed *ad libitum* outside starvation periods.

The starvation period completed two days prior to testing was designed so that the flies perceived themselves to be close to death with no food resources available. The starvation that older flies were exposed to at days two and three was early enough within their life that they had time to recover before testing, yet it still will have had a negative effect on their development. *Drosophila* can survive for more than ten weeks in laboratory conditions (Sohal 1970; Takahashi et al. 1970; Brigui et al. 1990); however many do not survive for this long (as found with deaths observed in the lead up to the experiment and the experiment itself) so it is likely that many of the older flies were also close to death. A small percentage of flies died before reaching the appropriate age for testing, resulting in a slight quality bias in the flies tested – only the stronger flies that survived to testing could be included within the analysis.

2. 4. 1 Weaker Males and Stronger Females Copulate for Longer

If the female was not starved, the pair was more likely to copulate (table 7). Females that have not been starved are more attractive, so males are more likely to bear the costs of courtship and reproduction in order to sire attractive and successful offspring (Weatherhead and Robertson 1979). It is most commonly suggested that younger females are more attractive (Cook and Cook 1975; Hercus and Hoffmann 2000; Lüpold et al. 2011; Dhole and Pfennig 2014); but there has been some evidence that older females are perceived to be more attractive due to superior genetics (Hansen and Price 1995) – however, direct benefits (such as increased egg production in younger females) are generally deemed to be more important than the indirect benefit of superior genes. It is likely that older females here are less discriminatory as they are in a poorer condition and can't afford the cost of choosiness (Bonduriansky 2001), and so are more willing to accept the male's courtship attempts. If this is the case, they will not exert as much energy into deterring and fleeing from the males' advances.

It was predicted that when males are more attractive and females less attractive, latency to court would be shorter because in both cases males are able to be choosier (Gowaty et al. 2003; Baxter et al. 2015) – but no effect was observed in females. This could be because the males were not given any other choice of mate. It could also be because other measures of attractiveness (as opposed to condition that was measured here) are causing an effect – such as the colour and pattern displayed in the flies' wings (Katayama et al. 2014). However, the results were as predicted in males (figure 4). Males engaged in courtship sooner when they were younger. It is expected that these males can afford the high energetic cost of courtship (Pitnick and Markow 1994) and do not need to be so cautious when choosing whether to initiate courtship or copulation. Weaker males will try to conserve energy for survival, rather than exert it on attempting to court a female – especially considering that copulation is not guaranteed (Ellers 1995; Flatt and Kawecki 2007; Marshall and Sinclair 2009).

Additionally, a longer courtship duration (or copulation latency) was observed when the female was starved (figure 6). Females often have more control over whether a pair copulates or not (Bastock and Manning 1955; Connolly and Cook 1973). When a female is weaker (older or starved) it is likely that they will have less energy to reject the courting males (Maklakov et al. 2008), however this would result in a shorter courtship duration. The result found is likely to be due to the male's unwillingness to copulate. Starved females will also be less attractive and they will produce fewer offspring due to their

previous exposure to limited resources. It is likely that males would be less willing to exert energy into copulating if it results in a poorer quality or quantity of offspring (Weatherhead and Robertson 1979; Lüpold et al. 2011). Copulation causes harm to females, especially in weaker females (Fowler and Partridge 1989). So it is possible that weaker females may actually reject more to avoid this, if they perceive themselves as being too weak to survive copulation and reproduction. It was predicted that high quality males would have shorter courtship durations because they are more attractive and therefore the female would be more willing to accept his copulation attempts (Steele 1986; Gavrilets et al. 2001). This was the case with increasing male age – although no effect of starvation was found (figure 5). This could be because some males are putting more effort into the amount of courtship behaviours they are initiating; making them more attractive to the female and convincing her that they are 'fitter'. It could also be because the high quality males are being choosier, and are therefore choosing not to mate with their female partner (Gowaty et al. 2003).

When copulation occurred, no effect of male condition was observed. However, it was predicted that poor quality males would trade-off courtship and copulation and practice terminal investment. These starved males would perceive themselves as being close to death, so instead of conserving energy for survival and potential future mates, they would exert all of their remaining energy into copulation (Ellers 1995; Flatt and Kawecki 2007; Marshall and Sinclair 2009). Longer copulation durations are more beneficial to a male because it enables a better transfer of sperm and increases the male's chances of inseminating the female (Bretman et al. 2009). Copulation duration was longer when females were more attractive (where females had not been starved) (figure 7). Copulation is an energetically expensive process (Fowler and Partridge 1989; Wigby and Chapman 2005; Kuijper et al. 2006), but when females are more attractive it makes the cost more worthwhile as it is likely that their offspring will be more attractive (Weatherhead and Robertson 1979). In addition to this, females that have been fed *ad libitum* have had better access to resources so it is likely that they will lay more eggs – increasing the male's reproductive fitness. Lengthened copulation duration also serves as a method of mate guarding, preventing other males from being able to copulate with the female, which is also seen more often when females are more attractive (Cook and Cook 1975; Mazzi et al. 2009; Lüpold et al. 2011).

2. 4. 2 Courtship Efforts Increase when Males are Stronger and Females are More Attractive

The energy that the male exerts in courtship is invariable until courtship is terminated or copulation begins, as shown by previous research completed within this laboratory (courtship effort observed in the first five minutes was found to be representative of total courtship effort ($r=0.612$, $d.f.=42$, $p<0.001$). It is possible that, due to the expense of courtship, once a male has committed to courting a female it would be a waste of energy to abandon his efforts in search of an alternative mate so his efforts remain relatively constant. Additionally, the results from this report suggest that the pattern of the various courtship behaviours is consistent (Bastock and Manning 1955), as all were positively correlated: there appears to be no significant trade-off between types of courtship when under stress. This could be because all responses have a similar expense, or those that are more costly also reap the most reward – a further investigation into the expense and reward of each response could be completed to determine this. In future investigations it would be possible to only measure wing extensions as a measure of courtship effort as they are representative of all courtship behaviours, and are well represented in video footage.

Total courtship effort declined with increasing male age as expected. Courtship behaviours are energetically expensive so weaker males cannot afford the expense – they trade-off energy required for courtship to conserve energy for copulation to ensure they sire offspring.

Courtship efforts also decreased when the females were less attractive – when they were younger and had been through a two day starvation period (figure 8). The ‘sexy sons’ hypothesis of (Weatherhead and Robertson 1979) could be applied to male investment here (although it is often used to describe female investment): more attractive females will produce more attractive. It is worth males spending more energy trying to court these females so any offspring they have will be more attractive and therefore more successful. Equally, females that have been starved will produce fewer offspring. This will reduce the reproductive success of any male who copulates with her, making her less likely to be chosen as a mate.

2. 4. 3 Females are More Likely to Reject Males when Males are Less Attractive

Rejection responses have been shown to cause the termination of courtship so it is important to address female rejection responses (Gromko and Markow 1993). Connolly and Cook (1973) studied a vast range of rejection responses (flicking, kicking, curling,

extruding and fending) to determine their cause and effect: they observed kicking to occur more often as a result of mounting attempts, whereas wing flutters were often linked to genital licking – or sometimes non-courtship behaviours. This could explain why wing flutters observed here were not correlated to the total rejection events, or why treatment of males and females did not alter the number of wing flutters initiated: they may have been affected by non-courtship behaviours too. Number of fleeing events and kicks were positively correlated with the total number of rejection responses, suggesting that as theorised with courtship, rejection responses are consistent throughout the courtship period.

As predicted, fewer rejection responses were observed when males were younger and fed throughout their life. Females are more willing to mate with more attractive males (Cook and Cook 1975; Gavrilets et al. 2001), so they perform fewer rejection behaviours.

When females are younger and did not experience any form of starvation, they performed more rejection responses – as expected. More attractive females are able to be choosier (Friberg and Arnqvist 2003) so they reject males more often. Younger females, and females that were not starved are also 'fitter' as they have more available resources. This results in them having more energy to be able to perform these behaviours, as they do not need to conserve energy for survival (Andrade and Kasumovic 2005). Older females spent significantly less time rejecting males, although they still completed a similar number of rejection responses. This suggests that these females trade-off duration of responses in favour of completing more responses (figure 8).

2. 4. 4 High Quality *Drosophila* Produce More Successful Offspring

The total number of eggs, and the number of successful offspring, is affected by both the maternal and paternal condition – caused by treatments (figures 11, 12 and 13). Spermatogenesis and egg production are energetically costly processes, so only high quality individuals can afford the cost (Dewsbury 1982; Ellers 1995; Galvani and Johnstone 1998; Terashima and Bownes 2004); for example both spermatogenesis and egg production declines with increasing age (Aigaki and Ohba 1984; Snoke and Promislow 2003). Therefore, as predicted, the number of successful offspring declined with male and female age; this result compliments previous research (Hercus and Hoffmann 2000; Jones and Elgar 2004; Lüpold et al. 2011).

Lüpold et al. (2011) found that males transfer more sperm when females are more attractive, which will further increase the negative effects caused by the female's poor

condition. It was shown that more attractive, high quality females (younger females that were not starved) produced more total eggs and more successfully hatched eggs, as they do not have to conserve energy to ensure their survival and are able to spend more on egg production (Clutton-Brock 1984; Hercus and Hoffmann 2000).

Older males produced less successful offspring, as predicted. Weaker males have to trade-off between survival and reproduction. They cannot afford the cost of spermatogenesis, so they produce less viable sperm cells, and therefore produce less offspring (Droney 1998).

2. 4. 5. Is Using Video Footage a Useful Method?

The number of wing extensions that were observed using both the video footage and direct observation were similar suggesting that it would be possible to use video footage to analyse wing extensions again in future experiments. It would also be possible to cross-compare them to data collected from direct observation. Wing extensions are the most useful courtship behaviour to analyse because it communicates clear intentions and obvious visual movements. They also account for approximately 80% of sexual stimulation in *Drosophila* (Ewing 1964).

As predicted, all behaviours analysed (except wing flutters) were positively correlated with each other, so although the exact numbers collected from each technique were not the same, the relationship was similar. Analysing video footage could prove to be a useful method in future experiments, but it would not be possible to compare data collected from videos with that collected using the human eye. Subtle behaviours however, such as wing flutters and abdomen taps could not be measured – as shown by the high difference in values collected from the two different methods.

The difference in results is likely to be caused by the different techniques used by the two methods. A video camera is stationary; unlike human eyes it is not able to move to ensure a constant focus is kept on the *Drosophila*. However, it is possible to pause and rewind video footage to help to eliminate human error. Additionally, the ability to film multiple mating pairs and fast forward through the footage enables more replicates to be collected in a shorter period of time. Yet more replicates can be collected if still photographs are taken from the footage to be analysed – for example, monitoring photographs taken every 30 seconds to observe the duration of copulation.

Video footage produces results of a slightly poorer quality than results that could be found using the human eye: there is trade-off between quality and quantity of data. However it is a useful tool which enables more replicates to be analysed in a shorter period of time; it

has been used in previous experiments (Full and Tu 1991; Schenk and Bacher 2002; Manoukis et al. 2014) and should continue to be used in future experiments.

Chapter 3: The Effect of Age and Starvation on Physiology and Reproductivity

3. 1 Introduction

3. 1. 1 Spermatogenesis

Spermatogenesis (the production of spermatozoa) is an energetically costly but essential process (Dewsbury 1982). *Drosophila* sperm is large compared to their body size, especially those of *Drosophila bifurca*, which produce the longest sperm on record (Pitnick 1996); it is over 55mm long, compared to human's which is merely 6μm (Smith et al. 2009). Additionally, sperm outnumber eggs in nearly all species (Bateman 1948; Andersson 1994) – another characteristic that increases their production cost. Males transfer seminal fluid proteins within their ejaculate (Wolfner 2002; Wigby et al. 2009) that perform a range of functions including inhibition of female remating and egg laying stimulation (Scott 1986; Herndon and Wolfner 1995). This trait evolved to help male sperm compete for the female's eggs when sperm competition is high. Additionally, viability of sperm transferred increases in a higher sperm competition risk (García-González and Simmons 2005; Moatt et al. 2014) but the opposite occurs in high sperm competition intensity (Simmons et al. 2007). Plastic responses are also observed with changing female mating status; such as an increase in sperm production or courtship behaviours (Droney 1996; Friberg 2006; Lüpold et al. 2011).

Its high cost means that sperm production may suffer when environmental conditions are poor or food is scarce (Droney 1996; Droney 1998); and in turn reproductive success may decline (Fricke et al. 2008). However, passing on genetic material is critical to any sexually reproducing organism, so sometimes spermatogenesis and other reproductive efforts are prioritised even when the individual is close to death (Clutton-Brock 1984; Creighton et al. 2009). This theory is referred to as terminal investment; an individual will exert all of their remaining energy into courtship and copulation for one final time (Clutton-Brock 1984; Andrade and Kasumovic 2005). However, the response is not immediate: spermatogenesis is a slow process so there is a delay in improved sperm quality, after the effort has been allocated (Dewsbury 1982).

Lifetime mating success of males is affected by their age and size (Partridge and Farquhar 1983; Jones and Elgar 2004; Dhole and Pfennig 2014). Larger male *Drosophila* are 'fitter' and can afford to allocate more energy to manufacturing and transferring more sperm (Pitnick and Markow 1994). *Drosophila* of an intermediate age are the most

discriminatory as they are the 'fittest' potential mates (Dhole and Pfennig 2014); too young and they will not have reached sexual maturity but too old and they will become weak. 'Fitter' *Drosophila* are able to discriminate more as they can afford to wait for a higher quality mate (Bonduriansky 2001).

3. 1. 2 Internal Egg Production

Female *Drosophila* experience a similar energetic cost to reproduction (Fowler and Partridge 1989; Wang et al. 2001; Wigby and Chapman 2005), although there is a limited cost to successful egg production (Partridge et al. 1986; Fowler and Partridge 1989). This is partially due to the fact that virgins also oviposit eggs (Partridge et al. 1986; Menon et al. 2014). After copulation, a female holds sperm in her sperm-storage organ until her eggs are ready to be fertilised (Pitnick et al. 1999).

Under nutrient-deficient conditions egg production, and thus female lifetime reproduction success, declines (Chapman and Partridge 1996; Terashima and Bownes 2004). However, stress can also be caused by a nutrient-rich diet, which could shorten a fly's lifespan and lower reproductive success (Fricke et al. 2008). It is possible that, like males, females commit terminal reproductive investment to enable them to improve their lifetime reproductive success. Younger and larger females are better able to cope with a variable environment, but regardless of environment they produce more successful offspring (Lefranc and Bundgaard 2000; Turiegano et al. 2012; Zajitschek et al. 2014). Males perceive younger and larger females to be more attractive, so their success could also be attributed to higher quality sperm (a male response to female status) (Lüpold et al. 2011).

3. 1. 3 Fat Reserve Levels

Fat stores enable organisms to cope with environmental nutrient availability fluctuations. If nutrient availability declines, organisms will utilise their fat reserves for energy (Wigglesworth 1949). It therefore follows that when nutrient availability declines, the amount of fat present in the fat reserves decreases. A fly's ability to store fat is affected by their condition: larger and younger flies have a higher fat content as they are in a better condition and have less need to utilise it (Ellers 1995).

3. 1. 4 Hypotheses

Body condition and environmental conditions have been shown to affect behavioural plasticity in a range of organisms including *Drosophila melanogaster* (Komers 1997). It is also important to understand the physiological effects of a variable environment, so this

chapter will investigate how *Drosophila melanogaster* physiology is affected by the same treatments as investigated in Chapter 2.

This investigation should enable better predictions into how a changing environment may affect an animal's physiology. It is likely that the behaviour they have exhibited in both previous and latter chapters will be inadvertently affected by how their physiology has been affected too. Therefore this information should further improve the ability to protect humans and other animals from stressful and variable environments.

The hypotheses are:

- Older and starved males will be weaker and so will have less energy to exert into spermatogenesis, so will produce fewer sperm cells
- Egg production rate will decline with increasing age and starvation as they will also not be able to afford the expense of sex cell production
- Young males and females will have the highest level of fat reserves – but this will decrease if the individual is exposed to a starvation period because they will need the energy to survive this

3. 2 Methods

3. 2. 1 Treatment Protocol

The method used to rear the flies, and the treatments tested in this experiment, were identical to that described in the Chapter 2.2: F1 Oregon-R and Canton-S crosses were subjected to eight treatments for each sex: aged to three, 14 and 28 days old; and either starved at days two and three, starved in the final two days prior to testing, or not starved – see table 8 for the number of replicates used.

Table 8. The number of replicates used in each treatment.
NS=Not starved SE=Starved in final two days S3=Starved at 2-3 days old
SE and S3 are the same treatment in three day old flies.
3=Three days old 14=14 days old 28=28 days old

Egg Count				**Spermatogenesis**			
	NS	**SE**	**S3**		**NS**	**SE**	**S3**
3	31	29	-	**3**	10	-	-
14	30	27	28	**14**	14	-	19
28	22	20	24	**28**	23	-	19
Male Individual Fat Reserves				**Female Individual Fat Reserves**			
	NS	**SE**	**S3**		**NS**	**SE**	**S3**
3	43	49	-	**3**	45	38	-
14	48	44	45	**14**	41	33	48
28	34	38	40	**28**	37	32	25
Male Combined Fat Reserves				**Female Combined Fat Reserves**			
	NS	**SE**	**S3**		**NS**	**SE**	**S3**
3	44	49	-	**3**	46	39	-
14	49	44	46	**14**	42	34	48
28	34	39	40	**28**	39	33	25

Once the flies reach the appropriate age, half were subjected to ether extraction to determine the level of fat reserves, and the other half were dissected to measure internal sperm and egg counts depending on their sex.

3. 2. 2 Fat Reserve Calculations

To calculate *Drosophila* fat reserves, they first had to be dried. After being frozen, they were stored for five days in 2.5ml Eppendorf tubes at 60°C, and combined and individual dry weights (with weighing materials) were recorded. All weights were calculated using a mean average of three measurements. The flies were then submerged in 2.5ml of diethyl ether ($(C_3H_3)_2O$) and left for 24 hours in a fume cupboard – this was then replaced with fresh diethyl ether. This was repeated a further three times before the specimens were dried for five days at 60°C (Pexton and Mayhew 2002). Combined and individual specimen weights were measured once more, and subtracted from the initial dry weights; enabling the difference to be calculated indicating the mass of each flies' fat reserves. The material weights were then measured and subtracted from the initial weights to calculate the flies' dry weights. The flies' fat reserve mass was then divided by the flies' total mass to give the proportion of fat reserves relative to the body mass; however this technique was not used in analysis as it produced wildly inaccurate values not within the zero to 100% margin.

3. 2. 3 Egg Production Counts

Internal sperm and egg counts were completed using dissections in phosphate buffer saline. Ovaries were removed from female *Drosophila,* and the eggs were separated and counted using a binocular microscope (Nikon Eclipse E200 with a graticule attached) at four times magnification. The maximum height of eggs included in the final internal egg count was 0.15mm. A paired t-test showed that the number of eggs found in each ovary were not significantly different ($p=0.96$, d.f.=165, $t=-0.045$) so when one ovary could not be included in the analysis, the other was doubled to achieve a total egg count.

3. 2. 4 Quantifying Spermatogenesis

Spermatogenesis is a slow and energetically expensive process (Dewsbury 1982); the effect of starvation on *Drosophila* spermatogenesis takes long enough that it would not be apparent two days prior to dissections, so these three starvation treatments were removed. In order to quantify the amount of sperm in male *Drosophila,* the testes and seminal vesicles were dissected and the sperm removed. DAPI was used to stain the sperm cells according to the manufacturer's instructions. A cover slip was added and set with mounting medium. The slides were viewed and photographed using a fluorescence

microscope (Zeiss 510 Meta Confocal 3) at 20x magnification. Two methods were used to quantify the amount of sperm. In the first, the photograph was divided into an eight by 10 grid, with each square measuring 17mm x 17mm. Each square was then assigned a number from zero to six based on approximately how much sperm was present in that square: zero if none were present; one if ≤10; two for 11-50; three for 51-100, four for 101-500; five for 501-1000 and six if >1000. More information about this method is provided in appendix 3. The second method was completed by inputting the image into Fiji (Schindelin et al. 2012); it was flattened using a z projection and analysed using a histogram. The pixel values calculated were then multiplied by the amount of times they occurred, and these values were then summed to acquire one overall value for each image. The fluorescing sperm cells resulted in lighter pixels which gave higher values, so those images with higher values contained more sperm cells.

3. 2. 5 Statistical Analysis

In all three investigations, left wings were removed (or right if the left was absent) and measured to the nearest 0.005mm using a binocular microscope (Nikon Eclipse E200 equipped with a graticule). This allowed an investigation into the effect of size.

Some of the flies died before they reached the required testing age. When this was the case, the age of death, treatment and size of the fly was recorded so statistical analysis can be completed to ensure there was no bias.

The statistical software package R version 3.0.2 (R Core Team 2013) was used to complete the statistical analysis on all data collected. One-way and two-way ANOVAs, ANCOVAs and Tukey HSD tests were used to calculate how age and periods of starvation alter egg production, spermatogenesis and proportion of individual and combined fat reserves.

To account for the effect of factors other than the treatment, the time and date of testing and the vial in which the fly originated (parent vial) was recorded to be included in the statistical analysis. Table 9 provides further information on the number of replicates and levels in each variable. The number of replicates and levels measured in each variable is described in table 3. The small sample sizes of the explanatory variables often caused some spurious significances. If this was the case they were removed from the analysis; the variables included are stated with the presentation of results.

Table 9i. The levels in each explanatory variable, and the total number of replicates measured for spermatogenesis.

Table 9ii. The levels and replicates of each explanatory variable used when investigating egg production.

Table 9iii. The levels and replicates of each explanatory variable used when measuring the levels of fat reserves.

NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing

3=3 days old 14=14 days old 28=28 days old

♂=male ♀=female

	3♂NS		14♂NS		14♂S3		28♂NS		28♂S3		TOTAL	
Explanatory Variable	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**
Date	1	10	11	14	8	19	4	23	3	19	21	85
Parent vial	2	10	5	14	4	19	9	23	10	19	23	85
Size	5	10	5	14	6	17	5	21	5	19	8	81
TOTAL	-	10	-	14	-	19	-	23	-	19	-	85

	3♀NS		3♀SE		14♀NS		14♀S3		14♀SE		28♀NS		28♀S3		28♀SE		TOTAL	
Explanatory Variable	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**
Date	6	31	6	29	8	30	8	28	8	27	5	22	6	24	5	20	12	211
Parent vial	18	31	20	29	17	30	17	25	18	27	13	22	14	24	12	19	86	207
Size	14	27	9	26	10	29	10	27	11	26	8	19	9	17	8	17	21	188
TOTAL	-	31	-	29	-	30	-	28	-	27	-	22	-	24	-	20	-	211

	3♂NS		3♀NS		3♂SE		3♀SE		14♂NS		14♀NS		14♂S3		14♀S3	
Explanatory Variable	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**
Parent vial	22	43	27	45	26	49	24	38	21	48	17	41	21	44	20	44
Size	12	32	15	40	9	40	11	34	10	28	10	28	8	33	11	40
TOTAL	-	43	-	45	-	49	-	38	-	48	-	41	-	45	-	48

14♂SE		14♀SE		28♂NS		28♀NS		28♂S3		28♀S3		28♂SE		28♀SE		TOTAL	
Levels	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**	**Levels**	**Replicates**
23	43	14	32	17	34	15	37	17	40	16	25	16	36	17	29	134	628
9	28	9	23	12	22	10	29	7	31	7	21	10	22	9	18	30	469
-	44	-	33	-	34	-	37	-	40	-	25	-	36	-	29	-	635

3. 3 Results

This experiment aims to further the research completed in Chapter 2 by investigating the effects of the combination of ages (three, 14 and 28 days) and starvation treatments on *Drosophila* physiology; including spermatogenesis, internal egg production and levels of fat reserves.

In total, 1587 flies were used for this experiment: 85 for evaluation of spermatogenesis, 211 for internal eggs counts, 640 (341 male and 299 female) to investigate individual fat reserves and 651 (345 male and 306 female) for combined fat reserves. Subsets of males and females were used to analyse both sets of fat reserves data.

3. 3. 1 Younger and Starved Males have a Lower Rate of Spermatogenesis

Older males, and males that were not subjected to a starvation period produced significantly more sperm cells (when taking the effect of parent vial into account) when investigating histogram values (age: $F_{2,\ 58}=9.893$, $p=2.01e^{-4}$; starvation: $F_{1,\ 58}=41.183$, $p=2.77e^{-8}$; parent vial; $F_{22,\ 58}=2.865$, $p=7.15e^{-4}$) and scores (age: $F_{2,\ 57}=44.841$, $p=1.20e^{-12}$; starvation: $F_{1,\ 57}=17.902$, $p=8.537e^{-5}$; parent vial: $F_{22,\ 57}=3.232$, $p=1.96e^{-4}$) (see figure 14). Post hoc Tukey HSD tests showed that three day old male sperm histogram values were significantly lower than 14 and 28 day old males' (three-14 days old: p=0.00125; three-28 day olds: $p=1.24e^{-4}$). Additionally, post-hoc TukeyHSD tests revealed a similar pattern in sperm score data: three day old male sperm scores were significantly lower than those of 14 and 28 day old males, and 14 day olds' scores were lower than those of 28 day olds (3-14 days old: p=0.00; 3-28 days old: p=0.00; 14-28 days old: p=0.00426).

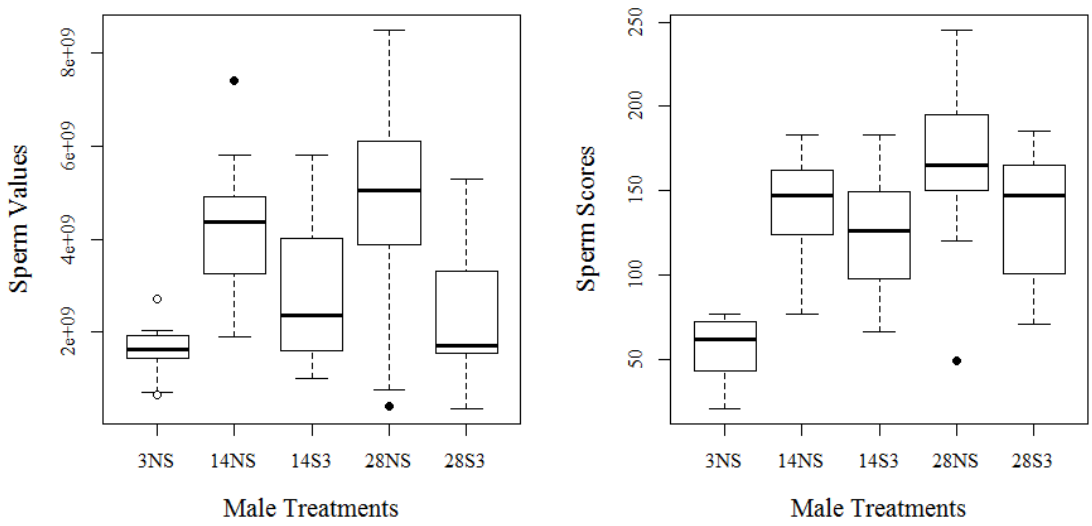


Figure 14. The effect of age and starvation on male spermatogenesis.

NS=Not starved S3=Starved at days 2-3

3=3 days old 14=14 days old 28=28 days old

Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

3. 3. 2 Internal Egg Production Declines with Age

The combination of increasing age, starvation periods and decreasing female body size significantly reduced the number of eggs that the female produced; when accounting for the confounding effect of parent vials (age: $F_{2,\ 179}=17.467$, $p=1.18e^{-7}$; starvation: $F_{2,\ 179}=3.165$, $p=0.0446$; body size: $F_{1,\ 179}=5.872$, $p=0.0164$); see figures 15 and 16. Post-hoc TukeyHSD tests showed that 28 day old females produced significantly fewer eggs than three day old ($p=1.91e^{-4}$) and 14 day old females ($p=2.00e^{-7}$). Further TukeyHSD tests identified that females that were starved immediately before being introduced to the male, produced significantly fewer eggs than those who were not subjected to a period of starvation ($p=0.0450$).

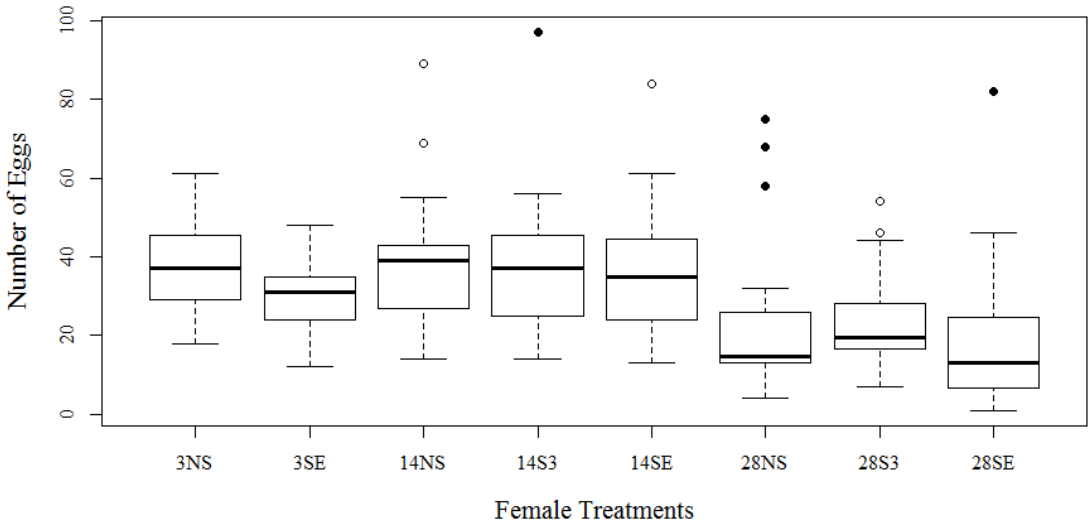


Figure 15. Older females produce significantly fewer eggs ($F_{2, 179}$=17.467, p=1.18e^{-7}). Egg production is also reduced in starved females compared to fed females ($F_{2, 179}$=3.165, p=0.0446).
NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing
3=3 days old 14=14 days old 28=28 days old
Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

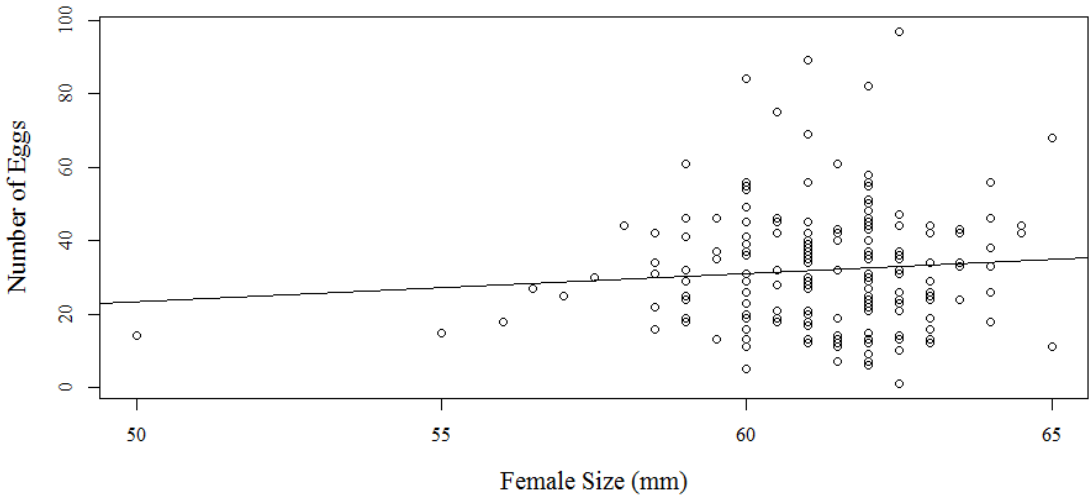


Figure 16. Larger females produced significantly more eggs ($F_{1, 179}$=5.872, p=0.0164).
Line of best fit is added to the scatter plot using the least square method.

3. 3. 3 Females Starved Early in Life have Larger Individual Fat Reserves

There was no significant effect of either age or starvation on the total level of fat reserves in males (age: $F_{2, 331}$=2.467, p=0.0864; starvation: $F_{2, 331}$=1.206, p=0.301); shown in figure 17.

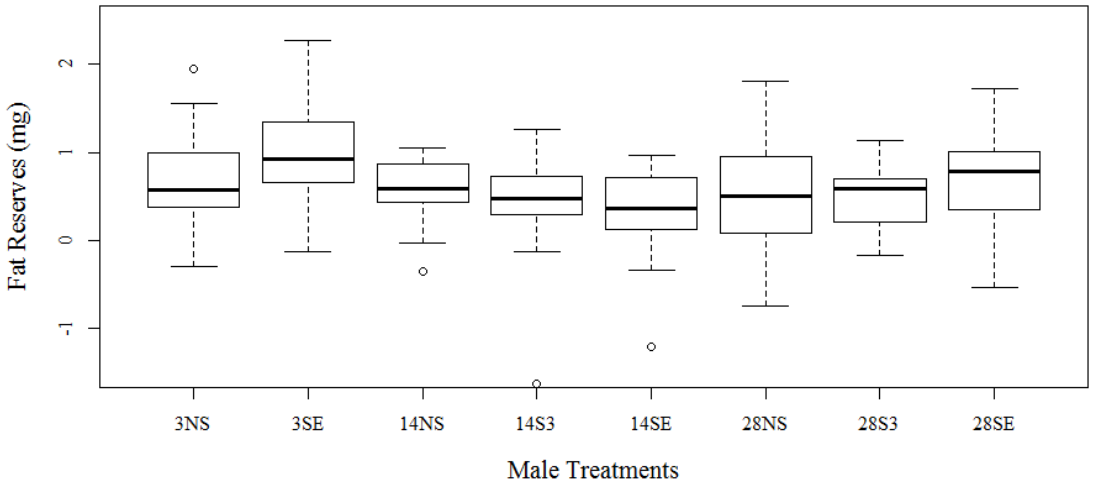


Figure 17. The effect of age and starvation on the amount of fat stored by male *Drosophila*. Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

The effect of starvation periods had a significant impact on the total amount of fat female *Drosophila* are able to store but age had no significant effect (age: $F_{2, 288}$=1.694, p=0.186; starvation: $F_{2, 288}$=5.447, p=0.00476). Post-hoc TukeyHSD tests showed that females starved at age two to three days old had significantly higher fat reserves than those starved prior to testing (p=0.0187), and those not starved at all (p=0.0173); see figure 18.

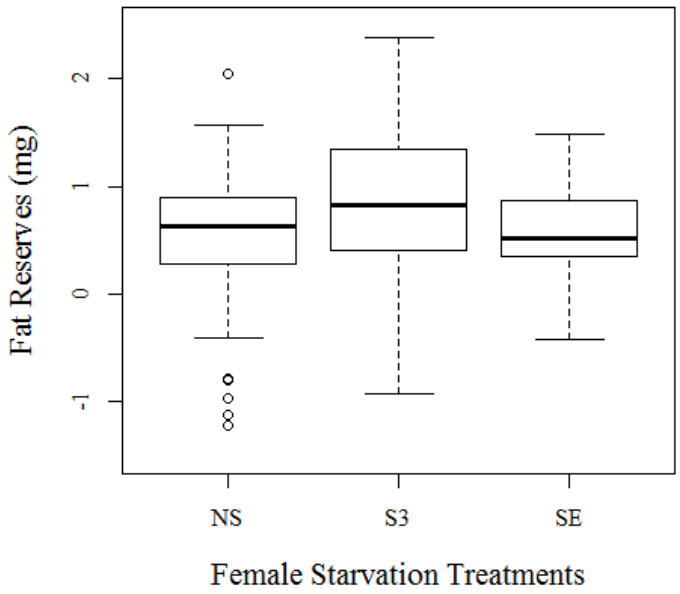


Figure 18. The impact of age starvation on the amount of fat stored by female *Drosophila* ($F_{2, 288}$=5.447, p=0.00476).

Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

3. 3. 4 Combined Fat Reserves Method

Age, starvation period and sex had no significant effect on the amount of fat stored by the *Drosophila* when combined measurements were analysed; see figures 19 and 20 (age: $F_{2, 20}$=1.196, p=0.323; starvation: $F_{1, 20}$=0.232, p=0.635; sex: $F_{1, 20}$=0.284, p=0.600).

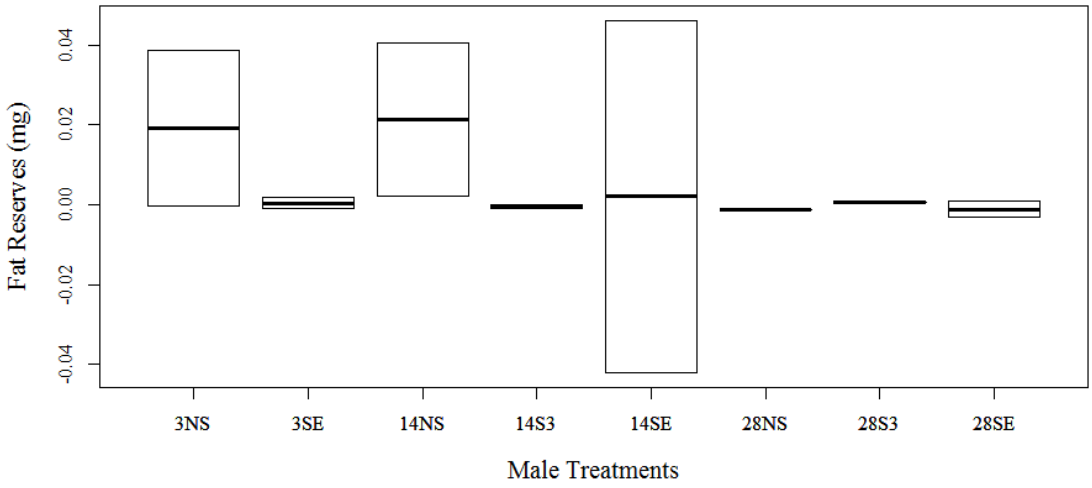


Figure 19. The impact of age and starvation on the amount of fat stored by male *Drosophila* when measured in combined treatment groups.

Boxplot shows median as a bold line and upper and lower quartiles.

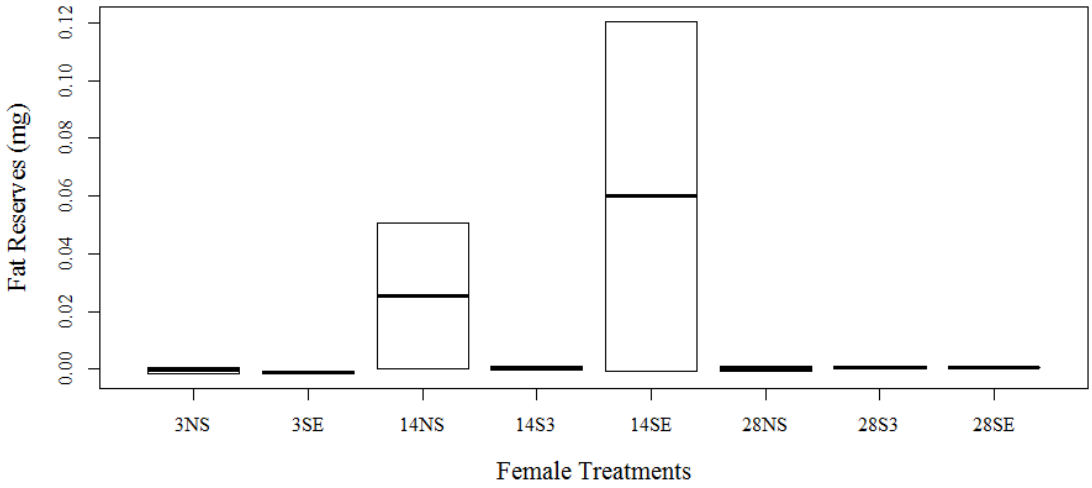


Figure 20. The impact of age and starvation on the amount of fat stored by female *Drosophila* when measured in combined treatment groups.

Boxplot shows median as a bold line and upper and lower quartiles.

3. 4 Discussion

3. 4. 1 Males that Experience a Starvation Period have a Lower Rate of Spermatogenesis

As spermatogenesis is so energetically costly, it is expected that weaker flies, and flies under additional stresses, will produce less sperm (Droney 1996; Droney 1998). However, it is crucial for all organisms to produce successful offspring to pass on their genes, so it is possible that a weak male will invest all his remaining energy into this, when close to death – termed terminal investment (Clutton-Brock 1984; Andrade and Kasumovic 2005; Creighton et al. 2009).

When male *Drosophila* were subjected to a two day starvation period early in their lifetime they produced fewer sperm cells than those that were fed throughout their life (figure 14). The quantity and quality of sperm produced declined for males in nutrition-poor environments (Gage and Cook 1994; Perry and Rowe 2010). It is likely that the starvation period stressed the flies and forced them to make a trade-off between spermatogenesis and other development; for example Dewsbury (1982) observed a shift in the composition of ejaculates with varying environmental conditions.

It was predicted that an increase in the age of a male would result in a decline in the amount of sperm he is able to produce. Mating success has been shown to decrease in older males (Jones and Elgar 2004; Dhole and Pfennig 2014). However, an increase in sperm cells produced was found in older males (figure 14). There are several possible explanations for this. This may be a result of a trade-off in current and future offspring (Simmons et al. 1992); younger males may be conserving energy for the potential future mates that older males would not get. However, this effect could also be because the flies may not be old enough to express the negative effects of age; flies maintained in laboratory conditions can live more than ten weeks, so although 14 and 28 days is old for wild flies, this may not be the case for the flies used in this experiment (Sohal 1970; Takahashi et al. 1970; Brigui et al. 1990).

Additionally, it is possible that these flies were still strong enough to practice terminal investment, causing a final attempt to increase their spermatogenesis rate so they are able to pass on their genes before their death (Clutton-Brock 1984; Creighton et al. 2009). However, this is unlikely because spermatogenesis is a slow process and it is possible that the male fly would die before receiving the benefits (Dewsbury 1982). These results could have been influenced by the method – perhaps if all of the sperm cells had been

individually counted, different conclusions would have been drawn. It is also possible that the number of dead sperm cells present affected the overall results. The number of viable sperm produced could have been counted: this could be done using a live/dead stain (Moatt et al. 2014). As well as sperm, accessory proteins are also transferred within the ejaculate (Wolfner 2002; Wigby et al. 2009); it could be that males produce less accessory proteins so they can afford to exert more energy in spermatogenesis. The method used in this experiment takes sperm size into account, as it measures the overall area coverage, so it could be that sperm size rather than sperm number was increased; although sperm size has also been shown to play an important role in the fertilisation of eggs (Gage and Cook 1994).

3. 4. 2 Internal Egg Production Declines with Female Age

Egg production is costly, so it is likely that it will decline under stressful conditions (Ellers 1995; Wang et al. 2001; Terashima and Bownes 2004). *Drosophila* will be forced to trade-off egg production in order to survive. Both mated and virgin female *Drosophila* produce eggs, so egg production can be examined internally before mating occurs.

Older and smaller females produced fewer eggs than younger and larger females as predicted (figures 15 and 16) (Zhao et al. 2008; Zajitschek et al. 2014). Older and smaller flies are weaker and more susceptible to damage (Sohal et al. 1995; Dubey et al. 1996), eggs therefore cannot be generated as quickly, so the total number found decreases.

Fewer eggs were counted in females that experienced a period of starvation – the fewest were found in females that had recently been starved rather than those starved early in their lifetime. These results align with results from previous research that found that egg production decreased under nutrient-deficient conditions (Ellers 1995; Wang et al. 2001; Terashima and Bownes 2004). When food resources are scarce, organisms rely on their stored energy to complete tasks such as producing eggs. This adds an additional stress, and they are forced to trade-off survival and reproduction.

