Cognitive mechanisms underlying responses to sperm competition in *Drosophila melanogaster*

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy

The University of Leeds

School of Biology

November 2016

The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Jointly authored publications

Rouse, J. Bretman, A. (2016) Exposure time to rivals and sensory cues affect how quickly males respond to changes in sperm competition threat, *Animal Behaviour*, 122, 1-8

All experimental work was carried out by the author of this thesis; manuscript preparation was jointly shared between the authors

Acknowledgments

I doubt many people will read this Thesis too closely, but from personal experience I know the acknowledgments are scrutinised, so I better make them good.

First and foremost, I would like to thank my supervisor Amanda for providing me with the opportunity to perform this work. From helping me understand statistics as a baby-faced undergraduate she has encouraged and cajoled me to the baby-faced 24 year old I am today. I couldn't have asked for a better supervisor.

I am also grateful to Tracey Chapman, who started me working on *Drosophila,* Tom Price for fly lines, Elwyn Isaac as my secondary supervisor and John Altringham as my assessor. I also wish to thank Grace Hoysted, Steve Laird and Liz Duncan for their help in unravelling the qPCR knots in my head and Zatul and Petra who have provided advice on fly lines.

I am lucky to have taught and worked alongside some very good undergraduate and Master's Students who have helped collect some of the data in this thesis. Katherine Watkinson, Luke Evans, Alice Gooch and Laura Tyndall, I hope I haven't scared you away from science forever.

I would particularly like to thank the people who have had to share a lab with me throughout some of these 3 years, Tom Leech and Laurin McDowall both have been extremely helpful and a source of constant support throughout the process. I would particularly like to apologise to Laurin for making her collect data in a dark cupboard for hours at a time.

Throughout my 3 years here I have met many people who have been brilliant. I want to particularly thank Roger, my fellow tiny ginger impersonator (cooookkkkkkiiiieee). Double A-ron, the man who taught me about flat décor (sort of). Charlotte Hopkins and Jamie and Claire, my PhD Mum and Dad. I would also like to thank Michael

Norman, for being my drinking partner whenever we actually manage to meet up, and all the office folk who have had to view the atrocity that is my desk.

To my family, especially Mum and Dad, I don't think I would be able to remember why I went to Leeds University every day if not for your continued questions, specifically the classic "so what are you doing again?" Also a shout out to my brother Edward, who has found hours of amusement in the fact I work on fly sex and has taken advantage with various witty birthday mugs. You'll have to find me a different present this year.

Last, but definitely not least I would like to thank Ruth Norman. She has kept me fed, focussed my mind, introduced me to cycling and provided a focal point for much of my craziness throughout the last two years, even though she herself has been doing a PhD. The whole thing would have been much less fun without you.

Abstract

In this thesis I use *Drosophila melanogaster* as a model organism to study the possible cognitive mechanisms controlling plastic behavioural responses to sperm competition. This plastic behaviour involves a male *D. melanogaster* responding to the presence of a rival male by increasing mating duration when housed with a female.

I provide a general context to the work (Chapter 1) before examining my model in more temporal detail by investigating how the length of time males were exposed to a high sperm competition environment affected maintenance time of the plastic behaviour. I show that for males to accurately portray the sperm competition environment in their behaviour over a useful timescale they must possess accurate sensory systems. Without these, behaviour is still fully plastic, but change occurs at a slower speed than males with full sensory ability (Chapter 3). I then show that extended mating duration is controlled by a suite of well-known learning and memory genes highlighting the need for specific memory pathways to reflect ecological change (Chapter 4). However, those same genes do not change in their expression due to increased sperm competition, potentially pointing to some other mechanism of temporal change underlying the behavioural change (Chapter 5). Due to this reliance on learning and memory, I show that an increase in sperm competition can affect cognitive ability, and increase expression of synaptic genes over a longer time period (Chapter 6). Finally, I summarise my thesis findings and discuss how future research can build on the research presented to develop the field (Chapter 7).

My research shows that learning and memory is paramount for males to react to changes in the sperm competition environment on a relevant timescale where behaviour and the environment have not become mismatched. In addition, I show that sperm competition pressures can cause an increase in male individual

cognitive ability, posing the question of whether competition is one of the main drivers of non-mammalian cognitive ability.

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Commonly used abbreviations

MBs**:** Mushroom bodies

STM: Short-term memory

MTM: Medium-term memory

LTM: Long-term memory

ASM: Anaesthesia sensitive memory

ARM: Anaesthesia resistant memory

Chapter 1

General Introduction

Plastic behaviour is a widespread and important component of fitness allowing organisms to constantly match the changing environment around them (Mery & Burns 2010). To be behaviourally plastic organisms must take information from the environment and integrate this with measures of current individual fitness. They must then be able to produce a relevant behaviour to environmental change; potentially a substantial amount of time after the change has occurred. To do this requires learning that acts to form representations of new information, and memory, that enables individuals to access this information at specific points in an organism's lifespan (Dukas 2008a). However, how learning and memory work together to control an adaptive plastic behaviour remains unclear. Studies on learning and memory paint a complex picture of different forms of learning depending on cues received from the environment, and of multiple forms of interacting memory types (Shohamy & Adcock 2010; Staddon 2016). Considering this, how organisms use these multiple learning and memory pathways to control plastic behaviour is of interest.

Here, I synthesise the field of behavioural plasticity and neuroscience to establish what role learning and memory has to play in controlling plastic behaviour. After this, I focus on plastic behaviour to changes in the sperm competition environment. These are important behaviours as they allow individuals to gain fitness benefits by plastically responding to the level of sperm competition, potentially requiring the integration of multiple cues and memory of previous environments. Finally, *Drosophila melanogaster* will be discussed as the ideal model for investigating cognitive control of plastic behaviour. The species is

genetically and structurally malleable allowing for in-depth investigation into this question.

1.1 Phenotypic plasticity

Phenotypic plasticity is integral to how organisms cope with fluctuating environments (West-Eberhard 2003). It is the ability of an individual to display more than one possible phenotype from one genotype when faced with different environmental conditions (Auld, Agrawal & Relyea 2010). Depending on the stage in an individual's life plasticity occurs it can work to modify development or act to change the phenotypic state of a fully developed individual in response to a changing environment (Garland & Kelly 2006). Phenotypes can also vary in the extent of their plasticity, ranging from environmental canalisation, where a phenotype will stay relatively invariant in response to environmental change, to continual flexibility (Flatt 2005). Plasticity in development modifies the developmental trajectory of an organism in response to early life environmental cues and can be influenced by parental behaviour, environmental conditions during development, and social situations of pre-adult developmental stages (Uller 2008). Once fully developed, a phenotype brought about by this type of plasticity is not entirely fixed but further plasticity induced later in development shows an associated cost (Hoverman & Relyea 2007). More flexible plasticity is seen within the life-span of an organism and includes behavioural plasticity, acclimation, metabolic changes and immune adaptation. These traits are a consequence of short term change, allowing for a more rapid plastic response to match pressures brought about by environmental fluctuations (Kelly, Panhuis & Stoehr 2012).

1.1.1 How do different types of plasticity evolve?

Environmental variation driving the evolution of plasticity can occur on different timescales, and hence be termed course or fine grained. These different environmental variations then lead to plasticity on different timescales that is either within or after development (Baythavong 2011). Course-grained environments remain constant throughout generations, but differ from one generation to the next and therefore promote plasticity acting in development (Moran 1992). Fine-grained environments are constantly fluctuating and select for reversible phenotypes that change within a short period of time. This is usually seen for longer lived individuals that encounter multiple environments through a lifetime, or short lived animals living within rapidly fluctuating environments (Via *et al.* 1995). For example, in a lot of short lived animals, predation pressures vary considerably over a small time-scale; this would be deemed fine-grained environmental change and would lead to the evolution of very labile phenotypic traits (Schoeppner & Relyea 2009). Environmental change causing plasticity can be from a variety of sources, such as a change in predation pressure, temperature shift or modification of social status. For example, temperature increase causes adaptive nesting behaviour in reptiles (Refsnider & Janzen 2012) and plastically of life history traits in fish (Crozier & Hutchings 2014), while African cichlids revert to one of two behavioural phenotypes depending on the social status achieved after fighting (Oliveira 2009). Overall, the environmental variation encountered defines the type of phenotypic plasticity evolved.

1.2 Behavioural plasticity

As one of the more flexible types of phenotypic plasticity behavioural plasticity can be evoked multiple times in a lifetime, usually as a consequence of short-duration environmental change acting as a stimulus (Mery & Burns 2010). Once a stimulus is

removed the behaviour again changes to match the environment (Foster 2013), usually reverting back to pre-environmental change levels. This ability to change behaviour rapidly is likely to be particularly important in highly changeable environments where it is thought to have fitness benefits over a more fixed phenotype (Komers 1997).