3. 4. 3 Fat Reserve Levels are Higher in Individuals Starved Early in Life

When environmental conditions are poor, food is less readily available and often the individual is no longer strong enough to search for food. When this is the case, they will metabolise fat reserves for energy – causing a decline in the amount of fat they have stored (Wigglesworth 1949; Aguila et al. 2007).

It was predicted that males and females that experienced a period of starvation would have less fat storage levels (Wigglesworth 1949; Chippindale et al. 1996) – especially those

which had only recently been starved and had not had time to recover. This however, was not the case in this experiment. Females starved early in life were found to have the highest levels of fat reserves (figure 18). It was expected that fed flies would have the highest levels of fat reserves as they have experienced less stress and had not had the need to utilise their reserves (Ellers 1995; Vermeulen et al. 2006). Some of the fat stored originates from the larval stage (Aguila et al. 2007), so it is possible that the early starvation period had a minimal effect on the fruit fly. It is also possible that this starvation period altered the flies' behaviour so that they maintained their fat levels in preparation for another starvation period. This is not the only evidence of starved *Drosophila* recovering from a starvation period and performing better than those fed *ad libitum*; Barker and Podger (1970) discovered that females recovered from a starvation period to produce ovarioles faster than those who were fed throughout their lifetime.

It was thought that younger flies would have greater fat reserves because they will have had less need to metabolise their fat reserves. It is possible that the expected results were not observed because of a trade-off in egg or sperm production (Ellers 1995; Marshall and Sinclair 2009; Stone et al. 2011); *Drosophila* may have used their stored fats to maximise their potential offspring. Or the variation in the flies' body size may have affected the pattern observed: smaller individuals are less able to cope with starvation, so it further reduces their fitness (Miller and Thomas 1958).

However, it is clear from the results that the method was not successful in both combined and individually assessed data. It is apparent that the flies themselves weighed less than the marginal error of the scales, and often negative values for the weight of fat reserves was found (figures 19 and 20). Also negative values and values greater than 100% were calculated for values representing the percentage fat reserves of individual body mass, hence they were removed from the analysis. These errors could be fixed if larger groups of flies had been measured – so that the weight differences were increased. However using this method would mean that male and female body size could not be included within the analysis.

Chapter 4: The Effect of Starvation and Sperm Competition on Courtship

4. 1 Introduction

4. 1. 1 Effects of Sperm Competition

As mentioned in Chapter 3, sperm competition occurs when more than one male's sperm compete for the same female's eggs (Parker 1970); when sperm competition is high, a male will increase his courtship efforts to improve his chances of siring offspring (Mougeov et al. 2001). Spermatogenesis incurs high energetic cost, but without it a male would not be able to pass on his genetic material (Dewsbury 1982). Seminal fluid proteins are transferred alongside the sperm (Wolfner 2002; Wigby et al. 2009) to enable sperm to outcompete other males' sperm for the same ova. In order for this to be an economical tactic, the benefit for the males must outweigh the costs of manufacturing these reproductive accessories. *Drosophila melanogaster* ejaculate contains a wide range of proteins (Chapman 2001; Wolfner 2002; Chapman and Davies 2004); including proteins involved in egg laying stimulation, more efficient sperm storage, inhibition of female remating, rival sperm eradication, regulation of female attractiveness and antibacterial proteins (Scott 1986; Clark et al. 1995; Herndon and Wolfner 1995; Tram and Wolfner 1998; Neubaum and Wolfner 1999; Lung et al. 2001). Under a high sperm competition risk, the viability of sperm transferred improves (García-González and Simmons 2005; Moatt et al. 2014); yet under high sperm competition intensity, it declines (Simmons et al. 2007). Plastic responses in male courtship and copulatory behaviour are observed when females, with varying levels of health and attractiveness, are present (Lüpold et al. 2011).

When nutrient availability is limited, sperm production quality and quantity may decline as it can no longer be afforded (Droney 1996; Droney 1998); causing a decline in reproductive success (Fricke et al. 2008). However, if an individual perceives themselves to be close to death, they may prioritise spermatogenesis for copulation under terminal investment (Clutton-Brock 1984; Creighton et al. 2009).

Male size affects his lifetime mating success; larger male *Drosophila* are in a better condition and can afford to allocate more energy to manufacturing and transferring sperm (Partridge et al. 1987a; Partridge et al. 1987b; Partridge and Farquhar 1983; Pitnick and Markow 1994). *Drosophila* age also has an influence; spermatogenesis declines with increasing age (Snoke and Promislow 2003). In addition to this, those of an intermediate

age are the most attractive mates and so are able to be the most discriminatory (Jones and Elgar 2004; Dhole and Pfennig 2014).

4. 1. 2 Copulation

Male *Drosophila* engage in courtship rituals using visual, olfactory and auditory cues to attract females – all of which are energetically expensive (Bennet-Clark and Ewing 1967; Spieth 1974; Markow 1987). Females respond to these advances using a range of repulsion behaviours, and often eventually acquiesce to copulation (Partridge et al. 1987b; Spieth 1952). Males exposed to sperm competition prior to copulation will mate for longer, to ensure more sperm as transferred and possibly as a method of mate-guarding (Friberg 2006; Bretman et al. 2009; Bretman et al. 2010). Furthermore, latency to mate is shorter when rival males are present (Abraham et al. 2015) which means that courtship duration is shorter.

Copulation and spermatogenesis are energetically expensive, so under nutritional stress, sperm quality declines (Gage and Cook 1994; Galvani and Johnstone 1998; Fricke et al. 2008; Perry and Rowe 2010). It is likely that males will need to trade these costs off against their longevity; an interaction that will probably be exaggerated under high sperm competition intensity. Weaker males will suffer more from this interaction: smaller males often have a shorter lifespan and a lower lifetime reproductive success (Partridge et al. 1987b; Partridge and Farquhar 1983) and require comparatively more energy to survive (Partridge and Farquhar 1983). Older males are also weaker, and also suffer from a reduced mating success (Lefranc and Bundgaard 2000; Zajitschek et al. 2014).

4. 1. 3 Hypotheses

A large amount of research has been completed into the effects sperm competition has on the quality and quantity of ejaculate. Yet, little is known about the effect it has on males' courtship behaviours, or how it is affected by poor environment quality. This research address these knowledge gaps, and should add to understanding of copulation and reproduction, and what the optimum population size may be. This may enable better conservation and preservation methods to be designed for endangered species.

The hypotheses are:

- Courtship and copulation will occur more often in larger individuals because they will be more attractive and will be better able to cope with the expense

- Equally, larger individuals will also begin courtship and copulation sooner – but copulation will last for longer as males will attempt to transfer more sperm and guard the female from rival males
- When males are exposed to high sperm competition, they will be more likely to court and copulate as they perceive themselves as having more competition for mates
- Additionally, they will also invest more into reproduction so will initiate both courtship and copulation sooner, and copulation will last for longer
- Likelihood of copulation and courtship occurring will decline when individuals have been starved because they will be weaker and will need to conserve energy for survival
- When starved individuals do opt to court or copulate, latency to do so will be longer due to less resource availability – however copulation duration will be lengthened if they invest in reproductive terminal investment

4. 2 Methods

4. 2. 1 Treatment Protocol

Drosophila melanogaster used in this experiment originated from an Oregon-R stock. This stock was reared at a constant temperature of 25°C (Imasheva et al. 1998) in a 12 hour light:dark cycle, in 550ml bottles with 50ml of nutritious agar (appendix 1.i). Virgins were collected and sexed from the stock bottles within six hours, then placed in 40ml vials containing Bloomington's standard cornflour agar-based medium (Lakovaara 1969) under 24 hour light.

There were nine treatments in this experiment: solitary males, two males, eight males, and solitary males placed in a vial containing pheromones from previous flies – then each of these were either starved (using the starvation agar in appendix 1.ii) or not starved. A higher male presence is used increase the probability of sperm competition occurring. The ninth treatment has starved females, maintained in a vial containing four individuals; these females were used to determine whether there was any change to the males' behaviour in varying female condition. Table 10 describes the number of replicates used in each treatment. Each of the flies was tested at four days old. They were maintained on Bloomington's standard cornflour agar-based medium (Lakovaara 1969), in a 40ml vial for two days. They were then switched to another identical vial, or one containing starvation agar (appendix 1.ii) for a further two days. Switching the fed flies ensured that the observed effects were caused by the starvation period and not the effect of translocation. A two day starvation period was selected based on results from a preliminary experiment: both males and females died after 48 hours (males: $\bar{x}$=50.13 hours, SD=15.06, n=26; females: $\bar{x}$=63.29 hours, SD=16.98, n=28).

To set up the pheromone treatment, eight flies were placed in a vial two days prior to the treatment fly entering, to deposit pheromones. The same flies were then moved to the second vial for a further two days. To increase the number of potential replicates, the individual was only discarded if more than two pheromone flies died. Similarly, in the eight male treatment, the vial was compromised if fewer than six individuals remained - and three out of four in the female vials.

Table 10. The number of replicates used in each treatment.

NS=Not starved S=Starved

1=Solitary 2=Pairs 8=8 males P=Solitary male with pheromones

Female=4 females

	NS	S
1	18	19
2	20	15
8	25	23
P	17	23
Female	-	22

4. 2. 2 Copulation Observations

Each of these individual males were then placed in the mating arena with one female (starved females were only paired with solitary fed males) to enable courtship and copulation to be observed. The female flies were anaesthetised using ice, and then added to the mating arena first – up to nine at a time, one in each compartment. Each compartment (1cm diameter, and 1.5cm deep) contained 0.01g of active yeast granules to promote egg laying and copulation; see figure 21. A sheet of acetate was placed over the females so the males could be added to the same compartment, without coming into contact with the females.

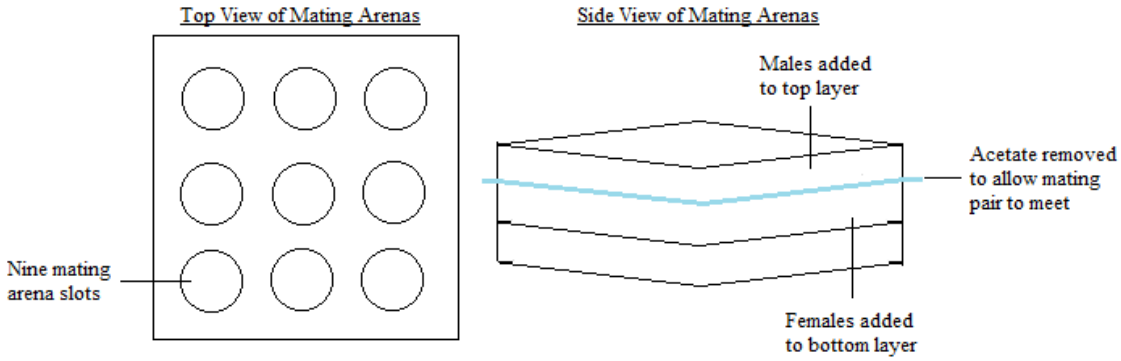


Figure 21. The mating arena with nine holes approximately 1cm in diameter and 1.5cm deep, with a sheet of acetate to separate them into two halves.

Once both the males and females had been added, and the arena had been secured, they were left for five minutes to allow them to awaken and acclimatise. The temperature was set to 27° (Imasheva et al. 1998) to increase activity, and therefore encounter rate. The acetate was then removed and the video began recording courtship and copulation.

The parameters for observing courtship and copulation were the same as stated in Chapter 2.2 (appendix 2.iii) (Eastwood and Burnet 1977; Nandy et al. 2012).

4. 2. 3 Statistical Analysis

Once the flies had died, their wings were removed and measured under a microscope (Nikon Eclipse E200 with a graticule attached) to investigate whether male or female size, or the size difference between the pair, had a significant impact on the final results. Additionally, the bottles from which the flies originated were noted (referred to as parent vials), to enable investigation as to whether this has an effect on the results collected, along with the date and time of both recording and analysing the data. Flies which died before reaching four days old were noted, along with their treatment and parent bottle, to ensure deaths were random, and not caused by the treatments or the individual's body size.

R version 3.0.2 (R Core Team 2013) was used to perform statistical analysis. Males and female data was analysed separately; using one- and two-way ANOVAs, binomial GLMs and post-hoc Tukey HSD tests, the effect of starvation periods and sperm competition perception on courtship and copulation durations were calculated. The time and date of testing, the parent vial, and the mating arena slots that the flies were placed in were also included in this analysis to check whether these variables also had an effect – more information is provided in table 11. It was often the case that the explanatory variables had small sample sizes and would cause many spurious significances, when this was true, they were removed from the model; which variables were included is stated with the presentation of results.

Table 11. The levels in each explanatory variable, and the total number of replicates measured.

NS=Not starved S=Starved

1=Solitary males 2=Paired males 8=Groups of eight males P=Solitary males in the presence of pheromones

♂=male ♀=female

Explanatory Variable	1♂NS		1♂S		2♂NS		2♂S		8♂NS		8♂S	
	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates
Date	10	18	7	19	9	20	7	15	4	25	4	23
Time	10	18	7	19	9	20	7	15	4	25	4	23
Parent vial one	9	18	9	19	6	14	4	14	1	6	1	14
Parent vial two	6	11	5	11	6	14	1	3	4	9	2	7
Mating arena	9	18	9	19	8	20	9	15	9	25	9	23
Male size	11	17	11	18	8	15	8	13	13	23	12	23
Female size	8	15	11	18	12	20	6	13	10	23	11	22
Size difference	12	15	13	17	12	15	10	12	13	21	12	22
TOTAL	-	18	-	19	-	20	-	15	-	25	-	23

P♂NS		P♂S		♀S		TOTAL	
Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates
10	17	12	23	6	22	28	182
10	17	11	23	6	22	23	182
9	16	7	19	2	18	12	138
5	8	9	15	7	22	14	100
8	17	9	23	9	22	9	182
9	14	9	16	14	20	25	159
12	17	12	19	9	21	24	168
12	14	12	16	11	20	38	152
-	17	-	23	-	22	-	182

4. 3 Results

This experiment aims to determine the combined effects of an increase in potential sperm competition, starvation and body size on *Drosophila* copulatory behaviours: courtship latency, courtship duration and copulation duration.

Of the 182 flies measured in this experiment, 149 (81.87%) were observed to court. Seventy three of these (40.11% of the total flies) went on to copulate.

Because not all flies courted and mated, the data was analysed both as a whole set, and using subsets of just those that courted and just those that mated. Time limits were set to determine maximum courtship latency and duration (1800s and 4500s respectively).

4. 3. 1 Sperm Competition and Starvation Do Not Alter Likelihood of Courtship or Copulation Occurring

Whether males initiate courtship was not affected by sperm competition or starvation, nor was it affected by male or female size, the difference in size between the mating pair, or position in the mating arena (results shown below in table 12).

Table 12. *Drosophila* response to a potential increase in sperm competition and starvation; none of the measured treatments had a significant impact on the likelihood of the male to initiate courtship.

	Estimate	Standard Error	Z Score	P Value
(Intercept)	-5.831	6.597	-0.884	0.377
Pairs	16.989	1652.720	0.010	0.992
Eight	-1.246	0.817	-1.526	0.127
Pheromones	-0.391	0.910	-0.430	0.667
Females	1.300	1.315	0.989	0.323
Starvation	0.921	1.029	0.895	0.371
Size Difference	-0.029	0.096	-0.301	0.763
Male Size	0.156	0.119	1.315	0.188
Mating Arena Slot	-0.062	0.100	-0.621	0.535
Pairs:Starvation	-16.974	1652.751	-0.010	0.992
Eight:Starvation	0.671	1.284	0.522	0.601
Pheromones:Starvation	0.523	1.710	0.325	0.745

Degrees of Freedom = 151

The perceived level of sperm competition had no effect on whether the pair successfully copulates; neither did the period of starvation in either males or females, or the mating arena slot (table 13). However, the likelihood of copulation occurring was increased when males were larger (t=2.594, d.f.=127, p=0.00947) and when the size difference between the mating pair was greater (t=2.510, d.f.=127, p=0.121).

Table 13. Increasing male size, and size difference between the pair, increased the likelihood of copulation occurring.

	Estimate	Standard Error	Z Score	P Value	
(Intercept)	-15.802	6.352	-2.488	0.013	*
Pairs	-0.034	0.834	-0.041	0.967	
Eight	-1.224	0.901	-1.358	0.174	
Pheromones	-0.821	0.914	-0.898	0.369	
Females	0.035	0.739	0.047	0.963	
Starvation	-0.361	0.827	-0.436	0.663	
Size Difference	0.203	0.081	2.510	0.012	*
Male Size	0.298	0.115	2.594	0.009	**
Mating Arena Slot	-0.091	0.077	-1.185	0.236	
Pairs:Starvation	0.848	1.197	0.708	0.479	
Eight:Starvation	1.716	1.172	1.464	0.143	
Pheromones:Starvation	1.211	1.206	1.004	0.315	

Degrees of Freedom = 127

4. 3. 2 Courtship Latency is Affected by High Sperm Competition

The perceived level of sperm competition significantly affected the latency of the male to court (see figure 22), although there was no effect of starvation period (sperm competition: $F_{4, 167}$=2.901, p=0.0236; starvation: $F_{1, 167}$=0.726, p=0.396). A post-hoc TukeyHSD test showed that males housed with seven other males took significantly longer to court compared to other treatment types (p=0.0397).

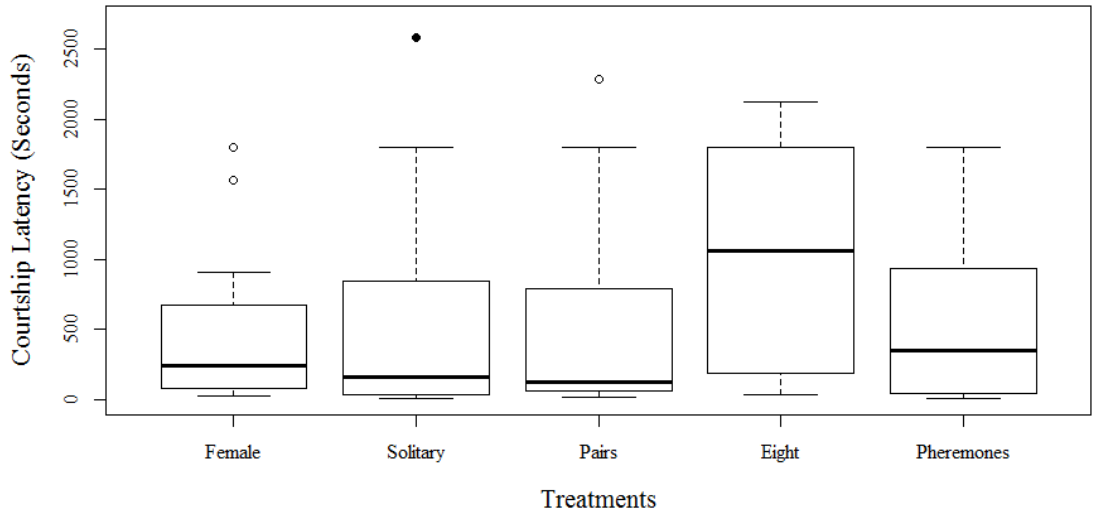


Figure 22. *Drosophila* response to a potential increase in sperm competition: mean courtship latency with upper and lower quartile ranges and standard error. Males from groups of eight took significantly longer to court their mates (TukeyHSD: p=0.0397).

Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

4. 3. 3 Increasing Female Body Size Reduces Copulation Latency

There was no significant impact of sperm competition or starvation period on courtship duration (or latency to mate) (sperm competition: $F_{4,\ 126}$=0.173, p=0.952; starvation: $F_{1,\ 126}$=0.547, p=0.461) but increasing female size causes a significant reduction in the pair's latency to mate ($F_{1,\ 126}$=11.031, p=0.00117); see figure 23.

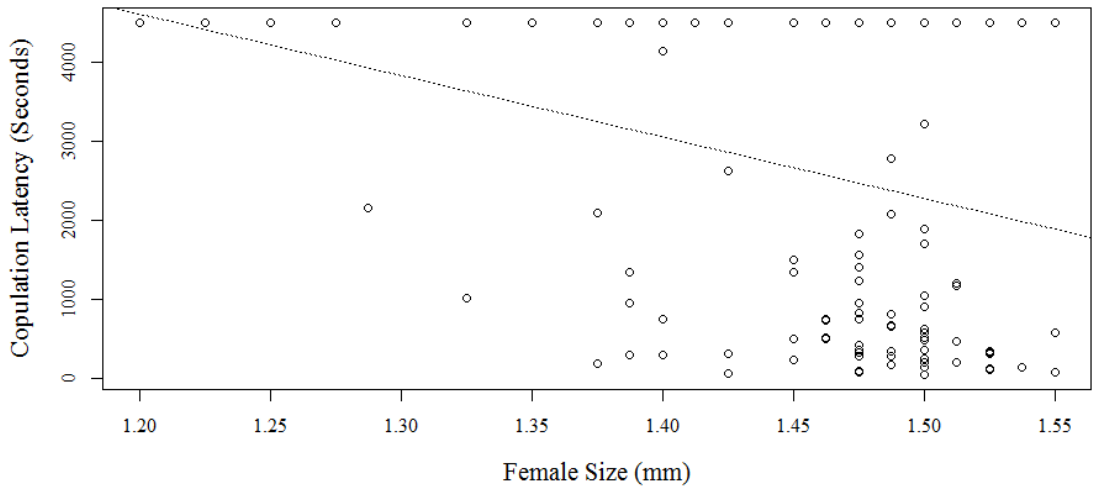


Figure 23. Increasing female size causes a decrease in latency to court ($F_{1, 126}$=11.031, p=0.0017). Line of best fit is added to the scatter plot using the least square method.

4. 3. 4 Increasing Female Body Size Increases Copulation Duration

Copulation duration was not significantly affected by sperm copulation or starvation (sperm competition: $F_{4, 125}$=0.754, p=0.557; starvation: $F_{1, 125}$=0.860, p=0.355). However, copulation duration did increase with increasing female size ($F_{1, 125}$=7.189, p=0.00832) (figure 24).

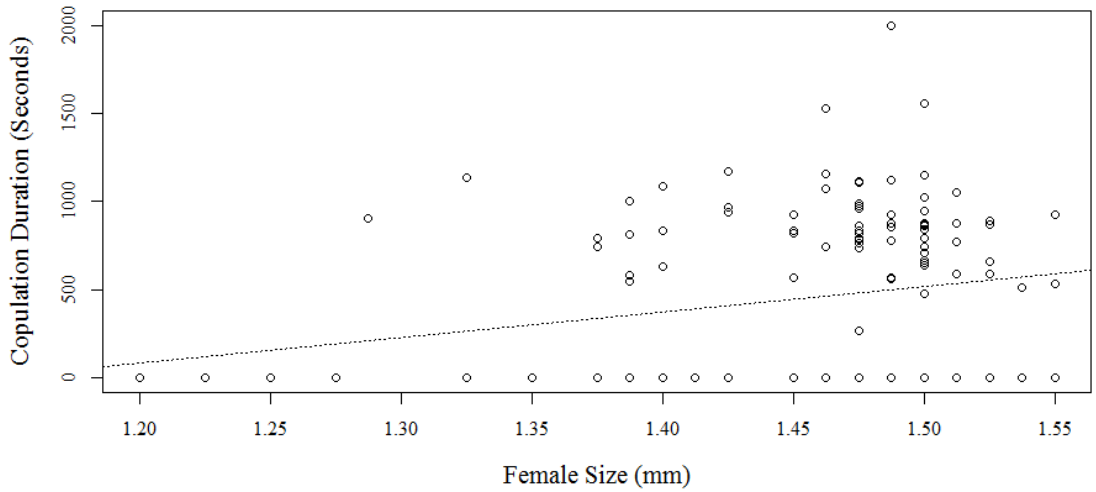


Figure 24. Increasing female size causes an increase in copulation duration ($F_{1, 125}$=7.189, p=0.00832).

Line of best fit is added to the scatter plot using the least square method.

4. 4 Discussion

When several males compete for the same set of ova, sperm competition occurs (Parker 1970). Despite the high energetic cost of spermatogenesis, courtship and copulation, a male will increase his efforts he perceives sperm competition to be high, to improve his chances of siring offspring (Bennett and Houck 1983; Fowler and Partridge 1989; Chapman 1992; Mougeov et al. 2001). When sperm competition risk is high the viability of the sperm transferred improves (García-González and Simmons 2005; Moatt et al. 2014). Plastic responses are also observed in male courtship and copulatory behaviour when female quality and attractiveness varies (Lüpold et al. 2011).

When male 'fitness' varies under changing environmental conditions, individuals are able to alter the quality and quantity of the sperm they produce (Droney 1996; Droney 1998). However this effect can be reversed if a male perceives himself to be close to death. They may invest in reproductive terminal investment and focus all of their remaining energy on spermatogenesis and copulation, in order to sire more offspring (Clutton-Brock 1984; Andrade and Kasumovic 2005) – this effect is also often observed in females (Clutton-Brock 1984).

4. 4. 1 Poor Quality Males Invest in Reproductive Terminal Investment

A male was more likely to mate with the female when he was larger, and when the size difference between the pair was greater (table 13). Larger and healthier males are more attractive and more robust so they are probably better able to cope with the stress of starvation (Sørensen et al. 2005). They have more fat stored, and have more energy to be able to perform an energetically expensive courtship ritual and copulation (Dewsbury 1982; Briegel 1990) so it was predicted that larger males would be more likely to copulate.. The likelihood of both courtship and copulation occurring did not differ with starvation periods or varying levels of sperm competition. It is likely that the treatments will have weakened the flies' body conditions, but despite this, there was no difference in behaviours. This could be because the treatments were not harsh enough to prevent the flies from aiming to reproduce and increase their lifetime reproductive success. However, if this was the case, I would be unlikely that a significant effect of body size was discovered. The result may have been detected in error however, due to small sample sizes (shown in table 7).

It was predicted that courtship effort and likelihood of courtship occurring would increase with the potential for high sperm competition. When males are faced with increased competition, they need to work harder to attract a female and persuade her to choose them as a mate (Tauber and Eberl 2002). This is an energetically costly process for a male, but the benefits often outweigh the costs as the result will often be that he sires offspring (Dewsbury 1982; Harshman and Zera 2007). However, in this experiment, males kept in pairs and in solitude seemed to court more quickly than males kept in groups of eight (figure 22). This could be because males in larger groups have traded-off courtship behaviours against spermatogenesis and production of accessory proteins. An increase in sperm quantity and quality increases the chances of the males sperm outcompeting other sperm, so the male will sire more robust offspring (Hosken et al. 2003; Pattarini et al. 2006). Accessory proteins that are transferred with the sperm cells within the ejaculate also improve the males' chances of siring offspring: they can promote more efficient sperm storage, eradicate rival sperm cells, stimulate egg laying, prevent the females from remating, regulate the females attractiveness and some have antibacterial properties (Scott 1986; Clark et al. 1995; Herndon and Wolfner 1995; Tram and Wolfner 1998; Neubaum and Wolfner 1999; Lung et al. 2001). Additionally, it could be because males in larger groups switch tactics to avoid courtship, and attempt to force matings upon the females to conserve energy (Markow and Wade 2000). It is also possible that the males perceive these females to be less valuable; if they perceive them to be kept in a high male environment then it would be likely that they have previously mated. However, this is unlikely because the males should detect the pheromones that the females are releasing to advertise that they are virgins (Tompkins and Hall 1981).

4. 4. 2 Males Housed in Low Sperm Competition Environments Initiated Courtship Faster

The results from this study show that latency to court is longer when males perceive potential sperm competition to be high (figure 22). When males experience potential high sperm competition they invest a large amount of their energy into spermatogenesis. This could potentially make them weaker and mean that initially they cannot afford the costs of courtship. The presence of other males could also be detrimental to their condition, due to direct physical interactions with the other males. When the female is larger and more attractive, the male often initiates courtship much faster. These 'fitter' females produce more eggs, so they are more attractive to males because mating with them will result in them having a higher reproductive success. Additionally, if a male mates with a more attractive female, their offspring will be more attractive and will therefore be more

successful at producing grand-offspring (Weatherhead and Robertson 1979). However in the presence of many other males, it is likely that males will be less choosy and attempt to sire offspring from multiple females (Tauber and Eberl 2002).

When females are smaller, copulation latency increases (figure 23). This effect may have been observed due to increased courtship vigour. It is likely that more attractive females were courted more vigorously, and were therefore more sexually stimulated (Ewing 1983). It could also be caused by weaker females rejecting more to avoid the costs of copulation (Wigby and Chapman 2005; Kuijper et al. 2006).

It was predicted that copulation duration would be longer when males were starved and have an experienced an environment with any potential rivals due to terminal reproductive investment (Clutton-Brock 1984; Andrade and Kasumovic 2005). Males that are exposed to potential high sperm competition perceive there to be many rivals that they have to outcompete, so they will copulate for longer. Longer mating duration enables a better transfer of sperm and improves the male's chances of inseminating the female (Edvardsson and Canal 2006). It is also a potential mate-guarding method as the female cannot remate if she is still mating with him (Alcock 1994). Copulation duration was longer in larger females (figure 24). When females are 'fitter' and more attractive, successful copulation and mate-guarding becomes even more important because the female will be able to provide him with more offspring. It was predicted that copulation duration would be longer in starved individuals when they were maintained (or perceived to be maintained) in high sperm competition. Under high sperm competition, successful copulation and mate-guarding becomes even more important because there are an increased number of potential rivals. It is possible that this result was not seen due to a trade-off for other expensive processes such as spermatogenesis (Pitnick 1996; Moatt et al. 2014); or because the weaker individuals were conserving energy for survival and meetings with other more suitable mates.

Chapter 5: The Effect of Age and Starvation on Activity and Survival in Males

5. 1 Introduction

5. 1. 1 Activity Levels in *Drosophila*

The activity of male *Drosophila melanogaster* varies through the day, an in common with many animals, they experience bouts of sleep where their brain activity declines in order to improve their overall performance (Cirelli and Bushey 2008). The condition of the individual affects how active it is and how much sleep it requires. Le Bourg and Lints (1984) observed that male *Drosophila* activity rates declined with increasing age. Additionally Koh et al. (2006) found that periods of sleep became more fragmented with increasing age. Body size also effects how active *Drosophila* are. Larger individuals use less energy to complete the same movements and actions as smaller individuals (Partridge and Farquhar 1983; Speakman 2005), which suggests that larger males would be more active. However, if any individual is faced with extremely poor nutrition, resting time will decrease as starvation suppresses sleep (MacFadyen et al. 1973; Keene et al. 2010). Additionally, Kopeć (1928) observed that *Drosophila* longevity declined when starvation intensity increased.

5. 1. 2 *Drosophila* Longevity

Using mutant strains, Trout and Kaplan (1970) showed that increased activity caused a decrease in longevity. The flies' average lifespan decreased with an increasing metabolic rate, and that increasing metabolic rate was directly proportional to total activity (Trout and Kaplan 1970).

It has long been established that the size of an individual effects the length of its lifespan: larger flies tend to live longer than smaller flies (Partridge et al. 1987b; Partridge and Farquhar 1983; Speakman 2005). Longevity will also be affected by whether or not the male courts the female, and then whether the pair mate. Courtship, repulsion and copulation are energetically expensive behaviours and thus if an male partakes in these activities their lifespan will be reduced (Bennett and Houck 1983; Fowler and Partridge 1989; Cordts and Partridge 1996; Harshman and Zera 2007; Creighton et al. 2009). Although Partridge and Andrews (1985) claim that 'the effect of reproductive activity on longevity is short-term and reversible'.

5. 1. 3 Hypotheses

Understanding how variable environments affect a male's activity and longevity is important to help conserve endangered species – to understand how individual's in a population move and react. This knowledge could even be expanded to help medical research in mobility-affecting conditions such as narcolepsy or neurologic disease (Siegel et al. 1991; Pearson et al. 2004); to help discover how their activity rates could be increased. An increased longevity will give an individual a better chance at a higher lifetime reproductive success (Rhine et al. 2000), and increased activity suggests an individual will have more energy to use, for example hunting, courting or parental care (Tucker 1975; Bennett and Houck 1983; Strohm and Marliani 2002). This study aims to investigate how male *Drosophila melanogaster* activity and starvation resistance is affected by their age and by periods of starvation.

The hypotheses are:

- Rate of activity and starvation resistance will be positively correlated as flies that can afford to be active will also have energy conserved for survival
- Young, large males, that do not experience any period of starvation will have a higher activity rate because they have more resources available to them and will be better able to afford the energetic costs
- These high quality males will also have an increased starvation resistance

5. 2 Methods

5. 2. 1 Treatment Protocol

This investigation was completed using the same treatments as used in Chapter 2.2; the same males were moved from the mating arena to this experiment. Oregon-R and Canton-S F1 crosses, used across sixteen treatment; see table 14 for the numbers of replicates used.

Table 14. The number of replicates used in each treatment.

NS=Not starved SE=Starved in final two days S3=Starved at 2-3 days old

3=Three days old 14=14 days old 28=28 days old

Males				**Females**			
	NS	SE	S3		NS	SE	S3
3	15	10	-	**3**	18	12	-
14	16	14	14	**14**	11	13	13
28	14	12	14	**28**	12	11	12

5. 2. 2 Activity and Survival Measurements

Once the males were introduced to the mating arena, as described in Chapter 2.2, they were removed and aspirated into a 3mm by 65mm tube with a small amount of starvation agar-based medium allowing them to uptake water (appendix 1.ii). These were then put into a *Drosophila* Activity Monitor (pictured in figure 25) (DAM, Trikinetics, Waltham, MA, USA) which measured the activity levels of the fly and recorded the time of death. The resistance to starvation was used as a proxy for lifespan – as has been done several times since Rose et al. (1992) established the link between the two factors (Bharucha et al. 2008; Moatt et al. 2013). An example of the code and files required to extract the data from the DAM can be found in appendix 4.

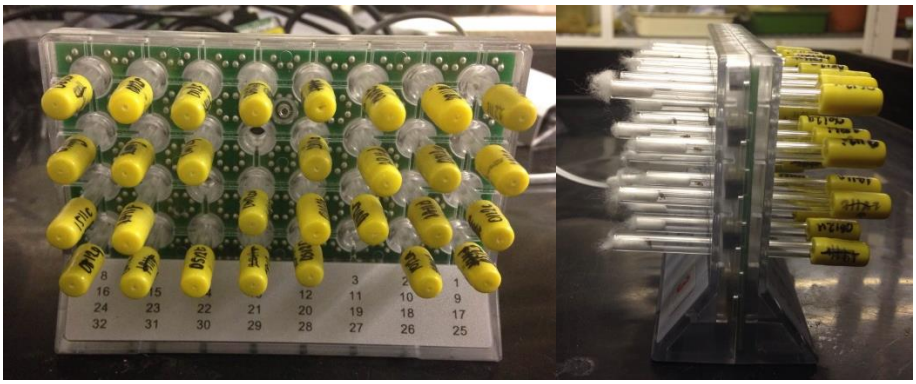


Figure 25. The male *Drosophila* in the DAM.

After death, their left wings were removed and measured under a microscope; as described in Chapter 2.2.

5. 2. 3 Statistical Analysis

The output from the DAM system was extracted using the statistical software package R version 3.0.2 (R Core Team 2013). This software package was also used to complete one-way and two-way ANOVAs, ANCOVAs and post-hoc Tukey HSD tests on the collected data, to understand whether age and starvation periods had a significant effect on the activity and starvation resistance of *Drosophila*. Pearson product-moment correlations were used to analyse the relationship between activity and starvation resistance.

Date and time of testing, the parent vials in which the fly eclosed and the slot number of the DAM that the flies were placed in were also recorded to enable an assessment of whether they had an effect on the final results. Table 15 describes the number of replicates and levels measured within each variable. The small sample sizes of the explanatory variables often caused some spurious significances. If this was the case they were removed from the analysis; the variables included are stated with the presentation of results.

Table 15. The levels in each explanatory variable, and the total number of replicates measured across all eight treatments.

NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing

3=3 days old 14=14 days old 28=28 days old

♂=male ♀=female

Explanatory Variable	3♂NS		3♀NS		3♂SE		3♀SE		14♂NS		14♀NS		14♂S3		14♀S3		14♂SE		14♀SE		28♂NS		28♀NS		28♂S3		28♀S3		28♂SE		28♀SE		TOTAL	
	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates
Date	12	15	14	18	10	10	9	12	13	17	11	12	12	14	9	13	14	14	12	13	13	14	9	12	10	15	10	11	10	12	11	12	48	214
Time	11	15	12	18	10	10	8	12	12	17	9	11	8	14	9	13	10	14	11	13	12	14	8	12	10	15	9	11	10	12	11	12	32	214
Parent vial	11	15	16	18	7	10	11	12	11	13	11	12	10	11	12	13	10	11	12	13	5	5	9	12	1	2	9	11	2	4	10	12	73	174
DAM slot	13	15	15	18	9	10	12	12	12	17	9	12	10	14	11	13	12	14	11	13	9	14	11	12	14	15	9	11	11	12	11	12	32	214
Size	6	15	7	17	6	8	7	12	9	17	5	9	6	14	8	12	4	12	7	12	7	13	6	11	6	15	4	8	5	12	7	11	17	193
TOTAL	-	15	-	18	-	10	-	12	-	17	-	12	-	14	-	13	-	14	-	13	-	14	-	12	-	15	-	11	-	12	-	12	-	214

5. 3 Results

This experiment aims to determine how the condition of a male *Drosophila* affects its activity and starvation resistance – and if male activity and starvation resistance is also affected by the condition of their female mating partner. Condition is manipulated using three ages (three, 14 and 28 days old) and three starvation treatments (none, at days two-three and two days prior to testing). To examine this, 111 males and 103 females were subjected to various aging and starvation treatments before being placed in a DAM.

5. 3. 1 Better Quality Males have a Longer Lifespan

Male *Drosophila* starvation resistance (as a proxy for lifespan) was significantly affected by starvation and age (see figure 26) – when the parent vial they originated in was considered) (age: $F_{2, 63}$=18.057, p=6.32e^{-7}; starvation: $F_{2, 63}$=4.211, p=0.0192; parent vial: $F_{40, 63}$=1.648, p=0.0372). Post-hoc TukeyHSD tests revealed that three day old males survived for significantly longer than both 14 and 28 day old males (14 days old: p=7.05e^{-5}; 28 days old: p=4.00e^{-7}). Post-hoc TukeyHSD tests showed that males that were starved early in life lived longer than those starved later (p=0.0198).

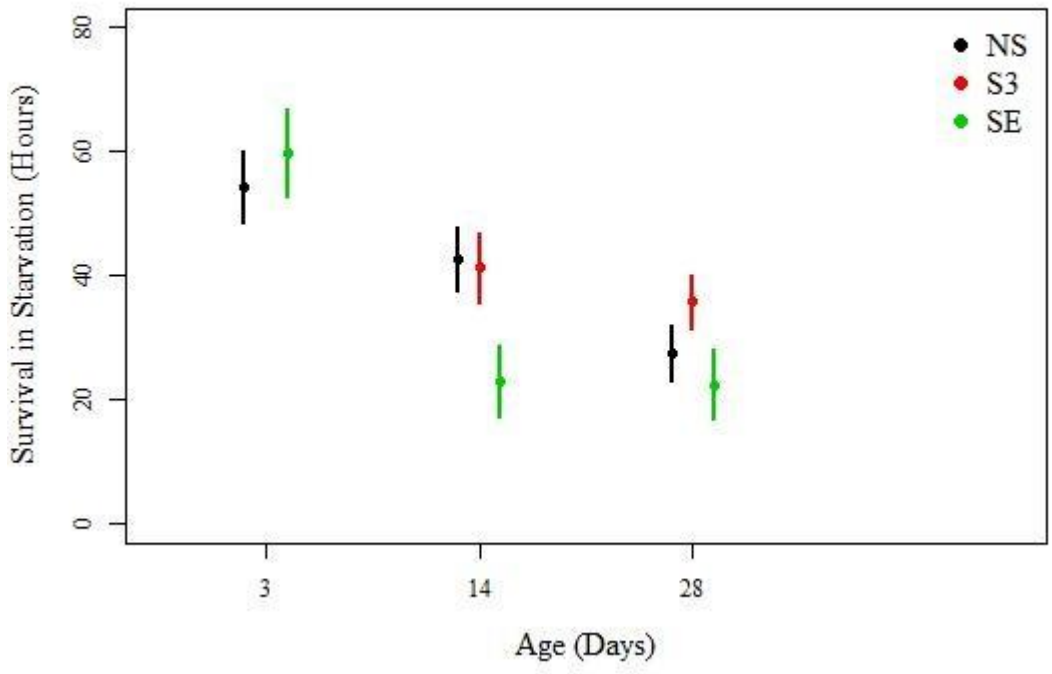


Figure 26. The effect of age and starvation treatment on male *Drosophila* starvation resistance. Activity rate is the number of times in its life, that the fly crossed the infra-red laser found in the DAM.

NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing

The bars on this plot show standard error.

The starvation treatment the males underwent also had a significant effect in their activity (see figure 27). Activity decreased under recent periods of starvation but increased when males were starved early in life; although age had no significant effect (age: $F_{2, 103}=2.517$, $p=0.0856$; starvation: $F_{2, 103}=12.480$, $p=1.40e^{-5}$). Post-hoc TukeyHSD tests revealed that males that were starved immediately before being placed in the DAM exhibited a significantly lower activity level than both fed and early starved males ($p=0.00302$; $p=2.00e^{-5}$ respectively).

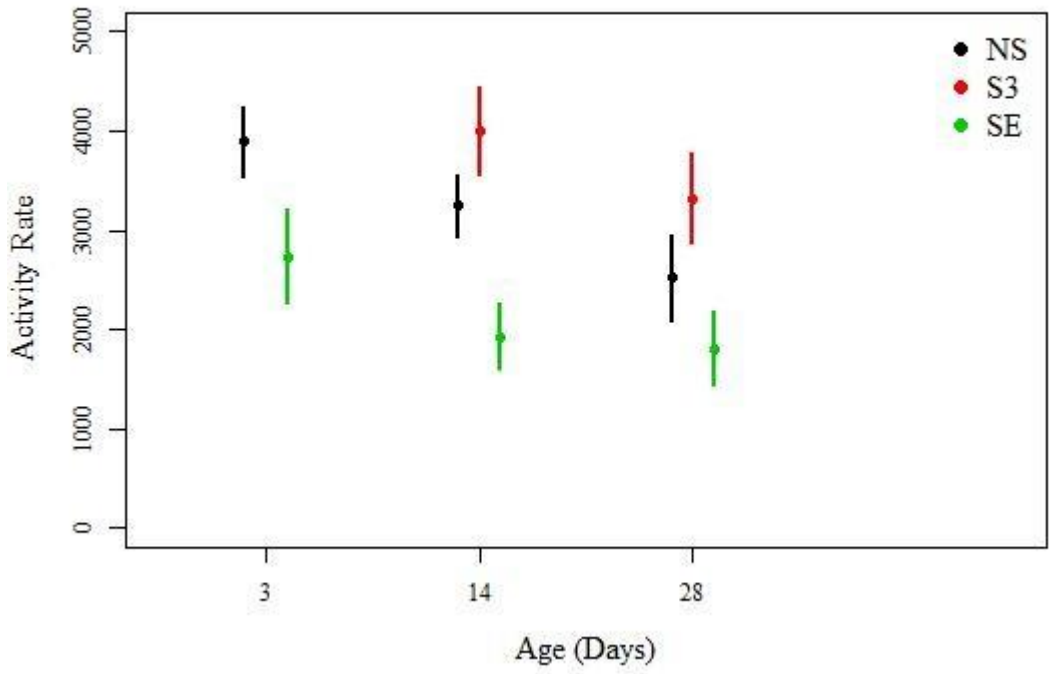


Figure 27. The effect of age and starvation treatment on male *Drosophila* activity rate. Activity rate is the number of times in its life, that the fly crossed the infra-red laser found in the DAM.
NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing
The bars on this plot show standard error.