Behavioural plasticity can be loosely categorised as developmental or activational, though in reality behaviour is a continuum between these two areas (Snell-Rood 2013). Developmental behavioural plasticity is similar to the traditional definition of developmental plasticity, in that it is a result of different developmental trajectories triggered by environmental change. This includes any change seen in the nervous system due to experience as well as morphological or physiological changes outside the nervous circuitry and can occur within the adult stages of an individual (Cardoso, Teles & Oliveira 2015) (Figure 1A). Activational plasticity is a change in behaviour that can be achieved through a switch between already existing physiological networks (Snell-Rood 2013) (Figure 1B). This is also referred to as behaviour as plasticity (Dukas 1998) and innate behavioural plasticity (Mery & Burns 2010). Differences between developmental and activational plasticity control how behaviour is expressed in a time sensitive manner (developmental plasticity is thought to take longer to occur) and so should be reliant on different mechanisms to control plastic behaviour. To fully understand how mechanisms act to control behavioural plasticity it is therefore important to distinguish between plastic behavioural types.

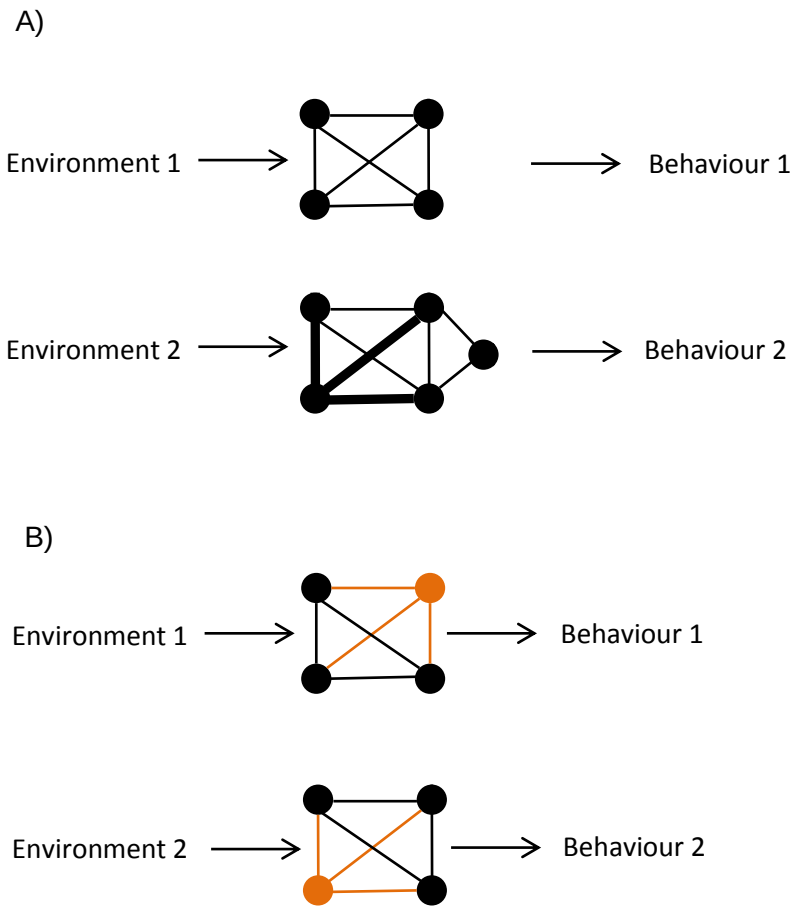

Figure 1: A neural network illustration of two forms of behavioural plasticity. A) Developmental plasticity refers to the development of reinforced (differentially weighted lines) or new network connections as a result of experience. This also leads to the development of modulated or new behaviour. B) Activational behavioural plasticity is controlled by differential activation of neural networks that control different behaviours in different environments. Here, activated networks can be seen in orange, compared to dormant networks shown in black.

1.2.1 Cost of behavioural plasticity

General phenotypic plasticity is limited by any costs that occur when a plastic individual's fitness is reduced in comparison to a non-plastic individual when both express the same trait value, as well as any physiological constraints that may prevent a plastic phenotype from being expressed (DeWitt, Sih & Wilson 1998). Both developmental and activational behavioural plasticity come with specific costs and limits that can constrain an individual's ability to be plastic. Developmental plasticity requires experience of an environment, meaning individuals undergo sampling costs by being naïve to their surroundings (Byers *et al.* 2005; Sasaki, Fox & Duvall 2009).This is also referred to as the exploration-exploitation trade-off (Kaelbling, Littman & Moore 1996). Developmental plasticity also requires an increase in neural circuitry and tissue (Dukas 1999; Huerta *et al.* 2004) and a potential increase in motor output (Sporns, Tononi & Edelman 2000).

Neural tissue is 10 times more expensive than muscular tissue (Laughlin, van Steveninck & Anderson 1998; Kaufman, Hladik & Pasquet 2003) and therefore this increase in neural circuitry comes with a substantial cost. Similarly, activational plasticity requires much of the neural circuitry associated with developmental plasticity. However, unlike developmental plasticity, activational plasticity may undergo additional costs on top of initial tissue investment if neural circuitry required for plasticity is not activated (Snell-Rood 2013). Considering this, activational plasticity may therefore be used to control innate plastic behaviour countering known changes in an environment. In comparison, developmental plasticity could be defined as learning, and would be more useful in a fluctuating environment where environmental change is more difficult to predict (Moran 1992). Selection may therefore act on both types of plasticity to optimize mechanisms underlying plastic behaviour dependent on the prevailing environment (Dunlap & Stephens 2009).

1.2.2 Behavioural plasticity and cognition

As seen above, the common factor linking both types of behavioural plasticity as a physiological cost is the neural tissue needed to develop a complex behaviour able to react to environmental change. As discussed above, neural tissue imposes a high energy cost (Kaufman, Hladik & Pasquet 2003). Despite this, neural tissue in the form of brains has continued to evolve both in size and complexity, with larger brains leading to more complex behaviours and increased propensity for rapid behavioural plasticity (Niemela *et al.* 2013). Although brain size is not a perfect proxy (Healy & Rowe 2007) for cognitive ability it has been shown to correlate with increased intelligence in multiple taxa (McDaniel 2005; Deaner *et al.* 2007; Reader, Hager & Laland 2011; Kotrschal *et al.* 2013; Benson-Amram *et al.* 2016). Considering this, the evolution of larger and more complex brains has continued due to the many advantages of increased cognition that can directly impact on fitness. For example, the ability to problem-solve is linked to reproductive success in great tits (Cole *et al.* 2012; Cauchard *et al.* 2013).

Once a relatively complex brain has evolved this can then act as a 'cognitive buffer' (Sol 2009) to any environmental change that develops, helping individuals create novel behaviours to deal with environmental uncertainty. This can be seen when species possessing increased brain size are more able to survive when introduced into novel environments (Sol *et al.* 2008; Maklakov *et al.* 2011). It can also be seen with an increased ability to forage across multiple environments and so exploit habitat flexibility (Edmunds, Laberge & McCann 2016). Behavioural plasticity and cognition are therefore inextricably linked and selection for plasticity has been implicated as playing a key role in the evolution of cognitive abilities (Morand-Ferron & Quinn 2015). In the next section I outline the evidence for the importance of one facet of the environment thought to be particularly critical in driving the evolution of cognition; social interactions.

1.3 Role of social environment in driving cognition

There have been many attempts to explain environmental effects on cognition but the idea to receive the most attention focuses on the social environment as a driver for brain size. Originally described in primates, the 'social brain' hypothesis theorises that an increase in group size has led to increases in brain size, and therefore increases in cognitive ability (Dunbar 1998). This increase in brain size is not seen across the brain, but is specific to relative neocortex size, the part of the mammalian brain responsible for higher mental functions such as learning and memory (Dunbar & Shultz 2007). An increase in size within this area of the primate brain also correlates with increased participation in social tasks such as prevalence of social play (Lewis 2000), tactical deception (Byrne & Corp 2004) and frequency of social learning (Reader & Laland 2002), all factors of group size and social complexity.

The hypothesis has been extended to other taxa such as carnivores (Perez-Barberia, Shultz & Dunbar 2007), ungulates (Shultz & Dunbar 2006) and birds (Beauchamp & Fernandez-Juricic 2004). Recently, the hypothesis has also been considered in the context of sexual interactions, with the discovery that monogamous species generally have larger brains than species that mate multiply (Schillaci 2006; Schillaci 2008). Monogamy stands out as a mating system due to the quality of the social relationship it can foster, namely pair-bonds. Pair bonds are strong affinities that develop between males and females, potentially leading to a lifelong bond. In primates, the ability to form multiple relationships akin to pair bonds in their complexity and longevity has enabled an increase in cortex size with increased sociality (Shultz & Dunbar 2007). This may also be true of birds, where brain size is affected by the ability to increase extra pair copulations (similar to a pair bond) while also maintaining a dominant pair bond (West 2014). However, for other taxa the quality of pair bonds is more important to the evolution of the social

brain than the quantity on these interactions. Therefore, the need for individuals to create a complex social bond acts as a stimulus for brain evolution in mammals. Although it is not yet known how pair bonds may act to effect the evolution of the brain, studies in monogamous rodents hint that pair bonding may be due to reward learning (Young & Wang 2004; Young *et al.* 2011), and that social behaviour is regulated by discrete neural circuits relating to pair bonds (Young *et al.* 2011).