5. 3. 2 Female Condition Affects Male Lifespan and Activity

Male starvation resistance (as a proxy for lifespan) was significantly lower when their mating partners were older (see figure 28), and when the males were smaller (figure 29), and when courtship or copulation occurred, although starvation had no significant effect (when accounting for effects of parent vial) (age: $F_{2, 32}$=103.802, p=1.02e^{-14}; starvation: $F_{2, 32}$=2.479, p=0.0998; courtship occurrence: $F_{1, 32}$=7.431, p=0.0103; copulation occurrence: $F_{1, 32}$=7.362, p=0.0106; size: $F_{1, 32}$=10.797, p=0.00247; parent vial: $F_{48, 32}$=8.111, p=8.44e^{-9}). Post-hoc TukeyHSD tests revealed that males paired with three day old females had a significantly longer lifespan compared to those paired with 14 day olds (p=1.52e^{-5}) and 28 day olds (p=1.00e^{-7}).

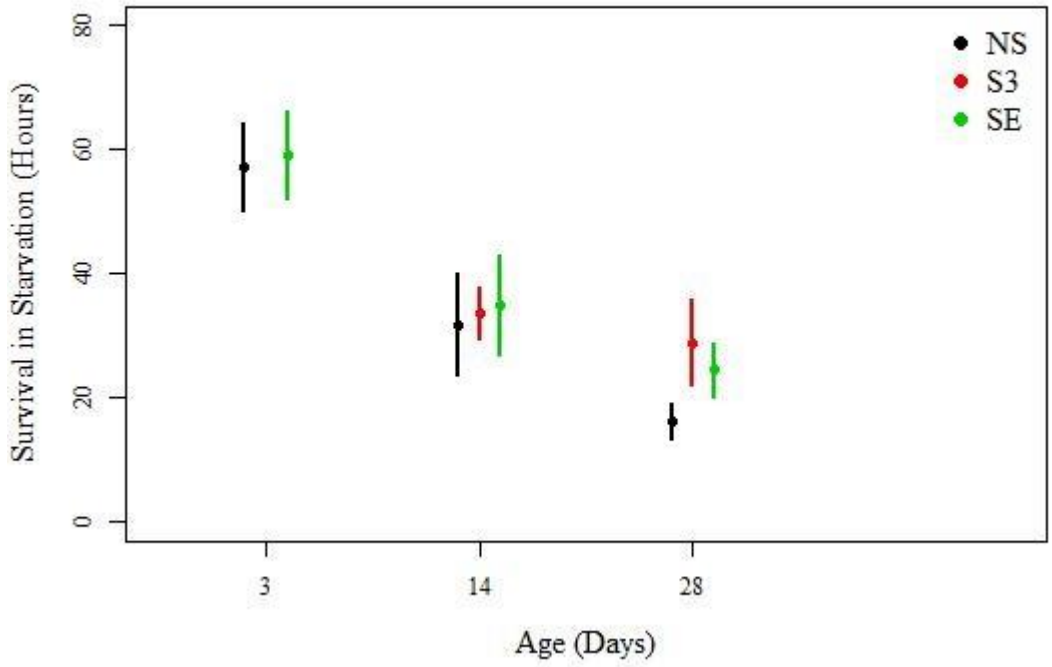


Figure 28. The effect of the age and starvation treatment of a male's mate, on male *Drosophila* survival – using starvation resistance as a proxy.

NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing

Bars show standard error.

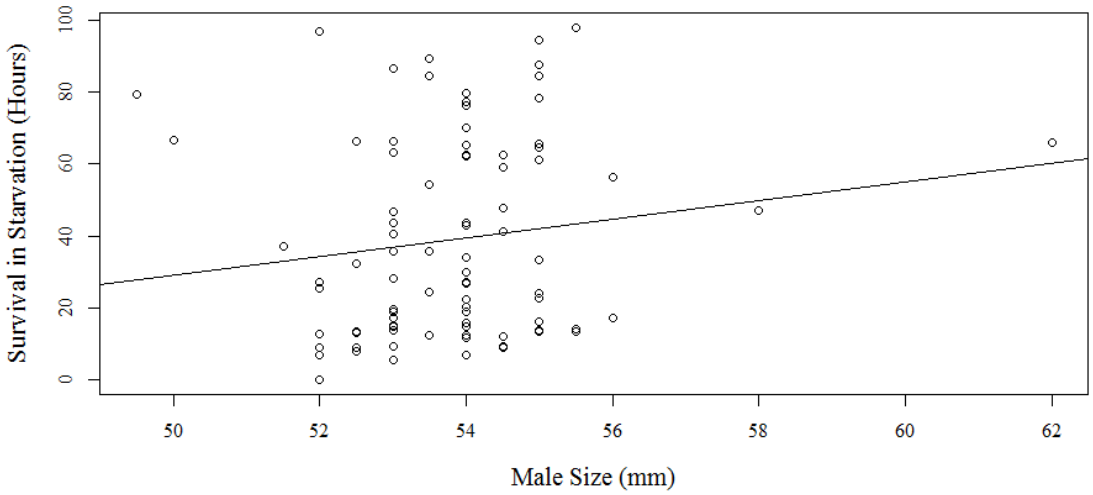


Figure 29. Survival in starvation is improved with increasing male size ($F_{1,\ 32}$=10.797, p=0.00247**).**

Line of best fit is added to the scatter plot using the least square method.

Increasing female age caused a reduction in male activity (shown in figure 30) although there was no significant effect of the starvation period (age: $F_{2,\ 95}$=10.622, p=6.87e^{-5}; starvation: $F_{2,\ 95}$=0.537, p=0.586). Post-hoc TukeyHSD showed that activity rate was significantly lower when their female mate was age 14 (p=0.00245) and 28 days (p=6.87e^{-5}) compared to three day olds.

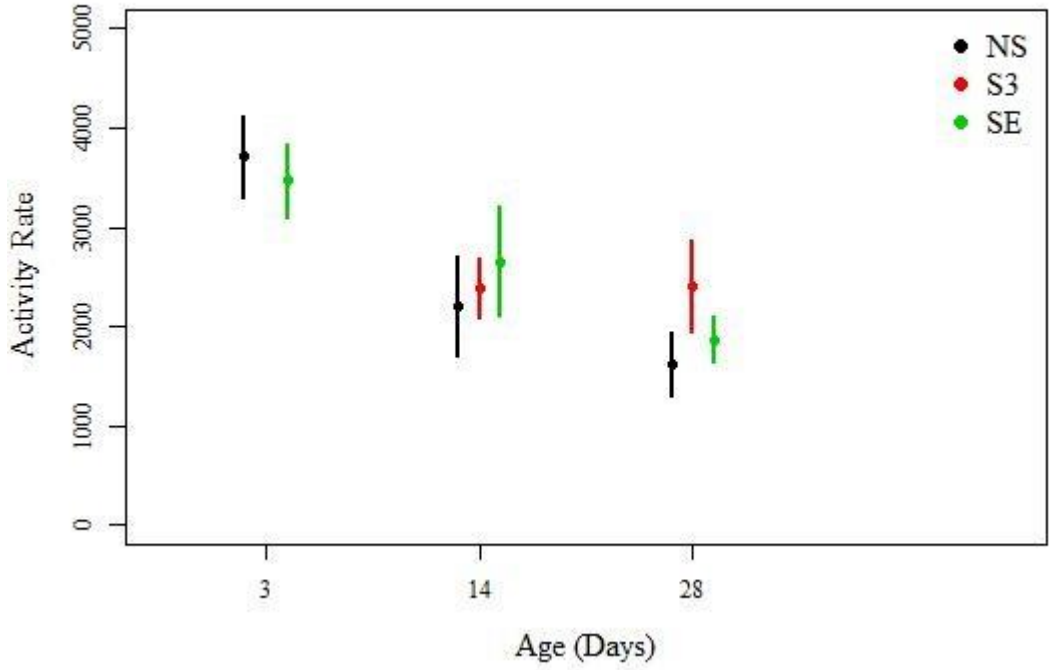


Figure 30. The effect of the age and starvation treatment of a male's mate, on male *Drosophila* activity rate. Activity rate is the number of times in its life, that the fly crossed the infra-red laser found in the DAM.

NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing

Bars show standard error.

5. 3. 3 Activity and Starvation Resistance have a Strong Correlation

Pearson product-moment correlation tests were used to identify a strong positive correlation between overall activity and lifespan ($r=0.706$, $d.f.=212$, $p=2.20e^{-16}$) (figure 31).

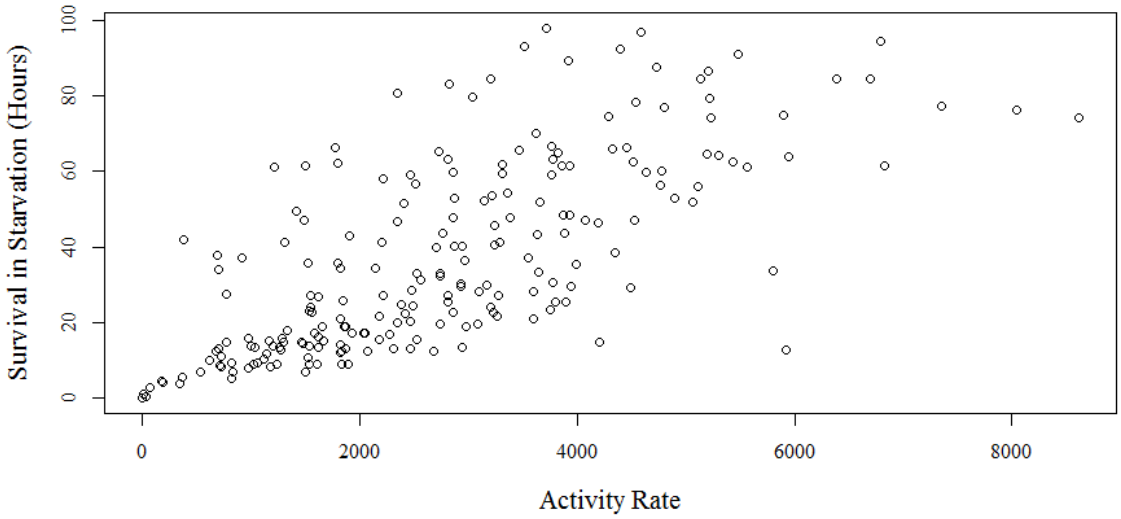


Figure 31. *Drosophila* activity and longevity is significantly correlated (r=0.706, d.f.=212, $p=2.20e^{-16}$**).** Activity rate is the number of times in its life, that the fly crossed the infra-red laser found in the DAM.

5. 4 Discussion

5. 4. 1. Activity Levels Increase with Male 'Fitness' but Decrease with Female Attractiveness

As predicted, males that were subjected to a period of starvation had a lower activity rate (figure 27). These males were in a poorer condition and were probably not able to conserve energy for survival so were closer to death (Vermeulen and Loeschcke 2007). Activity rate also declined when the male was paired with an older female, or a female that had not been starved (figure 30). This could be due to the attractiveness of females that have been fed. It is possible that males will exert more energy and effort into courting and copulating with these females as they will provide the males will more offspring (due to increased resources); this could reduce the male's activity later in their life. Although it is widely considered that younger females are more attractive, as they have better access to resources and can produce more eggs (Cook and Cook 1975; Hercus and Hoffmann 2000; Dhole and Pfennig 2014), it is possible that the males here perceive the older females to be more attractive due to their superior genetics (Hansen and Price 1995).

5. 4. 2 More Active Flies Have a Longer Lifespan

Male flies with a higher activity rate tended to live for longer (in starvation) than those who were less active (figure 26). This suggests that males only exert energy in movement when they can afford to, and conserve energy for survival when necessary (Djawdan et al. 1998). Older males that had been subjected to a period of starvation before testing were in a poorer condition, and so tended to die before better quality males, as expected. Older males have a poorer starvation resistance as they are weaker and have lower energy reserves (Marron et al. 2003). Additionally, when males have been starved, they have metabolised a larger proportion of their fat reserves so they have less energy remaining for survival (Djawdan et al. 1998).

These results showed that the treatments experienced by the female before mating also altered the length of the male's life: starvation resistance decreased when they were larger or their mating partners were older (figure 28). Survival was also decreased in males that completed courtship and copulation, compared to those that did not attempt to attract a mate. This is likely due to the energetic expense of muscular movement in courtship (Bennett and Houck 1983; Fowler and Partridge 1989). Furthermore, it has been found that sexual activity elevates metabolic rate (Giesel et al. 1989) which causes a reduction in lifespan (Miquel et al. 1976). In addition to this, there is a high cost to spermatogenesis and the production of accessory proteins (Dewsbury 1982). Larger flies are better able to

afford the cost of courtship and copulation, and have larger fat reserves, enabling them to survive for longer in starvation. It is likely that survival decreased when males were paired with older females because these females are more likely to accept any copulation attempts as it could be their final chance to increase their lifetime reproductive success; the cost could come from increased courting and copulation efforts in an attempt to attract these more attractive mates (Cordts and Partridge 1996; Clutton-Brock and Langley 1997).

Chapter 6: The Effect of Starvation and Sperm Competition on Activity and Survival

6. 1 Introduction

6. 1. 1 Activity and Survival in *Drosophila*

Drosophila melanogaster require periods of rest and sleep in order to survive (Cirelli and Bushey 2008). The amount of sleep an individual requires, or how active it is, depends on the state of their health: activity rates decline with increasing age (Le Bourg and Lints 1984). Furthermore, activity is inversely proportional to an individual's size based on the excess energy they have (Partridge and Farquhar 1983; Speakman 2005). Size of an individual also impacts on their longevity: smaller flies often have a shorter lifespan (Partridge et al. 1987b; Partridge and Farquhar 1983; Speakman 2005). Lifespan is further decreased with an increase in activity (Trout and Kaplan 1970).

In high sperm competition, reproductive activity and effort is increased (Mougeov et al. 2001). Reproductive activity is affected independently from other activity (Gromko and Pyle 1978); it is likely that when energy is expended on reproductive efforts all other activity suffers. When faced with the potential for sperm competition, it is probable that males will trade-off their activity in order to be able to maximise spermatogenesis and other reproductive behaviours.

Exposure to many males (and therefore sperm competition) increases the longevity of virgin males – likely because these males have completed a trade-off of movement versus tissue production, and are conserving all available energy for optimising spermatogenesis (Moatt et al. 2013). However, under high sperm competition, males are more likely to court and copulate with a female, which reduces their lifespan (Partridge and Farquhar 1981; Dewsbury 1982; Cordts and Partridge 1996; Lee et al. 2008).

6. 1. 2 Hypotheses

As well as age and bouts of starvation, sperm competition could also affect *Drosophila* activity and starvation resistance – as a proxy for lifespan. This experiment aims to further the findings of Chapter 5, by manipulating sperm competition and nutrient availability. Knowledge as to how to increase activity and longevity of a species should increase the chance of any managed populations growing and thriving. In addition to this, understanding how sperm competition affects individuals could help to determine the

optimum number of individuals in a population – which could also improve conservation efforts.

The hypotheses are:

- Rate of activity and starvation resistance will be positively correlated because flies that can afford the energetic expense of movement will also have energy for survival
- Smaller and starved female *Drosophila* will have a reduced activity and starvation resistance as they have reduced access to resources
- High sperm competition, starvation and decreased size will reduce activity and starvation resistance in males as they also have less available resources – and they need to invest more energy into outcompeting rival males

6. 2 Methods

6. 2. 1 Treatment Protocol

The second data set was collected at the same time that described in Chapter 4.2: Oregon-R flies maintained in ten different treatments: solitary males, two males, eight males, solitary males in vials that already contain pheromones, and four females per vial – each of which were either staved or not starved. The number of replicates observed in each treatment is described in table 16.

Table 16. The number of replicates used in each treatment.
NS=Not starved S=Starved
1=Solitary 2=Pairs 8=8 males P=Solitary male with pheromones
Female=4 females

	NS	S
1	29	38
2	36	33
8	33	35
P	28	28
Female	29	36

6. 2. 2 Activity and Starvation Resistance Measurements

Flies were aspirated into tubes which were then placed into the *Drosophila* Activity Monitor (DAM, Trikinetics, Waltham, MA, USA) as described in Chapter 5.2. Activity, starvation resistance and body size of the *Drosophila* was calculated.

6. 2. 3 Statistical Analysis

The statistical software package R version 3.0.2 (R Core Team 2013) was used to extract the data from the DAM output files and to complete all statistical analyses. One-way and two-way ANOVAs, ANCOVAs and post-hoc Tukey HSD tests were used to evaluate what impact sperm competition and starvation periods had on both male and female *Drosophila* activity and starvation resistance. Pearson product-moment correlations were used to establish the relationship between activity and starvation resistance.

The time and date of testing, the parent vial in which the fly originated, and which mating arena slot and DAM number, and DAM slot each fly occupied were recorded to account for any significant effect these factors had. The numbers of replicates and levels are shown below in table 17. It was sometimes the case that the variables had small sample sizes and would cause spurious significances, they were removed from the model when this was true; the variables included are stated with the presentation of results.

Table 17. The levels in each explanatory variable, and the total number of replicates measured.

NS=Not starved S=Starved

1=Solitary males 2=Paired males 8=Groups of eight males P=Solitary males in the presence of pheromones

♂=male ♀=female

Explanatory Variable	1♂NS		1♂S		2♂NS		2♂S		8♂NS		8♂S	
	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates
Date	9	29	7	38	7	36	9	33	4	33	5	35
Time	9	29	6	38	7	36	8	33	4	33	5	35
Parent vial	9	29	9	36	6	28	9	33	2	11	1	1
DAM number	4	29	3	38	4	36	4	33	3	33	3	35
DAM slot	18	29	25	38	24	36	16	33	20	33	23	35
Size	15	27	15	33	14	34	13	28	12	31	16	33
TOTAL	-	29	-	38	-	36	-	33	-	33	-	35

P♂NS		P♂S		♀NS		♀S		TOTAL	
Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates	Levels	Replicates
11	28	11	28	4	29	5	36	17	325
9	28	10	28	3	29	4	36	14	325
8	26	6	27	2	13	2	10	12	214
4	28	4	28	4	29	4	36	4	325
17	28	16	28	19	29	19	36	32	325
12	27	12	27	10	27	13	35	35	302
-	28	-	28	-	29	-	36	-	325

6. 3 Results

This experiment was designed to help determine how sperm competition and two day periods of starvation impact both male and females activity and starvation resistance. Sixty five females were used to investigate the effects of starvation – using four day old flies that were starved during the latter half of their life. Males were subjected to the same starvation periods, and effect of sperm competition was also investigated: 257 were used across the eight treatments. Sperm competition was manipulated using four categories: solitary males, two males, groups of eight, and solitary males placed in a vial containing pheromones of other males.

6. 3. 1 Sperm Competition and Starvation Affect Male Starvation Resistance

Increasing perceived sperm competition and starvation significantly reduces starvation resistance in male *Drosophila* (see figure 32) when accounting for parent vial and time and date of testing (sperm competition: $F_{3, 218}=9.896$, $p=3.82e^{-6}$; starvation: $F_{1, 218}=7.521$, $p=0.00660$; parent vial: $F_{15, 218}=3.556$, $p=1.69e^{-5}$; time: $F_{14, 218}=9.479$, $p=2.82e^{-16}$; date: $F_{2, 218}=7.296$, $p=8.57e^{-4}$). Post-hoc TukeyHSD tests revealed that solitary males had a longer starvation resistance than males that were, or perceived to be (using pheromones), maintained in groups of eight (solitary-eight: p=0.00900; solitary-pheromones: p=0.0325; pair-pheromones: p=0.0350).

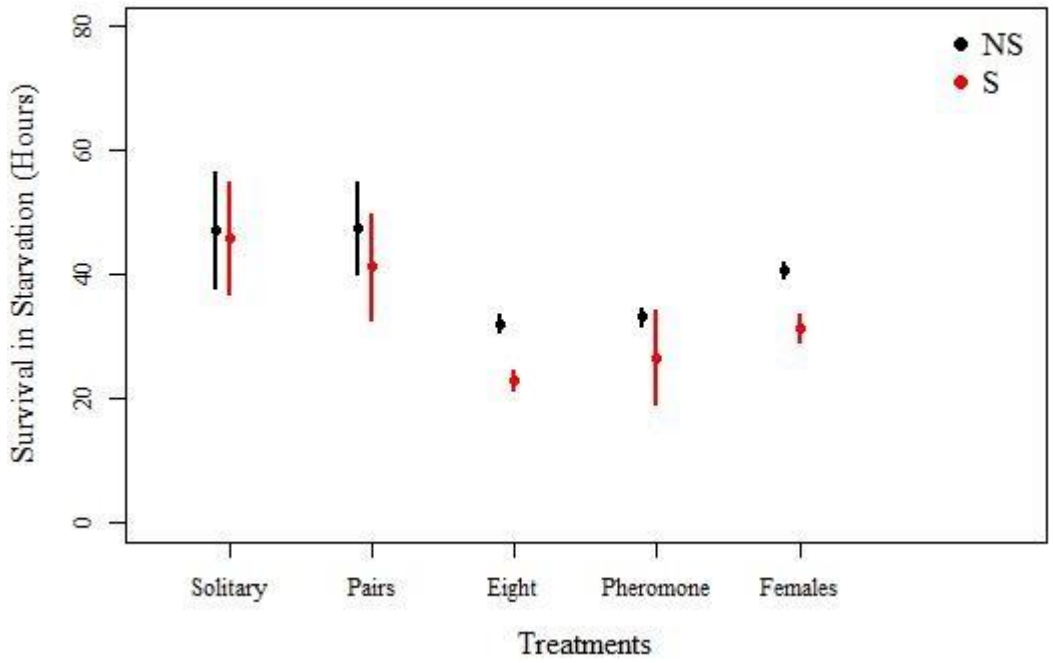


Figure 32. The effect of sperm competition and sex on *Drosophila* survival – when starvation resistance was used as a proxy.