Outside of mammals, and one paper on birds (Dunbar & Shultz 2007; West 2014), there is little evidence to support the social brain hypothesis. In insects, the mushroom bodies (MBs; see section 1.7.6) are analogous to the cortex in mammals by being a centre of higher-order learning (Devaud *et al.* 2015) that correlates an increase of cognitive ability with increases in neural complexity (Heisenberg 1998). If the social brain hypothesis applies to insects then this would predict an increase in MB complexity with increases in social contact (sociality). In insects differences in sociality are most easily seen with the switch to eusociality in groups such as Hymnoptera (Liao, Rong & Queller 2015). In Hymnoptera increases in MB size are exclusively seen in parasitoid species compared to non-parasitoid species, with no influence of sociality, a result in conflict with the social brain hypothesis (Farris & Schulmeister 2011). However, it is not parasitoidism itself that seems to drive an increase in MB size, but the requirement to locate hosts by learning spatial markers through vision and olfaction (Papaj & Vet 1990; Turlings *et al.* 1993), then memorise identified host locations for future egg laying opportunities (van Nouhuys & Kaartinen 2008). This ability to learn and memorise spatial information therefore seems to drive the evolution of insect brain complexity, as non-parasitic insects that rely on spatial feeding patterns show a similar comparative brain physiology to parasites. For example, *Heliconius* butterflies that repeatedly return to the same food sites have larger MBs than species that forage randomly (Sivinski 1989), and

solitary kleptoparasites that monitor burrows of hosts possess large, elaborate MBs (Rosenheim 1987; VanderSal 2008).

Eusociality presents a significant problem to the application of the social brain hypothesis to insects (Box 1) (Yan *et al.* 2014). Within these societies individuals are extremely specialised and therefore may only perform a restricted set of behavioural tasks throughout a lifetime. It has been suggested that this requires less cognitive ability than performance of multiple tasks by one individual away from a eusocial society (Gronenberg & Riveros 2009). Indeed, this pattern is observed across wasp species, in that eusociality is related to decreased MB complexity of workers (O'Donnell *et al.* 2015). This leads to the possibility that in eusocial insects brain investment is spread around the social group, leading to decreased individual brain complexity, but increased group cognition (O'Donnell *et al.* 2015). It has therefore been suggested that the social brain hypothesis should only be applied to insects which are not eusocial (Gronenberg & Riveros 2009).

1.4 Sperm competition

One of the ways an environment can change rapidly, therefore exposing individuals to experiences where behavioural plasticity may be advantageous, is the rapid and continual change in the social environment. This can lead to changes in mating opportunities, aggression and health of individuals within a group (Kappeler, Cremer & Nunn 2015; Ward & Webster 2016). One measurable change that can occur within the social environment and can be unpredictable in its variation is the socio-sexual context. This describes local sex ratios of males and females, which have an effect on mating opportunities or level of mating competition within a defined area, limited by an individual animal's sensory range (Kasumovic *et al.* 2008).

A rapidly changing socio-sexual environment can cause quick acting changes in the amount of sexual selection within an environment. Sexual selection commonly occurs when females have a choice between multiple males, causing males to compete for fertilisations. This can occur through males competing directly with each other for mates or territories, or can be a function of indirect competition where males display to attract female attention (Jones & Ratterman 2009; Kuijper, Pen & Weissing 2012). In polyandrous mating systems females mate multiply with a number of males, meaning male ejaculates can compete within a female's reproductive tract for fertilisations. This is deemed sperm competition and was first suggested by Geoff Parker as a post-copulatory mechanism of sexual selection (Parker 1970). Since its inception a large body of theoretical work has studied and modelled how sperm competition can be affected by changes in the environment, including social changes (Parker & Pizzari 2010; Kvarnemo & Simmons 2013; Edward, Stockley & Hosken 2015).

The simplest form of sperm competition is referred to as 'fair raffle', whereby all sperm ejaculated have an equal chance of fertilisation, hence male's fitness directly relates to the number of sperm transferred (Parker & Smith 1990). This most commonly occurs when fertilisation is external or female storage is unlimited. If mating once within a lifetime males following a fair raffle should invest maximally in sperm release to increase the proportion of successful fertilisations gained. However, considering sperm and ejaculates are costly (Pitnick, Markow and Spencer 1995) males encountering multiple lifetime mating opportunities must optimally allocate their resources to gain the greatest fitness benefits by trading-off current and future ejaculates (Parker & Pizzari 2010). Males must therefore react to environmental sperm competition changes to maximise lifetime fitness. These changes in sperm competition can fall into two categories, commonly referred to as changes in risk or intensity. Risk refers to the likelihood that a male's sperm will be

in competition (i.e. whether a female has or will remate) whereas intensity refers to the number of males competing for the same set of fertilisation opportunities (Parker *et al.* 1996). Males should be able to adjust ejaculate according to both risk and intensity (Simmons *et al.* 2007), with theory pointing to increases in ejaculate investment with increased risk, but a decrease in investment with increasing intensity (Parker *et al.* 1997; Engqvist & Reinhold 2005). Males therefore require a mechanism enabling them to accurately assess the risk and intensity of current sperm competition in the environment. This is likely to be cognitively challenging, as males need accurate sensory mechanisms to recognise what may constitute a rival (heterospecific v conspecific within an environment), as well as assessing the risk those rivals pose in terms of competing sperm. Indeed it has been suggested that the need to assess sperm competition intensity has driven the evolution of quantity estimation (Shifferman 2012). Shifferman (2012) argues that a male's response to sperm competition is driven by the ability to gauge sperm competition through quantity estimation, i.e. the ability to respond to a quantity aspect of stimuli, and that this ability is an aspect of an overall cognitive response. Therefore, to effectively respond to sperm competition risk a male may have to be able to learn rival signals, make a quantity estimation of the number of rivals and then respond to the environmental sperm competition risk. This is a complex process of events potentially requiring relatively high cognitive abilities. It is also in some ways similar to factors learnt when individuals forage in a spatial manner (VanderSal 2008) as discussed above. Therefore behaviours in reaction to changes in sperm competition could be influenced by cognitive ability or act as an evolutionary pressure on cognition in a similar manner to spatial learning.

Here, I will briefly cover sperm competition responses that males use when they are aware of sperm competition risk within the environment. For more

information see reviews (Bretman, Gage & Chapman 2011; Simmons & Fitzpatrick 2012).

1.5 Sperm competition responses to rival males

Plastic behavioural strategies males use to react to changes in sperm competition are extremely varied. As discussed above these are only favoured when the sperm competition environment is rapidly changeable and males have the option to mate multiply (Parker & Pizzari 2010). The mating system seems to define how males may react to sperm competition risk and intensity. It also controls whether plastic strategies increase or decrease under high sperm competition. Pre-copulatory plastic behaviours are more likely to decrease in frequency when expressed in a high sperm competition environment than behaviour occurring within or after copulation (Bretman, Gage & Chapman 2011). This follows with the theorised effects of sex-ratio on male competition. Using a meta-analysis it has been shown that males in an environment with a male biased sex ratio decrease courtship time but increase the time initiating post-copulatory behaviours (guarding and duration) (Weir, Grant & Hutchings 2011). This is thought to be due to the lack of females available to court and the subsequent need to protect females when males eventually manage to mate. However, many of these studies use operational sex ratio to change the extent of rival competition, a method that ultimately also changes the access males have to females and concurrently changes both sperm competition risk and intensity (Engqvist & Reinhold 2005). It is therefore necessary to study behavioural effects of sperm competition risk and intensity separately and without a female presence.

An example of male reactions to changes in the sperm competition environment where individuals are thought to associate rival stimulus with increases in sperm competition occurs in crickets (*Gryllus bimaculatus*). Males of this species

increase both intensity of courtship and the size and speed of spermatophore transfer when previously presented with a rival male, used within this investigation as a proxy for high sperm competition (Lyons & Barnard 2006). Yet another example of sperm response to increased risk of competition comes from the butterfly, *Pieris napi*, where males again respond to increased sperm competition by significantly increasing ejaculate investment, but this time without a behavioural change. Here, male reaction to sperm competition is also more subtle than in *Gryllus bimaculatus* as males are able to grade their response with fluctuations in sperm competition risk (Larsdotter-Mellstrom & Wiklund 2015). This seems to be a factor of the amount of male sex pheromone present at the time of mating (Larsdotter-Mellstrom & Wiklund 2009), supporting the idea that quantity estimation is a good signal of sperm competition risk. Sperm competition may also have an effect when female choice is the main basis of a mating system. In a leking insect, the lesser wax moth, where sperm competition would not traditionally be thought of as a component of competition, males allocate a higher proportion of sperm reverses when previously exposed to an increased sperm competition risk (Jarrige *et al.* 2015). An increase in sperm competition risk therefore seems to increase ejaculate investment in agreement with general sperm competition theory as discussed above (Wedell, Gage & Parker 2002). However, how males integrate the cues signalling the presence of increase sperm competition risk to modulate behaviour is still not known.

1.6 Learning and memory in plastic behaviour

In order to exhibit plastic behaviour, animals must be able to acquire new environmental information, or learn about the environment. Learning can be defined as the acquisition of neuronal representations of new information (Dukas 2008a) that can then become fixed (at least for a short period of time) as memory, enabling

an organism to modify future behaviour with experience from previous situations to better deal with an environment (Mery & Burns 2010). There are multiple types of learning characterised by how cues are received from the environment and how these cues interact with internal information already possessed by an individual.

1.6.1 Non-associative learning

Non-associative learning is the simplest form of learning and occurs when an animal learns about a singular stimulus in isolation to other factors. The animal can then either decrease (habituation) or increase (sensitisation) their original response to said stimulus after repeated exposure (Kirchkamp 2012). For example, bees show a proboscis extension reflex (PER) when their antenna is stimulated with a sugar solution. When continually stimulated with sugar solution bees cease proboscis extension, successfully habituating to sugar stimulation (Byrne & Hawkins 2015).

1.6.2 Associative learning

The most commonly used paradigm when testing learning and memory is pavlovian, or classical, conditioning. Here, animals learn that one stimulus predicts the occurrence of another (Pearce & Bouton 2001). Animals trained to one stimulus should therefore show a reaction to a second, previously unrelated stimulus. For example, *D. melanogaster* can learn to avoid an electric shock when paired with a particular odour (Tully & Quinn 1985), and rats can associate push levers with food (Brembs 2011). Associative learning is seen as ecologically relevant as in the wild conditioning allows animals to adapt to imminent biologically significant events in which they would otherwise struggle (Hollis 1982; Hollis 1997).

1.6.3 Operant conditioning

In operant conditioning an animal learns to associate the relationship between its own actions and a reinforcing stimulus by having the ability to control the reinforcement through a change in behaviour. For example, *D. melanogaster* can learn to avoid heated areas in a small chamber, which when memorised means individuals avoid the previously heated sections even when increased temperature is not present (Wustmann *et al.* 1996). In another example, male *D. melanogaster* can develop complex hierarchical relationships with other competitors depending on the outcome of previous fights. This shows learning from previous fight outcomes and the ability to associate a previous action with a reinforcing stimulus (winning or losing the fight). In addition, it raises the possibility of a complex recognition system that can assess whether an individual has previously encountered another competitor (Yurkovic *et al.* 2006; Trannoy, Chowdhury & Kravitz 2015).

1.7 Memory and plastic behaviour

Learning types go on to form different types of memory depending on the length, number and type of cues learnt from an environment. Memory types can be defined by the time memory lasts within an individual. These can be split, very basically, into short term, medium term and long term memory (STM, MTM and LTM). Although all organisms have slightly different memory traces the general features stay the same throughout taxa (Nader 2003). STM is a temporary representation of information, which when reinforced forms a MTM trace which after further reinforcement is then retained for an extended period of time as LTM. If STM is not reinforced it is forgotten and does not form as MTM or LTM (Jonides *et al.* 2008). It is commonly thought that there are 2 different memory traces that follow this basic pattern, anaesthesia sensitive memory (ASM) and anaesthesia resistant memory (ARM) (Reasor & Poe 2008; Davis 2011).

Different memory types are thought to allow organisms to gate memory depending on the cues received from the environment. For each learning type the number of times or length of time cues are received and reinforced has a direct impact on the length of time individuals can remember, with increased cue number leading to a longer form of memory (Margulies, Tully & Dubnau 2005; Giurfa 2015). This means, if a cue is transient in the environment it may not be important to the organism in question and so will only form STM. Conversely, if a cue is sustained this may signal that a cue is important and so longer term memory will develop (Healy & Jones 2002). In this way, an individual can develop sufficient memory to remember important environmental variables without using energy or neuronal space responding to unimportant associations (Mery 2013). In addition to the length of time a cue can last in an environment, importance of specific cues is thought to be different depending on species. For example, related species of parasitoid wasp (*Nasonia vitripennis* and *Nasonia giraulti*) form different types of memory from the same cue depending on the importance of this cue to the life history of the species (Hoedjes & Smid 2014). Some species may therefore react and form memory quickly to some cues associated with ecologically relevant information, whereas other cues will not induce memory as quickly. Overall, the formation of memory type is thought to be cue dependent, raising the possibility any plastic behaviour that may be controlled by memory is also cue dependent.

1.8 The study system

1.8.1 Socially induced extended mating duration

To explore the mechanisms behind plastic behaviour I am using a well-studied example of plastic mating behaviour that responds to the social environment. *D. melanogaster* males facultatively extend mating duration with females after previous exposure to a rival for 24 hours (Bretman *et al.* 2010). The behaviour tracks the

level of sperm competition in the environment, with males reducing mating duration again when kept without rivals for 3 days (Bretman *et al.* 2012). Extended mating duration is associated with an increase in the amount of sperm (Garbaczewska, Billeter & Levine 2013; Moatt 2014) and seminal fluid proteins (Sfps) transferred to females (Wigby *et al.* 2009), which modulate a reduction in remating rates and an increase in time till the next female remating (Bretman, Fricke & Chapman 2009). Males that extend mating duration increase both paternity and fitness across one mating, compared to males that are not exposed to sperm competition cues. The two main Sfps that modulate these effects are Sex peptide (SP) and ovulin that are strategically invested when males had been exposed to a high sperm competition environment i.e. the presence of another male (Wigby *et al.* 2009). It is not known whether other Sfps involved in *D. melanogaster* mating have a similar strategic allocation. Extended mating duration after exposure to rivals is repeatable across many of the *Drosophila* genus such as *D. pseudoobscura* (Price *et al.* 2012), *D. montana* (Mazzi *et al.* 2009) and even in species in which females do not remate (Lize *et al.* 2012) and so includes males not exposed to sperm competition.

1.8.2 Sensory cues involved in extended mating duration

To investigate the sensory inputs required for males to detect rivals and so increase mating duration sensory systems were removed individually and the reaction of males to rivals assessed. Males use multiple cues to detect rivals and in most cases needed any combination of two out of three cues from auditory, olfactory and tactile to extend mating duration. Out of these three cues touch elements were deemed to be the most important, with visual cues, that were also tested, considered to play no role at all (Bretman *et al.* 2011; Garbaczewska, Billeter & Levine 2013). However, some controversy remains, as it has also been reported that vision is the most important factor in detecting the presence or absence of other flies, specifying

moving red eyes as the modulator of this response (Kim, Jan & Jan 2012). The study used the Canton-S strain of *D. melanogaster* compared to the Dahomey used in the two previous studies. Both strains were housed in massed groups meaning evolutionary pressures were similar between the two strains, therefore discrepancies would not be expected, and are so far unexplained.

1.8.3 Lifetime costs and benefits of extended mating duration

In *D. melanogaster* extending mating duration is beneficial through one mating (as discussed above) but has no reproductive benefit if males are forced to undergo continually extended mating duration throughout a lifetime (Bretman *et al.* 2013). When compared to males exposed to an environment lacking rival males, males who continually extend mating duration undergo early mortality and decreased reproductive benefits later in life, leading to similar overall reproductive outputs between the two groups. This highlights the need for males to be plastic to benefit from extended mating duration rather than implement a beneficial behaviour regardless of environmental factors, and also highlights some possible costs associated with plastic behaviour. However, these results were based upon a lab study so the data did not take into account natural population dynamics. The natural ecology of wild populations of *D. melanogaster* is not well understood but they must undergo social variation, and so it is unlikely extended mating duration would be maintained throughout life (Carroll & Corneli 1995). In addition, wild insects generally have a shorter lifespan than their laboratory counterparts due to increased extrinsic mortality (Kawasaki *et al.* 2008). If this is the case, there would therefore be selection pressures for a short acting increase in fitness without any protection for later life fitness, supporting the pattern discussed above.

1.8.4 Types of learning in *D. melanogaster*

1.8.4.1 Associative learning

In *D. melanogaster*, one way associative learning can be tested is a T-maze choice test. In this test flies are alternatively exposed to two odours (conditioned stimulus) which when paired with electric or mechanistic shocks (unconditioned stimulus) create an association between one of the odours and shock. Flies can then be tested in a T-maze where memory is assessed with a choice between the two original odours, which will cause flies to reject the odour paired with the shock (Tully & Quinn 1985; Mery & Kawecki 2003). Within this paradigm differential training plans can create separate memory types in a fly. As previously mentioned, ASM and ARM are two different memory types separated by their response to anaesthesia (Reasor & Poe 2008). Within the T-maze paradigm, one training session causes ASM memory that can last up to 5 hours, followed by a consolidated ARM phase that can last up to a day (Margulies, Tully & Dubnau 2005). When training was repeated, massed training (10 training sessions one after another) successfully caused flies to remember for up to 3 days using ARM. In comparison, spaced training (10 training sessions split by 15 minute breaks) induced ASM long term memory (LLTM) and caused flies to remember an association for up to a week (McGuire, Deshazer & Davis 2005) (Figure 2). ARM does not require protein synthesis, whereas LLTM requires structural and functional modification of relevant synapses (Guan *et al.* 2011).