NS=Not starved S=Starved

The bars on this plot show standard error.

Activity declines with perceived sperm competition, starvation periods and decreasing body size (when effects of parent vial, time of testing and DAM position are controlled for) (see figures 33 and 34) (sperm competition: $F_{3,\ 169}=6.075$, $p=5.97e^{-4}$; starvation: $F_{1,\ 169}=76.486$, $p=2{,}16e^{-15}$; size: $F_{1,\ 169}=4.958$, $p=0.0273$; parent vial: $F_{15,\ 169}=2.953$, $p=3.42e^{-4}$; time: $F_{14,\ 169}=3.867$, $p=1.05e^{-5}$; DAM position: $F_{31,\ 169}=1.803$). Activity was significantly higher in solitary and paired males compared to those in the presence of pheromones (solitary-pheromones: p=0.0143; pair-pheromones: p=0.00921).

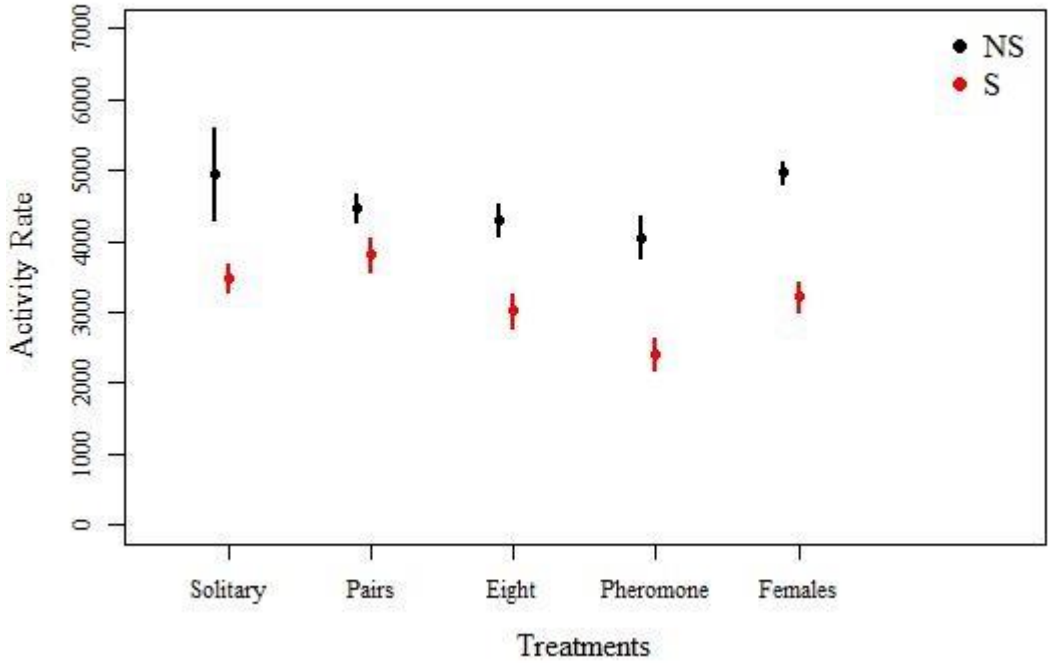


Figure 33. The effect of sperm competition and sex on *Drosophila* activity rate. Activity rate is the number of times in its life, that the fly crossed the infra-red laser found in the DAM.

NS=Not starved S=Starved

The bars on this plot show standard error.

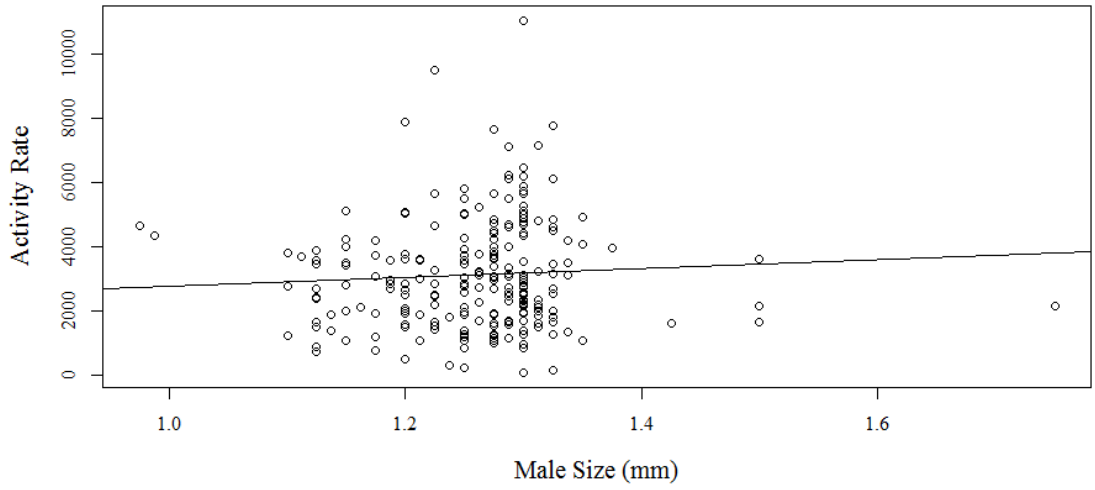


Figure 34. Larger males are more active than smaller males ($F_{1, 169}$=4.958, p=0.0273**).**

Line of best fit is added to the scatter plot using the least square method.

6. 3. 2 Starvation Reduces Female Activity and Starvation Resistance

Starvation resistance in female *Drosophila* was significantly reduced by approximately five hours, when the fly experienced a two day period of starvation when parent vial, and time and date of testing were accounted for; additionally, it was further reduced with decreasing body size (figure 35) (starvation: $F_{1,\ 50}$=17.880, p=1.00e^{-6}; body size: $F_{1,\ 50}$=9.818, p=0.00289; parent vial: $F_{5,\ 50}$=6.910, p=5.67e^{-5}; time: $F_{3,\ 50}$=7.198, p=4.15e^{-4}; date: $F_{1,\ 50}$=138.332, p=5.19e^{-16}) (see figure 22).

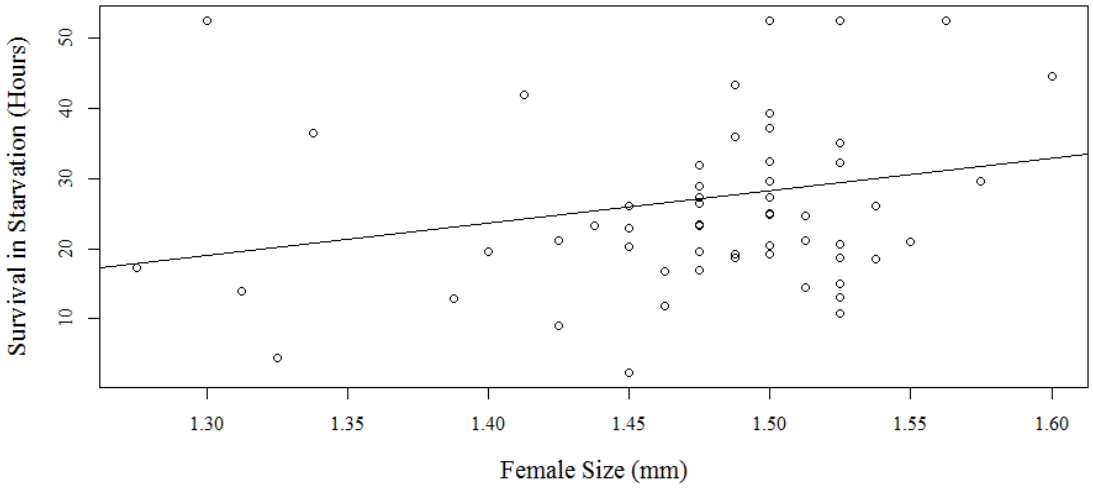


Figure 35. Larger females are able to survive for longer in starvation ($F_{1,\ 50}$=9.818, p=0.00289). Line of best fit is added to the scatter plot using the least square method.

Additionally, activity was also significantly reduced by a starvation period. And with decreasing body size, in females when date of testing was taken into account (starvation: $F_{1,\ 55}$=61.009, p=1.78e^{-10}; body size: $F_{1,\ 55}$=4.836, p=0.0321; date: $F_{4,\ 55}$=3.608, p=0.0111); see figure 36.

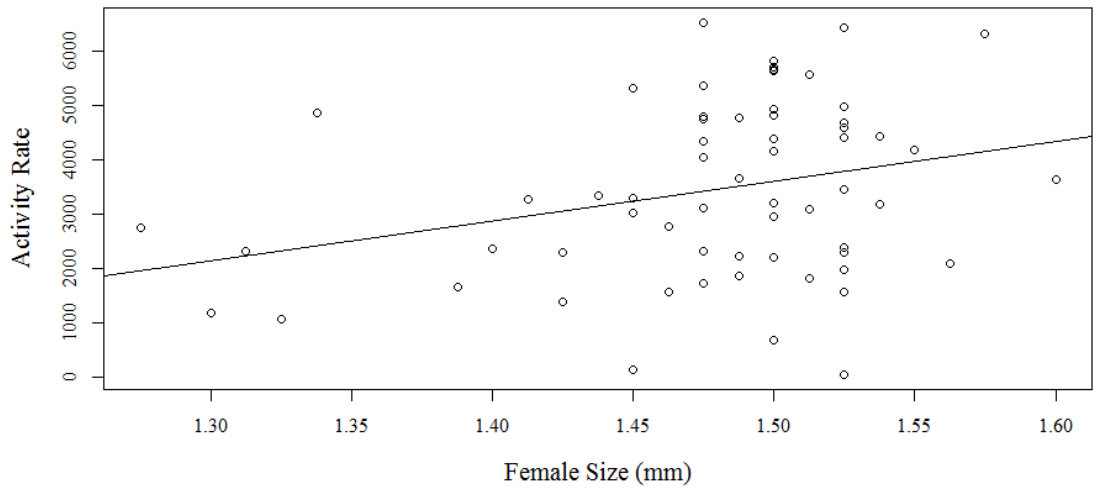


Figure 36. Larger females are more active than smaller females ($F_{1, 55}$=4.836, p=0.0321).
Line of best fit is added to the scatter plot using the least square method.

6. 3. 3 Activity and Starvation Resistance are Strongly Correlated

Pearson product-moment correlations were used to indicate the relationship between activity and starvation resistance. A strong positive correlation was found, regardless of sex, sperm competition or starvation treatment (r=0.535, d.f.=320, p=2.20e^{-16}) (see figure 37).

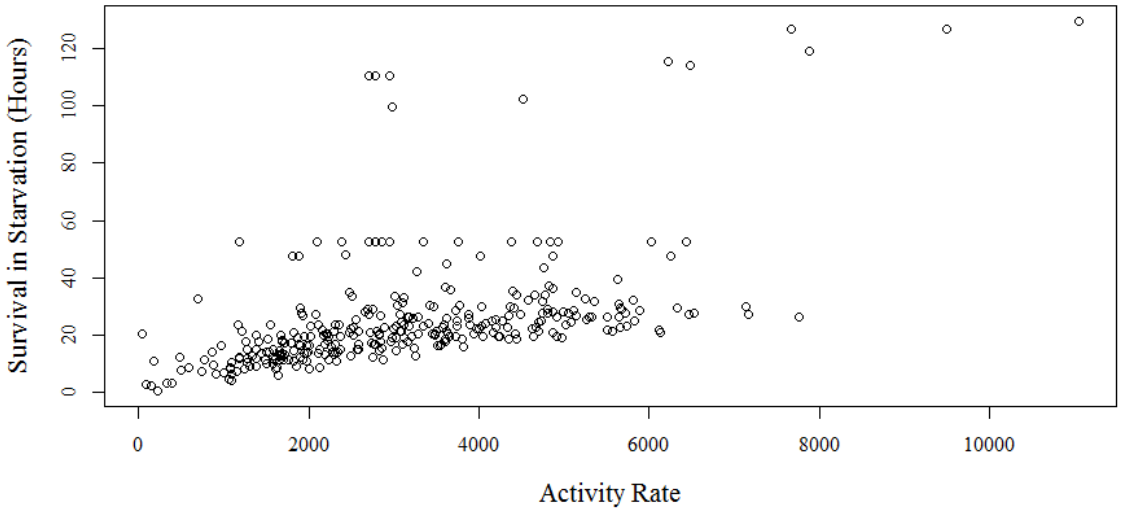


Figure 37. *Drosophila* activity and longevity is significantly correlated (r=0.535, d.f.=320, $p=2.20e^{-16}$**).** Activity rate is the number of times in its life, that the fly crossed the infra-red laser found in the DAM.

6. 4 Discussion

6. 4. 1 Activity and Survival Declined when Females were Smaller and Starved

Female activity and longevity (as measured by the proxy of starvation resistance) was reduced when the females were smaller, or were subjected to a starvation period (figures 35 and 36). When *Drosophila* are stressed, they use their fat reserves as source of energy (Marshall and Sinclair 2009). These females are less robust so it is likely that they have not been able to conserve energy for survival (Vermeulen and Loeschcke 2007). In addition to this, smaller females have less fat reserves to use for energy so their activity and lifespan declines further (Briegel 1990).

6. 4. 2 Male Survival was Shortened under High Sperm Competition

Male activity was reduced when males were smaller or starved, and male survival was also reduced by a period of starvation (figures 33 and 34). Like small females, small males also have lower levels of fat reserves. *Drosophila* use these fat reserves as a source of energy so smaller flies are unlikely to be as active. It is likely that males in this experiment were not able to conserve their energy for survival, causing a decline in their activity and starvation resistance.

When males are housed in perceived high sperm competition their starvation resistance decreases (figure 32). This was expected because in high competition, males often increase their rate of spermatogenesis in order to give their sperm a better chance to compete against other males' (Moatt et al. 2014). It is an energetically expensive process (Dewsbury 1982) so it is expected that they will not be able to conserve energy for the future. However, this result contradicts that found by Moatt et al. (2013):they found that virgin male survival increased with exposure to sperm competition. This could be because the trade-off is occurring elsewhere, so longevity is unaffected; for example, there energy could have been redirected away from other expensive tissues (Lemaître et al. 2009). Further research is necessary to determine the effect of varying sperm competition on *Drosophila* longevity.

Chapter 7: Discussion

'Fitter' organisms have a higher reproductive success and are better adapted to their environment. Natural selection drives any heritable characteristics they have to become more common within the population (Endler 1986). Sexual selection occurs when one sex has a preference for a particular characteristic – or set of characteristics (Bateman 1948; Andersson 1994). There are two mechanisms of sexual selection: the first is intra-sexual selection. This occurs when there is competition between two or more members of the same sex – often males. They fight for dominance, so they can overcome rivals and gain a mating advantage (Bateman 1948). The second mechanism is inter-sexual selection, which takes place when member of one sex have a preference for a certain characteristic of the opposite sex (Darwin 1871). Environment conditions are constantly changing, and organisms need to be able to adapt their survival strategy to cope with this. Phenotypic plasticity is an important fitness component; it is the ability of organism to respond to variable environmental condition by exhibiting different physiological and behavioural phenotypes (Agrawal 2001).

Drosophila melanogaster are a polygamous species (both sexes mate multiply) (Dobzhansky and Pavlovsky 1967) so it is essential that they are 'fit' and attractive to enable them to outcompete rivals to gain a mate and pass on their genes. Females benefit from mating with multiple males because it enables her to improve upon her mate choice. If she mates with a more attractive male, it is likely that her offspring will also be more attractive and therefore more successful – the 'sexy sons' hypothesis (Weatherhead and Robertson 1979). The good gene theory suggests that mating with a high quality mate will reap the benefit of offspring that inherit those genetic advantages (Yasui 1997). A fly needs to be robust and attractive to gain good quality mates and produce successful offspring.

7. 1 Levels of Fat Reserves

In extreme cases of nutrient deficiency, the lack of food will render flies too weak to be able to search for food; when this is true, they will breakdown the fats they have stored causing a decline in their storage reserves (Wigglesworth 1949; Aguila et al. 2007). This was seen in flies starved prior to testing. Although the results from this investigation are lacking, it has previously been shown that older flies, maintained in nutrient poor conditions, are able to store less fat (Wigglesworth 1949; Ellers 1995; Chippindale et al. 1996; Vermeulen et al. 2006). This could be for a number of reasons such as a

reproductive survival trade-off (Ellers 1995; Marshall and Sinclair 2009; Stone et al. 2011).

7. 2 Spermatogenesis and Egg Production

Spermatogenesis and egg production are both energetically expensive processes (Dewsbury 1982; Pitnick and Markow 1994; Ellers 1995; Pitnick 1996; Wang et al. 2001; Terashima and Bownes 2004). The quality and quantity of sperm that the males were able to produce declined when the males had been subjected to starvation (figure 14) (Gage and Cook 1994; Perry and Rowe 2010) – it is likely that there was also a shift in the composition of the ejaculate (Dewsbury 1982). Unexpectedly, older males had a higher rate of spermatogenesis (figure 14), as many previous investigations had found that lifetime mating success declines with age (Jones and Elgar 2004; Dhole and Pfennig 2014). This result could be due to a trade-off between current and future offspring, as younger males may be conserving energy for potential future offspring (Simmons et al. 1992). Equally, older flies may be investing in reproductive terminal investment; making one last attempt to sire offspring before their death (Clutton-Brock 1984; Creighton et al. 2009). Additionally, this effect may have been observed because of a shift in ejaculate composition: older males could be producing less accessory proteins because they have less energy to invest in spermatogenesis (Dewsbury 1982). However it could be that sperm number was not increased at all, and it was in fact the size of the sperm produced that was affected. Although sperm size has been found to play an important role in egg fertilisation (Gage and Cook 1994) so it is likely that number of offspring will be increased with either increasing sperm size or quantity.

Older and smaller females produced fewer eggs (figures 15 and 16); they are weaker and possibly cannot afford the expense (Dubey et al. 1996; Zhao et al. 2008; Zajitschek et al. 2014). Egg production also declined under starvation (figure 15) (Ellers 1995; Wang et al. 2001; Terashima and Bownes 2004) because flies were using their stored fats to be able to survive so it is unlikely that they would be able to produce eggs in addition to this.

7. 3 Activity and Survival

Courtship and copulation are energetically costly process as it uses many muscular movements (Bennett and Houck 1983; Fowler and Partridge 1989; Chapman 1992). This is likely to be the reason for lower activity levels observed in males that courted and copulated with their partner. Males that were subjected to a period of starvation had a decreased rate of activity (figure 27). These males were in a poorer condition and were less attractive, so it is likely that they exerted more energy attempting to attract a mate,

causing the decline in their activity (Cook and Cook 1975). The more attractive his mate was (younger, larger and continually fed), the more energy the male needed to expend to attempt to 'win' her (Droney 1996); therefore activity rate declined when the male was paired with a more attractive female.

More active *Drosophila* had a longer starvation resistance (figures 31 and 37), suggesting that flies only exert energy in courtship when they can afford the expense – otherwise they conserve energy for survival (Flatt and Kawecki 2007; Stone et al. 2011). However starvation resistance is decreased when the flies were observed to be engaging in courtship and copulation. Courtship and copulation are energetically expensive processes; it requires a large amount of muscle movement and females are often harmed by the male during copulation (Markow 1987; Fowler and Partridge 1989; Wigby and Chapman 2005; Kuijper et al. 2006). It places additional stress on the flies and this shortens their lifespan and activity.

Older males that were subjected to a period of starvation were less robust and often died sooner – they were weaker and had lower energy reserves (figure 26). However if the starvation period occurred early in life, the male recovered and had an increased starvation resistance. A similar pattern of recovery was found by Barker and Podger (1970) in the production of ovarioles in females. When the males' partner was more attractive (younger and had not been exposed to a period of starvation), survival was further decreased – this is probably due to increased courtship efforts. It is worthwhile for males to exert more energy attempting to court and copulate with more attractive mates, as it is likely that they will then sire more attractive, and therefore more successful, offspring (Weatherhead and Robertson 1979).

Females also experience a decline in longevity when the individual has been subjected to a period of starvation and with decreasing size (figures 28 and 29). Smaller insects have less fat storage reserves, and when they experience starvation it is necessary that these are used to ensure their survival (Briegel 1990). These females are of a poorer quality and have been unable to conserve energy for their survival, and die sooner.

Perception of high sperm competition has been shown to heighten the effort and energy that a male is willing to invest in his potential mate (Mougeov et al. 2001), although a large amount of that energy will be spent on spermatogenesis and production of accessory proteins (Dewsbury 1982; Wigby and Chapman 2005). Gromko and Pyle (1978) showed that reproductive activity varies independently from all other activity, so it is probable that

males complete a trade-off between reproductive activity and all other activity. Smaller individuals have less fat in their storage reserves, so they have less energy reserved and are expected to be less active (Briegel 1990).

Starvation resistance decreased in virgin males when they perceived themselves to be in high sperm competition environments (figure 32). Males in high sperm competition increase their spermatogenesis and production of accessory proteins (García-González and Simmons 2005; Simmons et al. 2007; Bretman et al. 2010; Moatt et al. 2014). These are energetically costly process so it is likely that this causes a decline in longevity, due to a reproductive-survival trade-off.

7. 4 Courtship Efforts

Courtship efforts remain invariable throughout the duration of courtship; it is likely that the expense of courtship is so high that once a male has committed to a female he does not want his efforts to be wasted. Additionally, the male appears to place a similar amount of effort into each type of courtship behaviour, as the number of behaviours observed were similar (Bastock and Manning 1955): there are no trade-offs occurring between the type of courtship behaviours completed when the fly is placed under stress.

Older, malnourished males were less active, so it follows that those males also had less energy for courtship. This was the conclusion drawn from observations of courtship behaviours. Weaker males appear to not be able to afford the expense of courtship behaviours, and possibly conserve their energy for copulation to increase their chance of siring offspring.