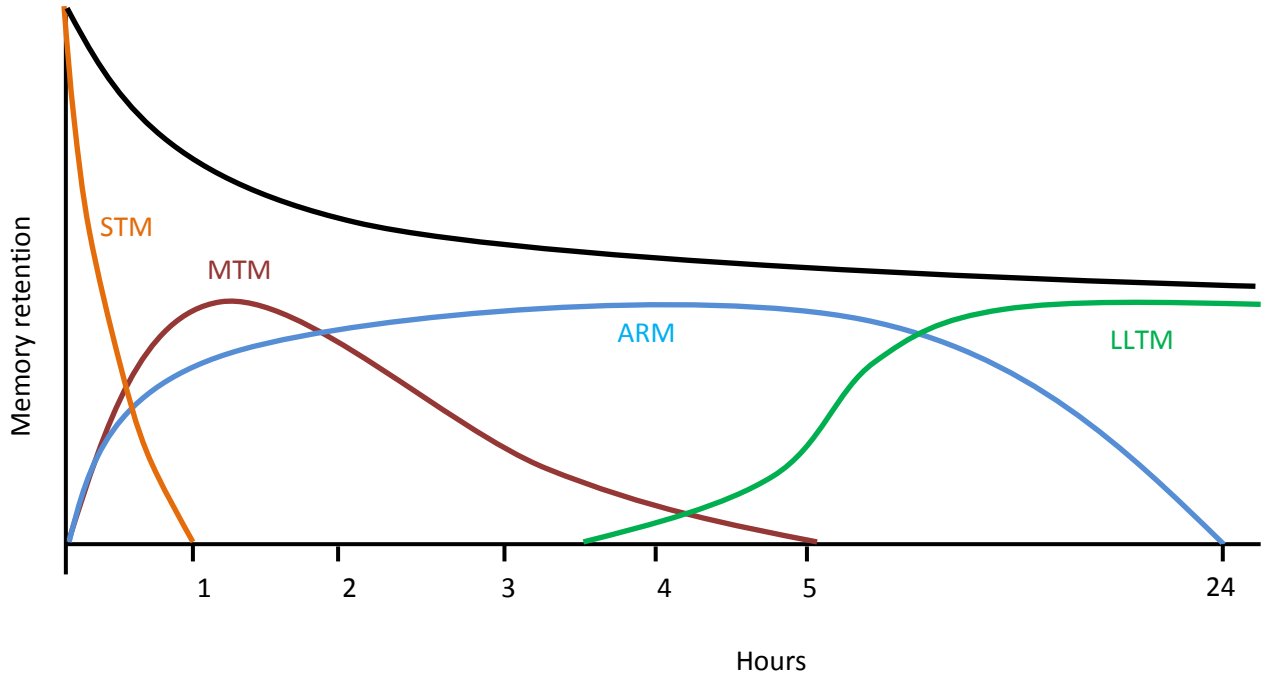

Figure 2: Memory phases in *D. melanogaster.* The black line represents memory decay at the behavioural level. In *D. melanogaster* memory can be split into four mechanistically distinct phases represented as part of the behavioural response. These are short term memory (STM; orange), medium term memory (MTM; Red), anaesthesia resistant memory (ARM; blue), and long-lasting long term memory (LLTM; green). Reproduced with modifications from Marguiles et al (2005).

1.8.4.2 Courtship suppression

Similar to testing learning and memory in the T-maze, another learning paradigm in *Drosophila spp*. focusses on courtship suppression as a type of associative memory. Here, exposure of males to a previously mated female for 1 hour causes courtship suppression towards virgin females that lasts for 2 to 3 hours (Siegel & Hall 1979). The conditioned stimuli is present on all females and allows a male to identify a female, the unconditioned stimuli is only present on previously mated females and acts to train the male to suppress courtship.

The *Drosophila* conditioned stimulus is 9-pentacosene, a compound present at higher levels on mature females than immature females, and enabling males to mark out females (Siwicki *et al.* 2005). The two main unconditioned stimuli within courtship suppression are 7-trocosene and *cis*-vaccenyl acetate (cVA), both part of the male cuticular hydrocarbon profile. 7-trocosene is a surface hydrocarbon transferred to a female during male courtship (Lacaille *et al.* 2007) that lasts for 24 hours before being groomed off, successfully suppressing male courtship during this time. cVA is a longer lasting compound that is instead transferred to females during copulation and retained in the reproductive tract. This can successfully cause generalized courtship suppression for an extended period of time, along with other less well known peptides (Ejima *et al.* 2007).

Male courtship suppression is adaptive within the mating environment, allowing experienced males who have 'learned' to court females depending on status to limit costs of courtship by targeting virgin females (Dukas 2005). This was evaluated using males who underwent an initial experience phase, and males who were naive. Both sets of males courted virgin and previously mated females, however, males with experience reduced time courting previously mated females and increased time courting virgin females compared to naïve males (Dukas 2005). This shows that courtship suppression, itself a plastic behaviour, can be further

modified by learning within a changing mating environment, leading to possible increased fitness benefits for individuals who invest in learning.

1.8.5 Genetics of learning and memory in courtship suppression and associative learning.

Using the two paradigms outlined above *D. melanogaster* has provided an ideal model to examine the genetics and mechanistic underpinnings of learning and memory. The main learning and memory pathway controlling learning and memory is Cyclic adenosine monophosphate signalling (cAMP) (Zhou *et al.*). In *D. melanogaster*, *dunce* (*dnc*) and *rutabaga* (*rut*) are particularly well studied cAMP signalling defective mutants. *dnc* encodes a phosphodiesterase that breaks down cytoplasmic cAMP (Dudai *et al.* 1976), and *rut* codes adenylate cyclase, a cAMP synthesing enzyme (Livingstone, Sziber & Quinn 1984). Both genes are preferentially expressed in the Kenyon cells that make up the MBs, which act as the centres for olfactory learning and memory (Campbell & Turner 2010). *Rut* is thought to be activated by stimulation from G-proteins and concurrent Ca^{2+} entry, meaning it can act as the detector allowing convergence of the two stimuli used in associative learning (Siwicki & Ladewski 2003). After this, *rut* is responsible for synthesising cAMP from ATP within the MBs, and *dnc* is responsible for that cAMP removal (Gervasi, Tchenio & Preat 2010). Mutants for *dnc* and *rut* therefore show an inability to learn or recall STM traces in both courtship suppression and associative learning (Ackerman & Siegel 1986).

Amnesiac (*amn*) is another gene that affects memory retention through stimulation of cAMP synthesis through release of its protein product, a preproneuropeptide (McGuire, Deshazer & Davis 2005). *amn* is mostly expressed in the dorsal paired medial (DPM) neurons that project over the lobes of the MBs. *amn* flies with expression blocked in the DPM neurons learn as well as wildtype flies but

suffer memory decay after 1 hour, with smell association undetectable after this time period (Waddell *et al.* 2000). *amn* is therefore thought to trigger prolonged cAMP cascade activation through *rut* required for the consolidation of permanent memory (Figure 3).

The only currently known gene to be totally specific to ARM is *radish* (*rsh*), encoding a protein thought to link with the cAMP pathway through PKA activation. *Rsh* mutants do not show ARM memory, but develop anaesthesia sensitive memory of similar length to wildtype flies (Folkers, Waddell & Quinn 2006). *Bruchpilot* (*Brp*) is a secondary gene that is involved in controlling ARM, but also has a role in ASM. *Brp* is homologous to ELK/CAST active zone proteins in humans and localises to presynaptic sites. Mutants show reduced ARM memory after 3 hours, though also show some signs of ASM recovery at this timepoint (Knapek, Sigrist & Tanimoto 2011).

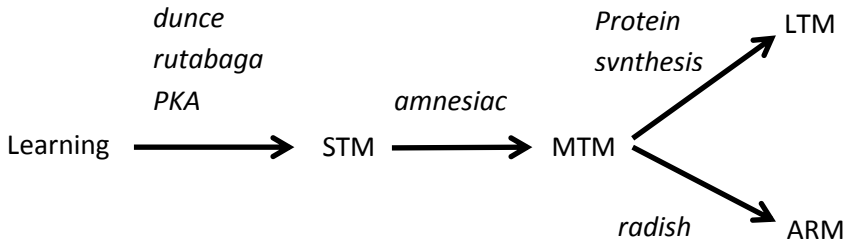

Figure 3: cAMP genes controlling memory phases in *D. melanogaster*. Memory phases with discussed genes controlling areas of memory formation in *Drosophila melanogaster*, whose memory formation can be considered representative of insect and mammalian learning and memory systems. All olfactory memory associated with courtship suppression has so far been reported to require the cAMP cascade for associative learning. PKA (cAMP-dependent protein cascade) is also an important molecule in the cAMP cascade, with targets including the transcription factor CREB needed for the development of transcriptionally controlled LTM, and *rsh*, needed for ARM. *Rsh* binds to a GTPase regulating neuronal and synaptic morphology (Folkers, Waddell & Quinn 2006). Reproduced with modifications from McGuire and colleagues (2005).

1.8.6 Neuroanatomy associated with associative learning

In addition to genetic dissection of memory *D. melanogaster* has provided an excellent model to elucidate the neuroanatomy underlying learning and memory. Experiments focussing on neuroanatomy are most commonly performed using the T-maze assay due to the increased power experimenters have over the timing and power of the stimuli used. When groups of flies are trained in associative learning paradigms olfactory stimuli are detected through very specific receptor neurons and then associated with shock in 3rd tier neurons thought to be within the MBs. Olfactory receptor neurons that project into the antennal lobes detect odours through antennae and maxillary palps (Waddell & Quinn 2001). Antennal lobes then project neurons to the dorsal protocerebrum, which synapses on the dendrites of the Kenyon cells that make up the MBs, as well as separately onto the lateral horn (Keene & Waddell 2007). The lateral horn is thought to act to modulate innate odor responses as removal of the MBs as larvae does not impair odour driven behaviour in adults (Heimbeck *et al.* 2001). The MBs are the site for the formation and expression of olfactory memories, expressing genes involved in cAMP synthesis at high levels, as discussed above. They comprise symmetrical clusters of 2500 Kenyon cells that have cell bodies in the MB calyx and extend axons through the MB penducle to form α/β, α'/β' or γ lobes (Fahrbach 2006). The α'/β' lobes seem to play a part in consolidating memories, and α/β lobes in the retrieval and expression of memory. The γ lobes are needed for STM and may play a role in detecting the conditioned and unconditioned stimuli used in a training protocol (Guven-Ozkan & Davis 2014).

To consolidate all the information *D. melanogaster* has provided us about learning and memory a model for associative learning and memory has been created. Inputs from antennal lobes and DPM neurons are thought to converge on the MBs, activating *rut* through coincident Ca^{2+} influx and monoamine binding to G-

protein coupled receptors. This causes a cAMP cascade that concludes in the case of LTM with changes in gene expression. To consolidate memory it is thought that MB neurons report back to DPM neurons, creating a feedback loop, which if activated multiply, leads to consolidated memory. An individual's memory output is finally expressed through lateral protocerebrum neurons, that project onto antennal lobe projection neurons, causing ecologically relevant behaviours (Figure 4) (Dubnau *et al.* 2001; Waddell & Quinn 2001; Keene & Waddell 2007; Smith, Wessnitzer & Webb 2008; Stopfer 2014).

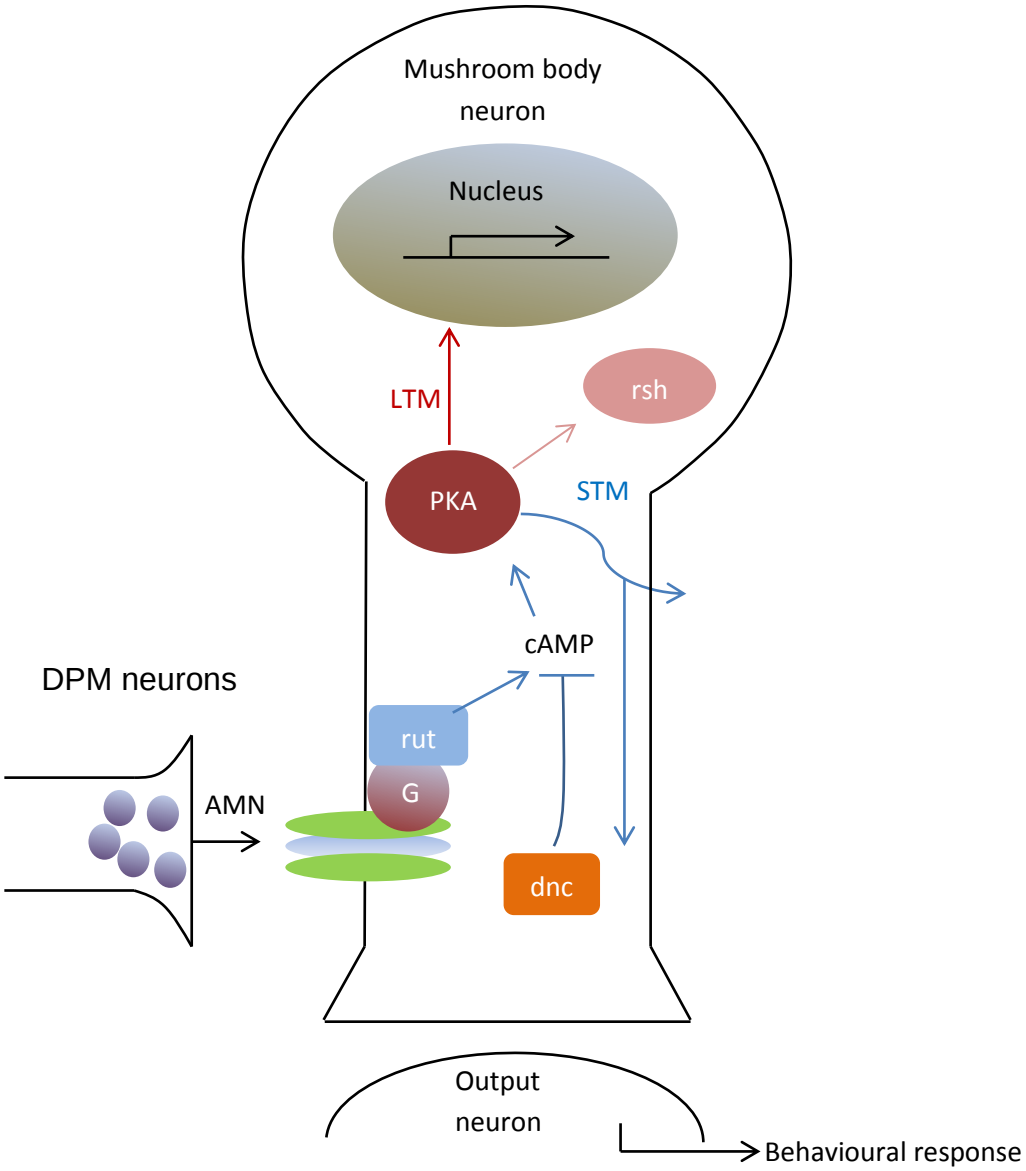

Figure 4: Possible model for associative learning in *D. melanogaster.* Mushroom body neuron receive inputs from antennal lobes and DPM neurons, activating *rut* through coincident Ca^{2+} influx and monoamine binding to G-protein coupled receptors. This causes a cAMP cascade and elevation of cAMP in relevant MB neurons. Depending on training conditions and duration of cAMP elevation this then results in long lived changes in gene expression (LTM) or modification in synaptic connectivity (STM and MTM). Figure with modifications from Waddell and Quinn 2001.

1.9 Outline of thesis

The overarching aim of this thesis is to address how behavioural plasticity in response to a changing sperm competition environment may be controlled by cognitive mechanisms through the use of a *D. melanogaster* model. This model is behaviourally well characterised and the species is fully genetically and neurologically malleable. In addition, similar patterns in response to increased sperm competition have now been shown to occur in other species of fly (*Merosargus cingulatus*) (Barbosa 2012), lekking moths (*Achroia grisella*) (Jarrige *et al.* 2015) and crickets (*Teleogryllus oceanicus*) (Simmons *et al.* 2007), raising the possibility any mechanism found to affect behaviour in this thesis could be conserved between species.

Chapter 2 asks how long male extended mating duration continues to last once males are removed from increased sperm competition. In addition, it examines how changing exposure time to a high sperm competition environment can influence how long plastic behaviour lasts. This is important to outline the temporal dynamics of extended mating duration for comparison to results in later chapters. It also allows theories of possible mechanisms to be drawn up, in this case, a theory of how learning and memory may contribute to extended mating duration.

Chapter 3 builds on previous work by Bretman and colleagues (2011) and uses information from Chapter 2 to look at the importance of different senses in controlling the temporal dynamics of a male's response to increased sperm competition. This is important to understand how cues needed to sense an environment may play a role in controlling the time frame of a plastic behaviour, and therefore how cues may impact on the mechanisms underlying plastic behaviour.

Chapter 4 investigated the possible cognitive mechanism behind extended mating behaviour using mutants of common genes associated with learning and memory in *D. melanogaster.* The aim was to disentangle whether learning and

memory were in part responsible for male extended mating duration, and if so, whether a specific type of memory modulated the reaction to the environment. This is important to help establish how extended mating duration may have evolved and may hint at possible interactions with other areas of behaviour, potentially controlled by the same mechanism. The original hypothesis based on the temporal dynamics of the behaviour investigated in Chapter 2 was that anaesthesia resistant memory, a form of long term memory, would control extended mating duration.

Chapter 5 investigates the molecular changes that might underpin behavioural state change in a male responding to increased sperm competition. Reversible behavioural plasticity similar to that shown in extended mating duration may parallel neurogenomic change where differential expression of an important subset of genes cause different behaviours (Cardoso, Teles & Oliveira 2015). I measured the expression of genes found to be important to extended mating duration (shown in chapter 4) that were also differentially expressed in a previous transcriptomics study. This enabled me to elucidate whether neurogenomic change was indeed underlying changes in behaviour. To focus on any transient changes in gene expression, qPCR was used to measure genes at time periods that paralleled changes in male behaviour and that I hypothesised would change in their expression levels with changes in behaviour.

An increase in sociality is theorised to increase neural tissue and so increase cognitive ability in mammals and birds. However, in insects the evolutionary pressures driving cognitive ability are not agreed on. In Chapter 6 I investigate whether exposure to a social environment affects within generation cognitive ability and/or expression of a suite of synaptic genes in male *D. melanogaster*. The aim was to establish whether changes in the social environment within generations could lead to differences in cognitive ability in an insect model and if so, to identify a possible evolutionary driver behind these differences.