It is worthwhile exerting energy to attract good quality mates as they will provide more attractive offspring (Weatherhead and Robertson 1979) which will be more successful at securing more potential mates and providing the fly with grand-offspring. However, it is not worth wasting energy mating with an unattractive or weak fly; they will produce unattractive offspring that may struggle to find a mate and produce grand-offspring. Equally, these ‘fitter’ females will be able to lay more eggs; if the male chooses to mate with her then he will sire more successful offspring, making the cost of courtship and copulation more worthwhile.

When males were maintained in high sperm competition, latency to court was longer (figure 22). This could be that males switched tactics to favour forced matings, to avoid the energetic cost of courtship (Spieth 1974; Bennett and Houck 1983; Markow and Wade 2000). It is possible that they cannot afford the cost of courtship because they have

previously invested in the expensive process of spermatogenesis (Dewsbury 1982). It is also possible that the males perceive these females to be less valuable as it is likely that they have already mated if they were also maintained in high male presence; however this is unlikely because virgin female pheromones have a different composition to those of mated females (Tompkins and Hall 1981).

7. 5 Rejection Responses

Females usually attempt to reject the male's advances using a series of rejection responses (Partridge et al. 1987b; Spieth 1952; Spieth 1974), which often result in the termination of courtship (Gromko and Markow 1993). Females performed fewer rejection attempts when males were more attractive (younger and fed continually throughout their life); figure 9. This could be because they were more willing to mate with attractive males to increase their chances of having more attractive and successful offspring (Weatherhead and Robertson 1979). It could also be because younger and fed males sire more successful offspring as they produce more viable sperm (as described in Chapters 1 and 2) (Droney 1998; Hosken et al. 2003). Females will be more willing bear the cost of copulating when the male will provide her with more sperm – and more successful offspring. Alternatively, it is possible that the increased resources and energy available to these males, enabled them to subvert female rejections and force them into copulation (Markow and Wade 2000).

This is also the case where females are less attractive. Females perform fewer rejection responses when they are younger and have been starved (figure 10); they are less attractive so it is likely that they cannot afford to be as choosy. Additionally, females that have not been starved are more robust so should be able to exert more energy on performing more rejection responses (Djawdan et al. 1998).

7. 6 Copulation Latency and Duration

The receptiveness of the female determines the duration of courtship (Bastock and Manning 1955). When females are more attractive, or males are less attractive, copulation latency increases; the more attractive a female is, the choosier she is able to be (Reynolds and Gross 1990).

The *Drosophila* pair are more likely to copulate when the females has not been starved (table 7). These females are stronger so are more likely to be able to withstand any damage caused by mating (Linder and Rice 2005). They are also more attractive, and will likely be able to lay more eggs, so males will be keener to mate. Additionally, the male is more

willing to engage in courtship when he is younger (figure 4). Younger males are in a better condition so are more attractive and better able to cope with starvation stress (Sørensen et al. 2005). They have larger energy reserves so it is expected that they will be able to spend more energy on courtship (Briegel 1990). When the males are maintained in solitude and pairs, it was predicted that they would be less willing to court (as all reproductive effort is likely to be low) because they perceive themselves to have little or no competition (Simmons et al. 2007; Bretman et al. 2009; Moatt et al. 2014). However, this was not the case figure 22. It is possible that males exposed to high sperm competition traded-off copulation, and invested more energy in the production of ejaculates: good sperm quality and accessory proteins can provide male sperm with a better chance of outcompeting other sperm (Hosken et al. 2003; Moatt et al. 2014).

Courtship latency was longer in older males and those that had experienced potentially high sperm competition environments (figures 4 and 22). Courtship is an energetically expensive process that weaker males do not have enough energy to engage in (Bennett and Houck 1983; Cordts and Partridge 1996). Instead they conserve their energy for survival (Marshall and Sinclair 2009). It is plausible that when the males were maintained in groups of eight, it weakened their condition. It is unlikely that this is due to resource competition, as they were fed *ad libitum*, but it could be due to direct physical interactions with other males. Males invest more in sperm production in the presence of sperm competition (Moatt et al. 2014), so this could further weaken their condition.

Males invest more energy into spermatogenesis and the production of accessory proteins when they have been exposed to more male competitors (García-González and Simmons 2005; Moatt et al. 2014). The result of this is that they have not conserved energy for survival, and they are weaker (Djawdan et al. 1998). It is likely that they initiate courtship quicker, because cannot afford to be so choosy about their potential mates. Males are more willing to court and mate with more attractive females as it will increase the attractiveness, and success, of their offspring (Weatherhead and Robertson 1979).

It was expected that copulation latency would be shorter when females are more attractive and males less attractive. In both situations, it is likely that females are able to be choosier. This was true of females (figure 6): latency to copulate was longer when the female had experienced a period of starvation. Female behaviour often determines whether the pair copulate (Bastock and Manning 1955; Connolly and Cook 1973). Weaker females are less likely to be able to afford the energy to reject males' courtship behaviours (Tucker 1975; Djawdan et al. 1998). They are also less attractive so it is likely that they will be more

willing to mate with any male that courts them. Better quality males should in theory have shorter courtship durations (or mate quicker) because they are more attractive and females would therefore be more willing to accept attempts to mate, and the male is more capable of overpowering the female (Dunn et al. 2002) – however no effect of this was found. This could be because some males are exerting more energetically demanding courtship behaviours; resulting in them being more attractive to the female and convincing her that they are 'fitter'. Additionally, it could be because the 'better' males are being choosier, and are therefore choosing not to mate with their female partner (Gowaty et al. 2003). On the other hand, it could be that the effect of body size was dominating the results found: courtship duration was found to be longer when both males and females were smaller. Smaller males are less attractive, so their mate will require more convincing to copulate. It is possible that less attractive males court with more vigour so the female perceives them to be more attractive. If this is the case, courtship cannot be an honest signal, as these males are 'lying' (Johnstone 1995). This was an unexpected result for females: smaller females are less attractive so it is expected that they are not able to be as choosy. They also have less energy to initiate movements required for rejection (Dunn et al. 2002). It is possible that they are not courted as vigorously as more attractive females, so they are less sexually stimulated (Ewing 1983). Finally, it could be that females assessed male attractiveness on a trait that was not measured in this experiment. For example, wing area was not measured but Ewing (1964) showed it influenced courtship behaviour. Additionally, the seemingly transparent *Drosophila* wings have been shown to display vivid 'wing interference patterns'. Katayama et al. (2014) discovered that these patterns (based on saturation and hue) effect female mate choice.

It was expected that weak males would invest more in reproductive terminal investment (Ellers 1995; Flatt and Kawecki 2007; Marshall and Sinclair 2009): instead of conserving energy for survival when they perceive themselves to be close to death, they exert all of their remaining energy to ensure they produce offspring. This effect was observed in older males. A better transfer of sperm is achieved with longer copulation durations, which will increase the male's chance of successfully fertilising the female's eggs (Edvardsson and Canal 2006; Bretman et al. 2009). Copulation is energetically costly (Fowler and Partridge 1989; Wigby and Chapman 2005; Kuijper et al. 2006), but is worth investing more energy on a longer copulation duration when the female is more attractive. The effect of this was observed, as copulation duration was shorter in females that had been subjected to starvation. Increased copulation durations are also used as a method of mate-guarding (which also occurs more frequently when their mates are more attractive) (Cook and Cook

1975; Mazzi et al. 2009; Lüpold et al. 2011). More attractive females tend to provide the male with more attractive, and therefore more successful offspring (Weatherhead and Robertson 1979). It is possible that reproductive terminal investment was not observed because the flies were placed under too much stress; they may not have attempted to copulate if they perceived themselves to be so weak that they would die during copulation – or in females, before the oviposition of eggs. It is unlikely that the reason that this effect was not observed was due to too little stress, as previous experiments showed that a two-day starvation period was the mean average duration that a fly could survive without food. Additionally, it was predicted that males housed in potentially high sperm competition would invest more in spermatogenesis and longer copulation durations – although no effect on the duration of mating was observed. This could be because they exerted so much energy into spermatogenesis that they had too little left for a lengthened copulation.

7. 7 Fecundity Costs

Producing offspring comes at a cost, as spermatogenesis and egg production are energetically expensive processes (Dewsbury 1982; Ellers 1995; Galvani and Johnstone 1998; Snoke and Promislow 2003; Terashima and Bownes 2004). Male treatment appeared to have no effect on the number of eggs that the female laid, but it did influence the number of offspring that hatched (figure 11). When males were younger, more successful offspring hatched. This could be because younger males are in a better condition; they have more energy so do not need to make survival-reproduction trade-offs and they also produce more viable sperm cells, further increasing their chances of siring offspring (Marshall and Sinclair 2009; Stone et al. 2011).

Younger and fed females produced significantly more eggs, and significantly more offspring, than their older and starved counterparts (figures 12 and 13). Younger males also produce more successful offspring. When the flies are starved, they have to use their energy stores to search for food, and there is not enough for them to do this and produce energetically costly eggs or sperm. Males have been found to transfer more sperm during copulation when the females are more attractive (Stone et al. 2011), which will further increase the number of successful offspring found (Lüpold et al. 2011). Both females and males often have to make a reproductive survival trade-off in order to maximise their lifetime reproductive success.

7. 8 Applications for Findings

All organisms aim to increase their lifetime reproductive success – increasing their longevity will improve their chances of doing so (Rhine et al. 2000). Individuals with a higher activity rate will also have a better chance of surviving; they will be able to hunt or court for example, much faster (Tucker 1975; Bennett and Houck 1983; Strohm and Marliani 2002). Age, size and condition will impact how active an individual will be, or for how long it can survive (Miller and Thomas 1958; Trout and Kaplan 1970; Partridge and Farquhar 1983; Zajitschek et al. 2014; Travers et al. 2015). Additionally, sperm competition could also affect an organism's activity and longevity (Dewsbury 1982; Moatt et al. 2013), but more research is required to fully understand these relationships.

Variable environmental conditions have been found to affect behavioural plasticity in a range of organisms, including *Drosophila melanogaster* (Komers 1997). Many investigations have addressed how male characteristics alter copulatory behaviours (Partridge et al. 1987a; Partridge et al. 1987b; Jones and Elgar 2004; Fricke et al. 2008); yet despite several studies indicating the importance of female characteristics (Lefranc and Bundgaard 2000), comparatively less research has been completed into female characteristics and responses. The ever-changing environment also impacts *Drosophila* physiology (Miller and Thomas 1958; Gage and Cook 1994; Galvani and Johnstone 1998; Terashima and Bownes 2004; Markow et al. 2009; Perry and Rowe 2010); physiology could trade-off behavioural responses. Trade-offs are also observed between reproduction and longevity (Marshall and Sinclair 2009; Stone et al. 2011).

Sperm competition alters the relationship between reproduction and survival; males often invest more in spermatogenesis to increase their chances of siring offspring (Ellers 1995; Marshall and Sinclair 2009; Stone et al. 2011). Although sperm competition theory is relatively recent, a wide range of studies have been completed to attempt to understand the effect it has on ejaculate composition. However, there has been comparatively less research into how it is affected by variable environmental conditions, or the effect this has on males' copulatory behaviours.

This research has addressed some of these knowledge gaps, providing useful information on how *Drosophila* might react to a variable environment – such as loss of food resources as caused by a changing global climate (McMichael et al. 2007). This, combined with an improved knowledge of copulation and reproduction, could help determine optimum population size, and birth and death rates. If males exert more energy into reproduction when more males are present, this suggests that a few large populations could be more

successful than many smaller ones. It could enable better conservation and preservation plans to be determined by other methods too, if this research (or similar) is repeated in a wider range of species. Understanding how organisms react to stress will help organisations to protect animals from variable environments. In addition to this, it could provide useful information on providing help for human civilisations. Knowing how food distributions will help protect populations could influence the distribution of human aid in natural disasters: for example what type of food, and where and when it should be distributed during a crisis. Furthermore, knowledge of activity rates could even be expanded to help medical research in mobility-affecting conditions such as narcolepsy or neurologic disease (Siegel et al. 1991; Pearson et al. 2004).

7. 9 Future Investigations

This research has provided additional details on behavioural and physiological plastic responses of *Drosophila melanogaster*, but there is still a great deal more to be found. All male courtship behaviours, and female rejection responses, vary according to the behaviour of their mating partners – for example, if males court a female more often, it is likely that she will initiate rejection responses more often. To avoid the effects of this in future investigations, the mating partners could be decapitated (so they initiate fewer courtship or repulsion behaviours) (Spieth 1966; Cook and Cook 1975; Moon et al. 2009). However, decapitated flies are less attractive and so it is likely that males will court decapitated females less – or females will reject decapitated males more (Spieth 1966; Cook and Cook 1975).

Courtship effort is often measured by the number of wing extensions performed (Bennet-Clark and Ewing 1968; Bennet-Clark and Ewing 1970). The work completed here showed that the time the individuals spent courting (or rejecting) also has a significant effect on the responses of their mate. It is also possible that the distance travelled during chasing and fleeing events would significantly affect courtship durations (or latency to mate), so this should be investigated.

The effects of perceived sperm competition were investigated by placing several virgin males in one vial, as has been done in previous experiments (Moatt et al. 2013). But it would also be interesting to determine whether that effect differs when the male competitors have previously mated. This research suggests that exposure to pheromones alone induces a similar response in males – they perceive themselves to be in the presence of potential sperm competition. More experiments in the future could be completed using pheromones. This would be beneficial because the treatment fly would be more easily

identified. Bretman et al. (2010) suggest that length of time exposed to sperm competition is a more important factor than number of competitors. Pheromones do not lend themselves to investigations on exposure time as the presence of cuticular hydrocarbons declines over time.

There is also a lack of research into the effect of female presence on male copulatory behaviours. These experiments could be completed by separating the males from the females or mated males (to ensure the test flies all remain virgins) using thin plastic dividers with holes added to allow visual, auditory and olfactory contact.

Additionally, Hercus and Hoffmann (2000) showed there were effects of maternal and grandmaternal age on offspring fitness, but little else is known about epigenetic effects. A potential future experiment could be to place *Drosophila* through similar treatments as completed in this project and allow them to mate and reproduce. The offspring could then be tested in a similar way as described in this thesis.

Finally, all of these experiments could be completed again with different species to determine whether the effects are unique to *Drosophila*. Although, experimental methods would have to be altered for larger animals to ensure the experiment is ethical and does not cause any permanent harm to any animals.

Appendices

Appendix 1: Recipes

1. i. Nutritious Agar-Based Medium

Ingredients for 100ml of agar:

- 5g live Brewer's yeast
- 5g sucrose
- 1.2g technical agar
- 100ml distilled water
- 4ml nipagin solution
    - 0.4g nipagin
    - 4ml 95% ethanol

Method:

1. Combine the live Brewer's yeast, sucrose, technical agar and distilled water in a conical flask and autoclave
2. Once cooled to 40-50°C add the nipagin solution and ensure it has dissolved
3. Pipette 2ml into each bijoux tube

1. ii. Starvation Agar-Based Medium

Ingredients for 100ml of agar:

- 3g technical agar
- 100ml distilled water
- 4ml nipagin solution
    - 0.4g nipagin
    - 4ml 95% ethanol

Method:

1. Combine the technical agar and distilled water in a conical flask and autoclave
2. Once cooled to 40-50°C, add the nipagin solution and ensure it has dissolved
3. Pipette 2ml into each bijoux tube

1. iii. Egg Counting Agar-Based Medium

Ingredients for 100ml of agar:

- 5g live Brewer's yeast
- 5g sucrose
- 1.2g technical agar
- 3ml red food colouring
- 100ml distilled water
- 4ml nipagin solution
    - 0.4g nipagin
    - 4ml 95% ethanol

Method:

1. Combine the live Brewer's yeast, sucrose, technical agar and distilled water in a conical flask and autoclave
2. Once cooled to 40-50°C add the nipagin solution and red food colouring and ensure it has dissolved
3. Pour 50ml into each clear plastic box – so it can later be 'stamped out' using empty vials

Appendix 2: Copulatory Behaviours

Further information on how behaviours were defined and measured is provided here.

2. i. Male Courtship Behaviours

Wing extensions: A wing extension was counted when one of the wings was extended past a 45° angle, and was recorded as having stopped once it was lowered below this marker.

Abdomen taps: An abdomen tap was recorded when the male's front legs were observed to be touching the female's abdomen. The taps were considered to be too fast to count each one individually, so each series of tapping was counted as one – a series lasted until there was a pause of over two seconds.

Genital licks: When a male's head was observed to be touching the female's genitals, one genital lick was recorded.

Mounting events: A male will climb onto the female, placing his front legs on her back, when this was observed it was counted as one mount. If the male fell, and climbed back, a second mounting event was recorded.

Chasing: If the male is following in the same direction as the female in front, and is within 1cm from her, this was recorded as chasing. The chasing event was only defined to have ended once the movement stopped or the distance increased to greater than 1cm.

2. ii. Female Rejection Responses

Kicks: A series of backwards leg movements out towards the male was named kicking. As these kicks are fast, a series of kicks was counted as one – with a series lasting until there was a pause of more than two seconds.

Flutter of wings: A flutter was recorded when the female moves her wings, and the result is that the male is forced away.

Fleeing: The female will constantly move away from the male; fleeing is recorded when there is a movement away from the male, and the female is no further away from the male than 1cm.

2. iii. Copulation Duration

Courtship latency: Courtship latency starts when the mating pair first meet and the arena is secured, until the male initiates his first wing extension.

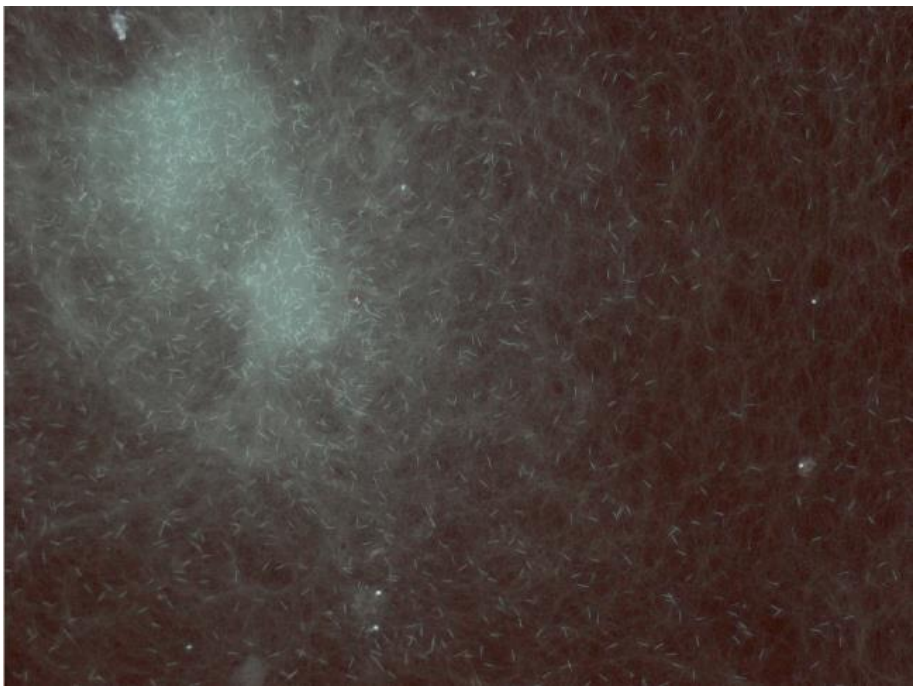
Courtship duration: The period of courtship duration is also the latency to mate. Courtship begins when the male initiates his first wing extension, and ends when the male mounts the female and their genitals touch. When observing these behaviours through video footage, it is possible to be more precise; copulation is recorded as having begun when female reluctance decreases (when kicks and other movements cease).

Copulation duration: Copulation begins when courtship ends – when the male mounts the female and their genitals touch. It ends when the male and the female have separated. If observations are being noted from video footage, the footage is rewound and mating is counted to have ended at the beginning of the movement before actual separation occurred.

Appendix 3: Spermatogenesis

3. i. Image Scores

Each photograph was divided into eighty smaller squares measuring approximately 17mm x 17mm. The squares were then assigned a number from zero to six according to how much sperm was observed in the square: zero if none were present, one if ≤10, two for 11-50, three for 51-100, four for 101-500, five for 501-1000 and six if >1000; shown in figure 38.



2	3	3	2	2	2	2	1	1	2
3	3	3	2	2	2	2	1	1	1
2	3	3	3	2	2	2	1	1	1
2	2	3	3	2	2	2	1	1	1
2	2	2	3	2	2	2	1	1	1
2	2	2	2	2	2	2	1	1	1
2	2	2	2	2	2	2	1	1	1
2	1	1	2	2	2	2	2	2	1

TOTAL SCORE = 148

Figure 38. An example of a sperm photo and the corresponding score.