Heterospecific social environments were also investigated to elucidate whether specific types of sociality were important for the evolution of cognition in *D. melanogaster.* I hypothesised that social differences would not drive within generation differences in cognitive ability, though social differences would contribute to the evolution of cognition over multiple generations as shown by Hollis and Kawecki (Hollis & Kawecki 2014). This work was carried out with the help of Laurin McDowall who assisted with behavioural experiments.

Chapter 7 is a general discussion of what this thesis has found and the wider implications of those findings. I discuss the mechanisms controlling male sperm competition responses and how these can impact the wider literature in informing work investigating how other plastic responses are controlled. I then go on to discuss how the competitive environment may have impacted cognitive abilities in line with theories of brain evolution. I conclude with suggestions for future work to further our understanding of cognitive ecology, in particular, mechanisms controlling plastic behaviour.

Appendix I details supplementary experimental methods and preliminary data associated with the main chapters. I show how concentrations for associative learning experiments used in chapter 5 and 6 were calculated. I also show that male's extended mating duration responses do not differ depending on the time of day they were tested, something that may have impacted experiments performed in chapter 2, 3 and 4.

Maintenance of extended mating duration in response to sperm competition threat is determined by exposure time to rival males

2.1 Summary

Phenotypic plasticity can increase fitness in rapidly changeable environments, but may be limited if the underlying mechanisms cause a lag-time between environmental change and individual response or if the information individuals receive is unreliable. Hence to understand the evolution of plasticity we need to assess whether individuals respond to fine-scale variation in environmental cues. In this study we use a *Drosophila melanogaster* fruit fly model to investigate factors that determine how quickly males alter their behaviour in response to changes in sperm competition cues. Male *D. melanogaster* respond to exposure to rival males prior to mating by extending mating duration and increasing ejaculate investment. It has previously been shown to build-up the response; males need ~ 24h exposure to a rival. We reasoned that this lag-time was necessary to increase ejaculate production, but this physiological limitation should not apply when moving from high to low competition environments, hence we predicted that males should immediately decrease their investment when competition is removed. Here we test this by measuring how long rival-exposed males maintain an extended mating duration phenotype after removal of the rival. I also assessed whether exposure time to a rival male affects the speed of change in behavioural state. Males maintain extended mating duration for hours after a rival is removed, but this is dependent on time of exposure to a rival. Our results suggest that males use exposure time to assess whether the threat of sperm competition is transient (so unlikely to translate into realised competition) or sustained (requiring a response). Therefore, lag-times between environmental changes and responses may buffer animals against making hasty decisions in fluctuating environments.

2.2 Introduction

Phenotypic plasticity is the expression of different phenotypes from the same genotype in response to an environmental cue (West-Eberhard 2003). In animals, behavioural plasticity is predicted to be a particularly potent form of phenotypic plasticity due to its rapid flexibility and low production costs (Parker 1982), and hence flexible behaviour can enable animals to cope with rapidly changing environments (Komers 1997). However, to be adaptive, behavioural plasticity must track the environment accurately and on a similar timescale to the environmental variation to which it responds (Gabriel *et al.* 2005). If it does not, mismatches between behaviour and the environment are predicted to be costly (Auld, Agrawal & Relyea 2010). This is most readily seen in environments that have recently undergone mass changes to which behavioural plasticity fails to respond correctly. For example, snowshoe hares (*Lepus americanus*) fail to modify hiding or fleeing behaviour in environments where snow cover has decreased, raising the likelihood of predation (Zimova *et al.* 2014) and red squirrels (*Tamiasciurus hudsonicus*) must breed earlier in the season to take advantage of increasing spring temperatures and food (Reale *et al.* 2003). Other phenological mismatches to climate change include changes in the timing of hibernation emergence in mammals (Ozgul *et al.* 2010; Lane *et al.* 2012) and migratory journeys in birds (Both & Visser 2001). Depending on the type of environmental variation, proximate cues might change more quickly than the prevailing population conditions, and so animals might need to judge if the change is transient or sustained enough to warrant a response. Mismatches with the environment driven by time-lags in production of new trait values are therefore crucial to the range of plasticity an individual can exhibit, but are rarely quantified (DeWitt, Sih & Wilson 1998; Auld, Agrawal & Relyea 2010). In order to understand these limits, how plasticity evolves, and why not all traits are plastic, it is crucial to

examine factors that impact the speed with which environmental inputs are translated into plastic responses.

One rapidly changing facet of the environment is the socio-sexual context, as sex ratio can vary locally and over short time scales (Kasumovic *et al.* 2008; Punzalan, Rodd & Rowe 2010). This is particularly important for males as they are predicted to allocate reproductive resources strategically, trading-off current and future mating opportunities depending on the competitive environment (Parker *et al.* 1996; Parker *et al.* 1997). To respond to changing socio-sexual environments males could change mating strategies either as an immediate response to another male (or cues of other males), or using a response that requires a period of exposure to changes in the social environment (Bretman, Gage & Chapman 2011). We currently have very little understanding of how males assess and assimilate environmental information and how this is translated into altered behavioural and physiological states. One of the best studied examples is the response of male *Drosophila melanogaster* fruit flies, whereby males exposed to a rival male before mating subsequently mate for longer than males held alone (Bretman, Fricke & Chapman 2009). This leads to increased short-term reproductive success compared to males who have not been exposed to rivals (Bretman, Fricke & Chapman 2009), mediated by alterations in ejaculate contents (Wigby *et al.* 2009; Garbaczewska, Billeter & Levine 2013; Moatt 2014). Mating duration tracks the level of sperm competition anticipated, increasing when males are exposed to a rival and reducing when that rival is removed (Bretman *et al.* 2012). Males kept with rivals die sooner and become progressively less successful at obtaining matings over life, supporting costs of responding to rivals (Bretman *et al.* 2013).

In this study, I explore how quickly males can match a new competitive environment and whether exposure time to rivals affects the speed of adjustment. It has previously been shown that males require ~24h exposure to a rival to increase

mating duration and gain fitness benefits, with this time lag thought to allow an increase in production of ejaculate components (Bretman *et al.* 2010). However, males moving from high to low competition environments should not be constrained by the same physiological limitation and so should not require any adjustment time. If this is the only consideration in the speed of response then I predict that males moved from high to low competition should quickly change their strategy and not mate for longer than males that have never perceived competition. To investigate this prediction, I measured how long rival-exposed males continued to extend mating duration after a rival had been removed. In addition, in order to assess whether males use exposure time as a proxy for the likely persistence of the sperm competition threat, I measured whether initial exposure time altered the maintenance of extended mating duration behaviour.

2.3 Material and Methods

2.3.1 General experimental set-up

Experiments were conducted in a 25°C humidified room with a 12 hours light: 12 hours dark cycle (9 am to 9pm light cycle), using plastic vials (75x25mm) with 7 ml standard sugar-yeast-agar (SYA) medium (Bass *et al.* 2007). All wild type flies were the Dahomey strain as in our previous studies. Larvae were raised at a standard density of 100 per vial. At eclosion, flies were collected and sexed using ice anaesthesia, and stored 10 per vial. Females were supplemented with live yeast granules. Males were aged for 24h before being haphazardly assigned to a social environment treatment i.e. plus-rival or no-rival. In different experiments we manipulated “exposure time” (time from introduction to removal of the rival) and “maintenance time” (time from removal of the rival to mating) (Figure 1 and Table 1) to gain an understanding of how long the mating duration response is maintained for and how it could be affected by exposure time. At mating, males were aspirated

singly into a vial containing a single female and allowed to mate, and mating duration recorded. If no mating occurred within 3 hours the vial was discarded. This gave sample sizes of at least 30 males for each time period, in line with previous work (Bretman, Fricke & Chapman 2009; Bretman *et al.* 2010).