Appendix 4: Transform DAM Data to Output Files

4. i. R Script Created by Dr. Michael D. F. Thom to Transform R Data to an Output File

```
setwd("E:/") #insert appropriate working directory

nfiles<- 5 #number of DAM files needed to be analysed

last<-function(s,column){
 flag<-0
 final<-NA
 prev<-NA
 startrow<-(s-min(dat[,1]))+1
 for(i in startrow:nrow(dat)){
  if(dat[i,column]==0 & flag==0){
   flag<-1
   prev<-final
   final<-dat[i,"index"]}
  else if(dat[i,column]!=0) flag<-0
 }
 final<-final-1
 prev<-prev-1
 if(!is.na(prev) &! is.na(final)){
  if(final-prev > 60) final<-prev #if gap >60 (30 mins) use previous as time of death
 }


 return(final)
}


for(set in 1:nfiles){
 dat<-read.table(paste("Monitor",set,".txt",sep=""),sep="\t")

 dat<-dat[,-c(4:10)]
 y<-c("index","date","time",paste("hole",(1:32),sep=""))
```

```
 colnames(dat)<-y

 layout<-read.csv("layout.csv",h=T) #read layout file
 layout<-layout[layout$set==set,]
 layout$act<-0
 layout$lastindex<-0
 temp<-0

 for(j in 1:length(layout[,1])){
  if(layout[j,"index"]>0){
   temp<-last(layout[j,"index"],j+3)
   layout$lastindex[j]<-temp
   layout$act[j]<-sum(dat[dat$index>=layout[j,"index"] & dat$index<=temp,j+3])
  }
 }

 layout$lifespanh<-(layout$lastindex-layout$index)/240
 layout$relact<-layout$act/layout$lifespanh

 write.table(layout,paste("all_longevity_activity",set,".csv",sep=""),sep=",",row.names=F)
 #save data as a separate output file for each input file

}


#To produce figures


d2<-read.csv("fall_longevity_activity1.csv",h=T)

d2$age<-as.factor(d2$age)

se<-function(x) sd(x)/sqrt(length(x)) #calculate SEs

means<-with(d2,tapply(lifespanh,list(starv,age),mean)) #tabulate means
```

```
ses<-with(d2,tapply(lifespanh,list(starv,age),se)) #tabulate SEs

x1<-(1:3)-0.1 #3 sets of x-axes for the 3 treatment groups
x2<-1:3
x3<-(1:3)+0.1

jpeg("longevity1.jpg",height=400,width=550)

plot(means[1,]~x1, #first plot
    xaxt="n",
    xlab="",ylab="",
    xlim=c(0.5,4.5),
    ylim=c(0,80),cex.axis=1,font.axis=6,
    pch=19,
    cex=2,
    col=1)

points(means[2,]~x2, #second plot
      pch=19,
      cex=2,
      col=2)

points(means[3,]~x3, #third plot
      pch=19,
      cex=2,
      col=3)

#error bars

segments(x1,means[1,]+ses[1,],
        x1,means[1,]-ses[1,],
        lwd=2,
        col=1)

segments(x2,means[2,]+ses[2,],
```

```
        x2,means[2,]-ses[2,],
        lwd=2,
        col=2)

segments(x3,means[3,]+ses[3,],
        x3,means[3,]-ses[3,],
        lwd=2,
        col=3)


axis(1,at=1:3,c("3","14","28"),cex.axis=1,font.axis=6)
title(xlab="Age (Days)",cex.lab=1.3,font.lab=6)
title(ylab="Longevity in Starvation Hours)", cex.lab=1.3,font.lab=6)

legend("topright",pch=19,col=c(1,2,3),c("NS","S3","SE"),cex=1.3,bty="n")

dev.off()
```

4. ii. Example of a “layout” file

	A	B	C	D	E	F	G	H	I	J	K
1	hole	set	date	index	sex	starvation	age	act	lastindex	lifespanh	relact
2	1	1		-1							
3	2	1	2111a	676	m	0	3				
4	3	1		-1							
5	4	1	2211a	2506	f	0	3				
6	5	1		-1							
7	6	1	2111a	676	m	3	3				
8	7	1		-1							
9	8	1	2311a	5446	f	3	3				
10	9	1	2211a	2506	f	0	3				
11	10	1	2311b	5909	f	3	3				
12	11	1	2311a	5446	m	3	3				
13	12	1		-1							
14	13	1	2311a	5446	m	0	3				
15	14	1		-1							
16	15	1	2111a	676	m	0	3				
17	16	1		-1							
18	17	1		-1							
19	18	1	2211a	2506	f	3	3				
20	19	1		-1							

Figure 39. A screenshot of the layout file in Microsoft Excel.

Appendix 5. Effect of Male and Female Age and Starvation on Male Courtship Behaviours

5. i. Male Treatment Affected the Number of Courtship Behaviours Initiated

The number of wing extensions completed by the male was significantly affected by the male's age but not starvation period (age: $F_{2, 92}$=16.997, p=5.225e^{-7}; starvation: $F_{2, 92}$=1.701, p-0.188). Post-hoc TukeyHSD tests showed that increasing age from three (p=0.00109) and 14 (p=7.00e^{-7}) to 28 days old reduced wing extensions; see figure 40.

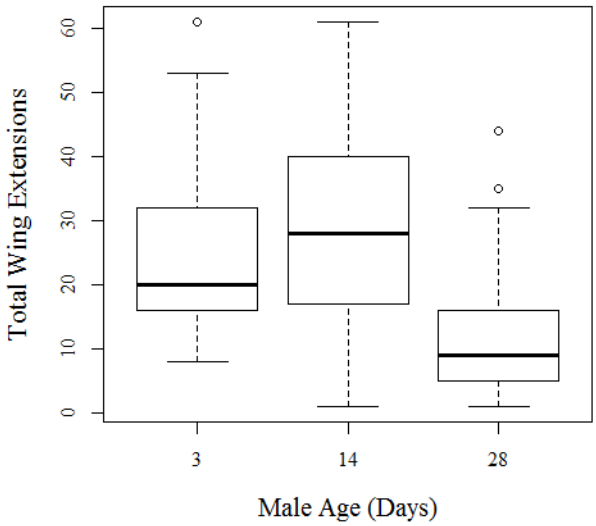


Figure 40. Number of wing extensions decreased in 28 day olds compared to three and 14 day olds ($F_{2, 92}$=16.997, p=5.225e^{-}7).

Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

Chasing events decreased with increasing male age, but starvation had no effect (age: $F_{2, 89}$=9.381, p=2.01e^{-4}; starvation: $F_{2, 89}$=0.666, p=0.516). Twenty eight day olds chased significantly less often than three day olds (TukeyHSD: p=0.00588) and 14 day olds (TukeyHSD: p=5.05e^{-4}). The time the males spent chasing also decreased with male age but not starvation periods (age: $F_{2, 88}$=11.961, p=2.54e^{-5}; starvation: $F_{2, 88}$=1.790, p=0.173). TukeyHSD tests showed that 28 day olds spent less time chasing females than 14 day olds (p=1.400e^{-5}) – as seen in figure 40.

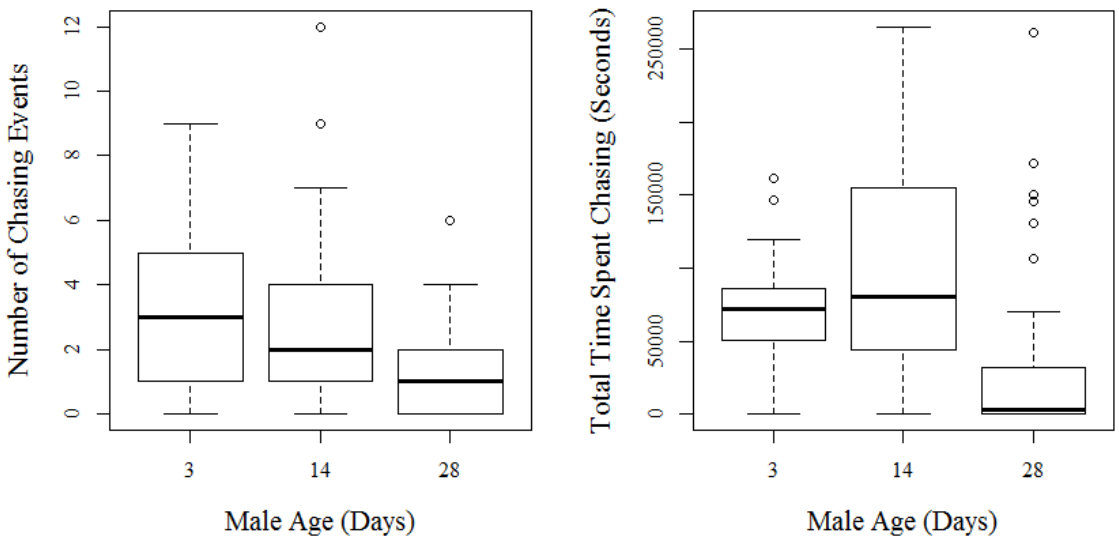


Figure 41. The effect of male treatment on the number of chasing events ($F_{2, 89}$=9.381, p=2.01e^{-4}) and the total time the male spent chasing ($F_{2, 88}$=11.961, p=2.54e^{-5}).
Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

The number of times the male attempted to mount the females was significantly lower in older males (age: $F_{2, 89}$=3.662, p=0.0296; starvation: $F_{2, 89}$=1.276, p=0.284); see figure 42. Post-hoc TukeyHSD tests found that 28 day old males mounted significantly less often than 14 day olds (p=0.0247).

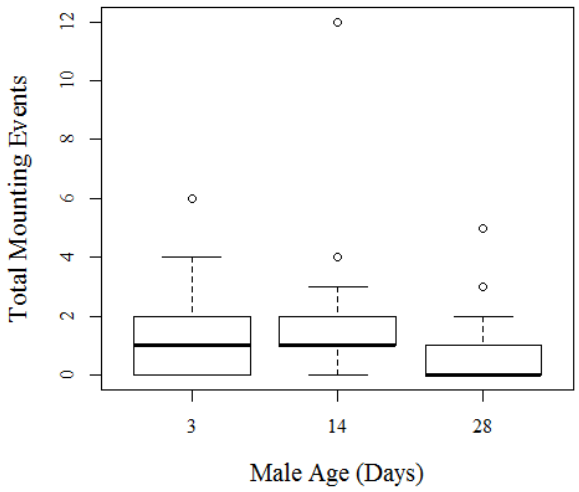


Figure 42. The effect of male age on the number of mounting events ($F_{2, 89}$=3.662, p=0.0296). Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

The number of genital licks initiated by the male declined with increasing age, but no effect of starvation was found (age: $F_{2, 89}$=4.439, p=0.0145; starvation: $F_{2, 89}$=1.586, p=0.210); figure 43. Post-hoc TukeyHSD tests identified that a 28 day old male initiates less genital licks that a three day old (p=0.0844) and 14 day old (p=0.0217).

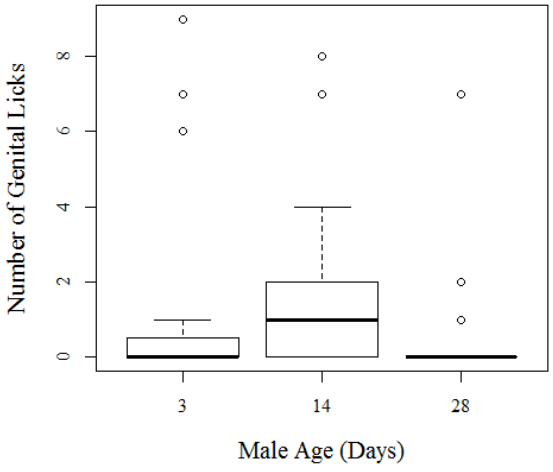


Figure 43. The effect of male age on the number of genital licks completed ($F_{2, 89}$=4.439, p=0.0145). Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

Male starvation and age had no significant effect on the number abdominal taps he completed (age: $F_{2, 89}$=1.743, p=0.181; starvation: $F_{2, 89}$=0.113, p=0.893).

5. ii. Female Condition (by Treatment) Affects the Males' Courtship Behaviours

Female age and the starvation period she was exposed to had no significant effect on number of wing extension the male completed (age: $F_{2, 82}$=0.684, p=0.507; starvation: $F_{2, 82}$=1.677, p=0.193).

The number of times the male initiated a chasing event was significantly reduced with female age but not starvation (age: $F_{2, 82}$=6.355, p=0.00272; starvation: $F_{2, 82}$=0.165, p=0.848); figure 44. TukeyHSD tests also revealed that chasing events declined when 28 day old females were present compared to three (p=0.0111) and 14 day olds (0.00906). However there was no effect of female age or starvation on the amount of time the male spent chasing her (age: $F_{2, 82}$=0.463, p=0.631; starvation: $F_{2, 82}$=1.950, p=0.576).

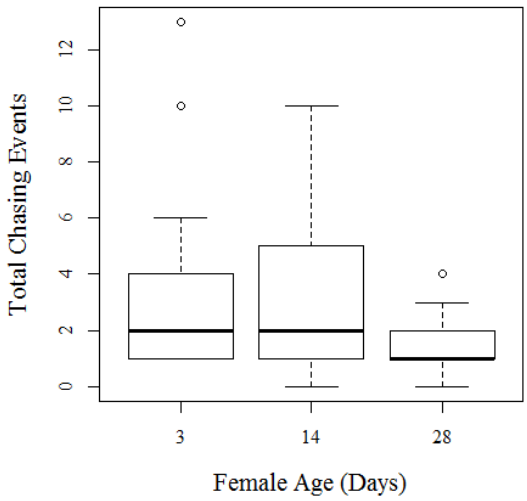


Figure 44. The effect of female treatment on the number of chasing events.
Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

Female starvation had no significant effect on the number of mounts attempted by the male, but they increased with increasing age (age: $F_{2, 82}$=4.806, p=0.0106; starvation: $F_{2, 82}$=2.319, p=0.105). TukeyHSD tests showed that the presence of 28 day old females

caused an increase in the number of attempted mounts compared to 14 day olds (p=0.00785); figure 45.

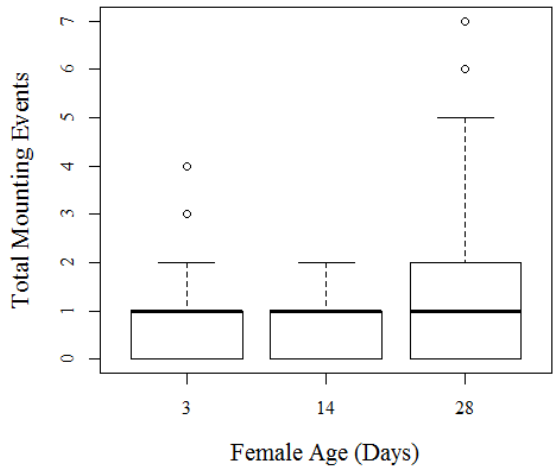


Figure 45. The effect of female age on the number of mounting events ($F_{2, 82}$=4.806, p=0.0106). Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

Female treatment had no significant effect on the number of genital licks performed by males (age: $F_{2, 82}$=0.629, p=0.535; starvation: $F_{2, 82}$=0.434, p=0.649).

The number of abdomen taps completed by the male differed significantly with varying female age (but not starvation) (age: $F_{2, 89}$=4.343, p=0.0161; starvation: $F_{2, 82}$=0.782, p=0.461) – shown in figure 46. Males completed significantly more abdomen taps in the presence of 28 day old females compared to three (p=0.0389) and 14 day olds (p=0.0414).

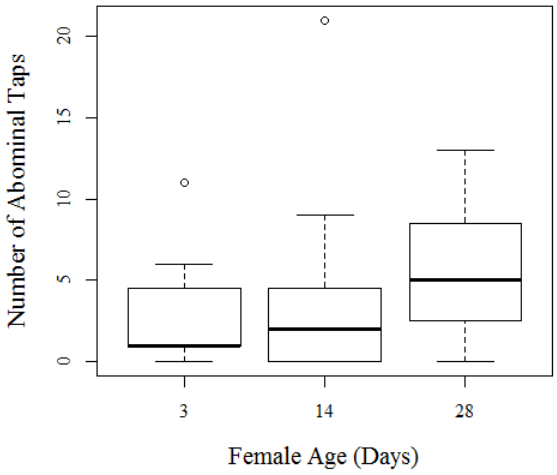


Figure 46. Number of abdominal taps increase with increasing female age ($F_{2, 89}$=4.343, p=0.0161). Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

Appendix 6: Effect of Male and Female Age and Starvation on Female Rejection Responses

6. i. The Number of Rejection Responses Completed was Affected by Male Condition

The starvation period that the male was exposed to had a significant effect on the number of fleeing events completed by the female, but male age did not (age: $F_{2, 91}$=1.915, p=0.153; starvation: $F_{2, 91}$=3.699, p=0.0285); see figure 47. Post-hoc TukeyHSD tests highlighted that the female fled less when the male was starved early in life compared to when he was fed *ad libitum* (p=0.0281).

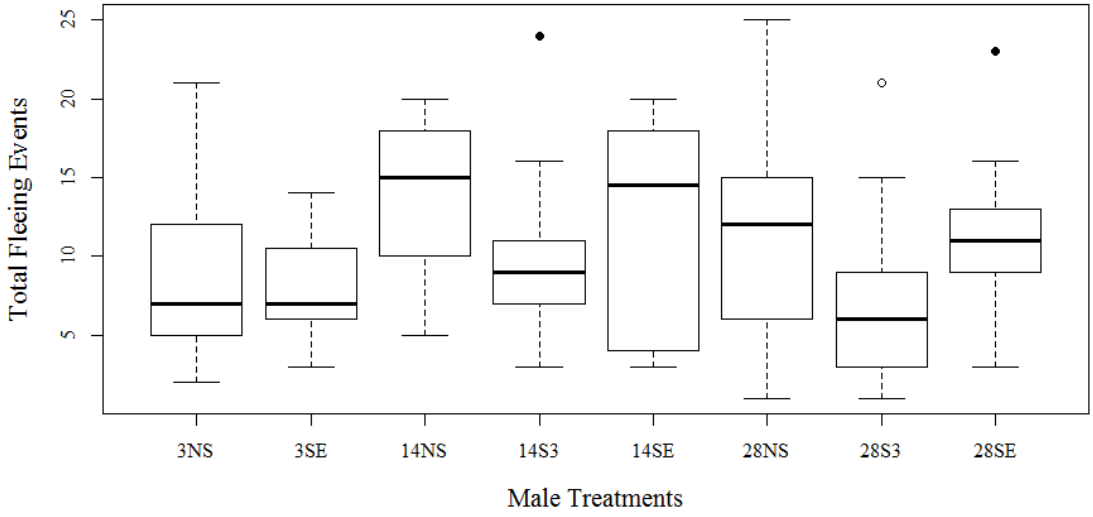


Figure 47. Male treatment effected the number of fleeing events completed by the female ($F_{2, 91}$=3.699, p=0.0285).

NS=Not starved S3=Starved at days 2-3 SE=Starved for two days prior to testing

3=3 days old 14=14 days old 28=28 days old

Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

The male's age significantly affects the number of kicks the female initiates as a rejection method, however starvation does not (age: $F_{2, 91}$=3.505, p=0.0342; starvation: $F_{2, 91}$=1.811,

p=0.169). Post-hoc Tukey tests showed that females kicked significantly less when the male was 28 days old compared to 14 day olds (p=0.0364) – shown in figure 48.

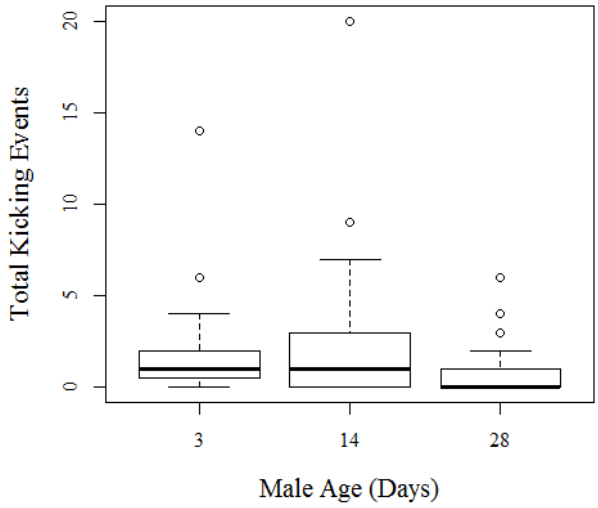


Figure 48. Male age affects the number of kicking events observed ($F_{2, 91}$=3.505, p=0.0342). Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

6 ii. Female Treatment Affected the Number of Rejection Responses

There was no effect of female treatment on the number of fleeing events completed (age: $F_{2, 84}$=1.419, p=0.248; starvation: $F_{2, 84}$=0.0335, p=0.967).

Increasing female age (but not starvation) lead to an increase in the number of kicks initiated by the female (age: $F_{2, 84}$=8.391, p=4.76e^{-4}; starvation: $F_{2, 84}$=1.753, p=0.180); figure 49. Post-hoc TukeyHSD tests showed that 28 day old females kicked significantly more than 14 (p=0.00240) and three day olds (p=0.00274).

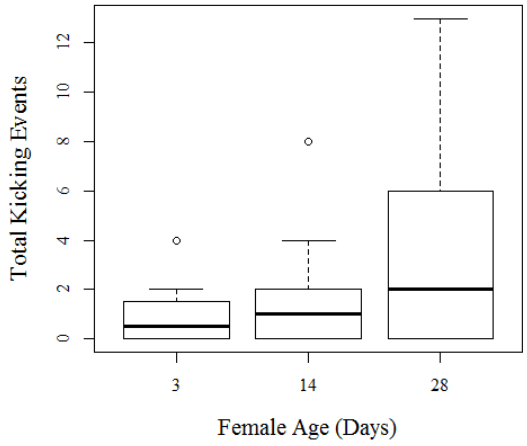


Figure 49. Increasing female age causes a decrease in the number of kicking events observed ($F_{2, 84}$=8.391, p=4.76e^{-4}).

Boxplot shows median as a bold line, upper and lower quartiles, the interquartile range (the whiskers) and outliers (data above the upper quartile, or below the lower quartile, by 1.5 times the interquartile range).

Glossary

Good gene theory – Females should choose a high quality mate so offspring will inherit their genetic advantages (Yasui 1997)

Inter-sexual selection – Occurs where members of one sex (often females) have a preference for a particular characteristic on the opposite sex (Darwin 1871)

Intra-sexual selection – Occurs when there is competition between two or more members of the same sex (usually male) (Bateman 1948)

Lifetime reproductive success – The number of successful, fertile (grand-)offspring produced in an individual's lifetime; or the amount of genetic material passed on to future generations (Fisher 1915)

Plasticity – The ability to produce different phenotypes (phenotypic) or responses (behavioural) in response to environmental fluctuations (Via et al. 1995; DeWitt et al. 1998)

Reproductive terminal investment – The process whereby an individual exerts all of its remaining energy into producing successful offspring as opposed to conserving energy for survival (Clutton-Brock 1984)

Sexy sons hypothesis – More attractive males will sire more attractive sons so females should opt to mate with the most attractive male where possible (Weatherhead and Robertson 1979)

Sperm competition intensity – An increasing presence of rival males (starting with at least two males) (Byrne 2004)

Sperm competition risk – The introduction of a rival male, where previously there was just one solitary male (Byrne 2004)

Trade-off – Where an individual has to compromise one quality in order to gain another quality; one cannot increase without a decrease in the other occurring

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