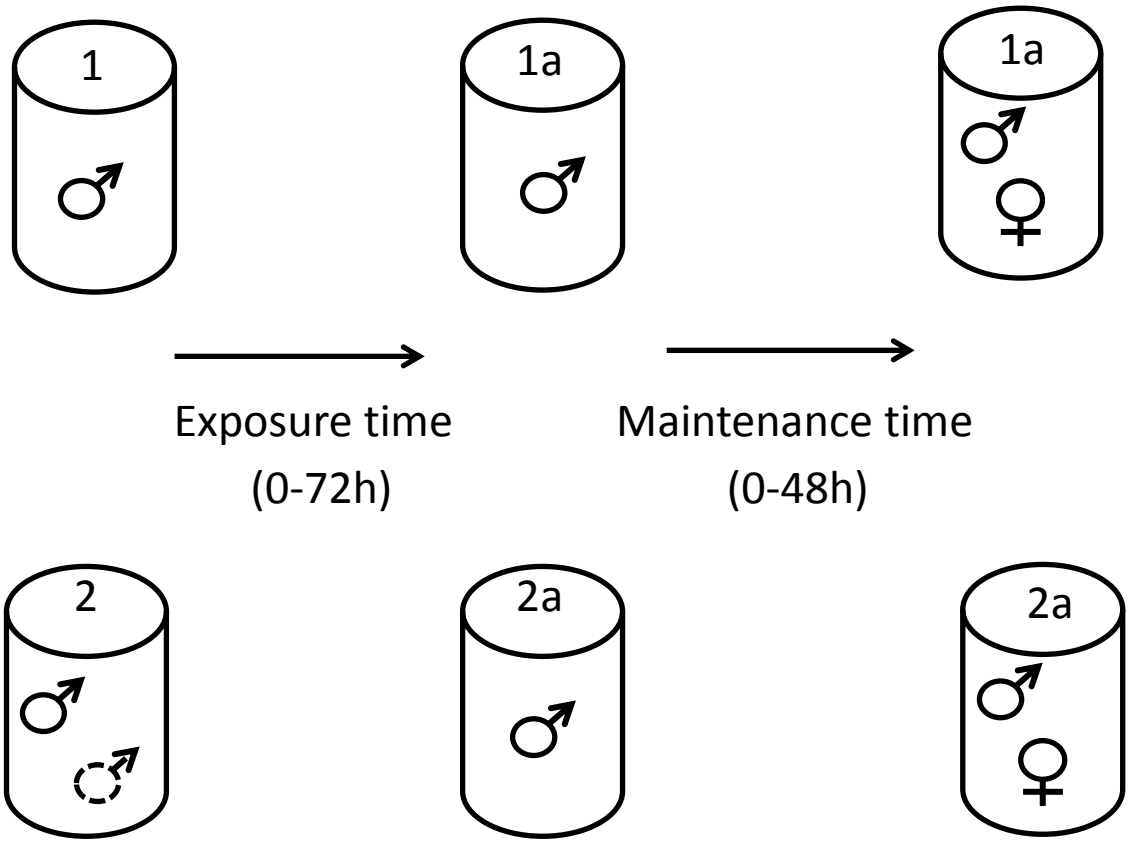

Figure 1: Experimental design. Focal males (solid symbols) were separated at eclosion and haphazardly assigned to no-rival (vials 1) or plus-rival (vials 2, rival is the dotted symbol) treatments, handled in exactly the same way except for the presence of absence of the rival. In different experiments we varied exposure time (time kept with the rival male) and maintenance time (time from removal of the rival male until mating), as described in Table 1. Focal males were transferred to new vials for isolation (vials 1a and 2a) and females were added to these vials to record mating duration.

Table 1: Description of experiments 1-5 providing information about the exposure times, maintenance times and paired set-ups for each experiment

Experiment	Paired treatments?*	Exposure time (h)	Maintenance time (h)
1	y	72	0, 12, 24, 36, 48
2	n	72	0, 9, 12, 15, 18, 24
3	y	36	0, 12, 24
4	y	24	0, 12, 24
5	n	24	1, 3, 6, 9, 12

* y = each plus-rival treatment has a corresponding no-rival treatment, n = multiple plus-rival treatments compared to one no-rival control treatment

2.3.2 Maintenance of the response after 72h exposure

In Experiment 1 (Table 1), we investigated how the response to a rival in extended mating duration was maintained over 48h after a rival was removed (maintenance time). This required offsetting the introduction of the rival and therefore the day on which males were mated. Hence, we set up paired treatments, whereby each plus-rival treatment had a corresponding no-rival treatment handled in the same way. The plus-rival treatments were exposed to a rival for 72h to make sure a full response was realised (Bretman *et al.* 2012), and then isolated from any social interaction for 0, 12, 24, 36 and 48h before mating. In Experiment 2 we further narrowed down the maintenance time. Here we were able to mate all males at once, hence had one no-rival treatment and 6 plus-rival treatments exposed to a rival for 72h and then isolated for 0, 9, 12, 15, 18 or 24h before mating.

2.3.3 Effect of exposure time on maintenance time

To test whether the amount of time males spent with rivals (exposure time) affected the maintenance time of the response we repeated Experiment 1, but this time plus-rival treatments were exposed to a rival for either 36h (Experiment 3) or 24h (Experiment 4). After exposure, focal males were isolated for 0, 12 or 24h prior to mating. Finding that 24h exposure reduced maintenance time to under 12h we further narrowed this down as in Experiment 2, this time giving plus-rival treatments 24h exposure and isolating them for 1, 3, 6, 9 or 12h before mating (Experiment 5).

2.3.4 Statistical analysis

Statistical analysis was performed using SPSSv14. If data were normally distributed, comparisons between three or more treatments were made using ANOVA with Dunnetts post-hoc tests. Where there were only pairs of treatments

comparisons were made using t tests. If data did not meet the assumptions of normality Kruskall-Wallis (K-W) or Mann Whitney U tests (MWU) were used (as indicated in the Results section). To reiterate, where the design permitted, the key comparisons were between males kept singly or with a rival but treated the same in all other respects, as this controlled for any other manipulation effects. Bonferroni corrections were made where multiple tests were used; this was relevant only when multiple groups of males in a plus-rival treatment were compared to a single no-rival treatment group.

2.4 Results

2.4.1 Maintenance of the response after 72h exposure

In Experiment 1, after 72h exposure to a rival, males extended mating duration for 12h (MWU: Z = -3.722, N = 77, $p < 0.001$), but not for 24h (T-test: t_{70} = -1.597, p = 0.115) or more after removal of the rival (Figure 2A). In Experiment 2, we narrowed maintenance time down further, again finding that mating duration was affected by time since isolation from a rival (K-W: X^2_7 = 15.862, p = 0.026). Post-hoc tests showed that males continued to significantly increase mating duration after 12h of isolation (MWU: Z = -3.136, N = 77, p = 0.014), but failed to do so after 15h isolation (MWU: Z = -2.349, N = 75, p= 0.133, Figure S2B).

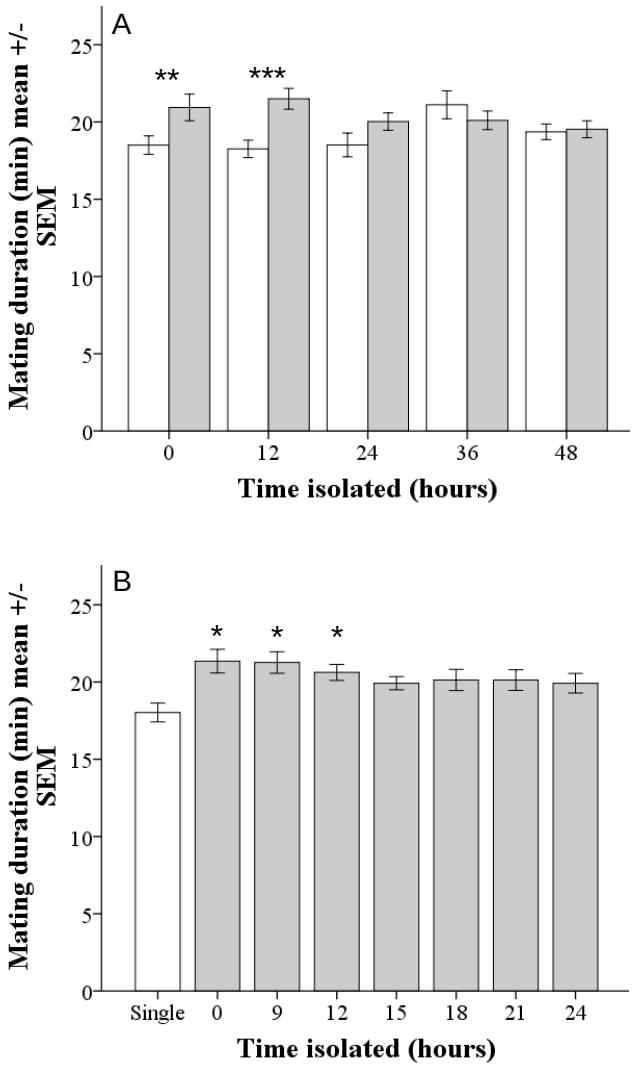

Figure 2: Effect of maintenance time on mating duration. Mating duration (mean +/- SEM) of males held singly or exposed to rivals for 72h. A) Males were held singly (white bars) or exposed to a rival (grey bars) then separated for 0-48h before mating. * indicates a significant difference between paired treatments (** < 0.01, *** < 0.001). B) Males were held singly or exposed to a rival and separated for 0-24h before mating. * indicates a significant difference compared to the single treatment, after Bonferroni correction.

2.4.2 Effect of exposure time on maintenance time

Length of exposure to a rival affected the maintenance of extended mating duration. Males exposed to rivals for 36h showed a similar pattern to those exposed for 72h (Experiment 1) and extended mating duration for at least 12h after removal of the rival (Experiment 3 MWU Z = -3.294, N = 76, p = 0.001; Figure 3A). This was not the case for males that had only been exposed to a rival for 24h before isolation (Experiment 4 MWU Z = -0.985, N = 71, p = 0.324; Figure 3B). We explored this further, finding that when males had been exposed to a rival for 24h (Experiment 5) only males isolated for 0h (MWU Z = -3.292, N = 75, p = 0.006) and 1h (MWU Z = -3.406, N = 72, p = 0.006) before mating mated for significantly longer than males never exposed to a rival (Figure 3C).

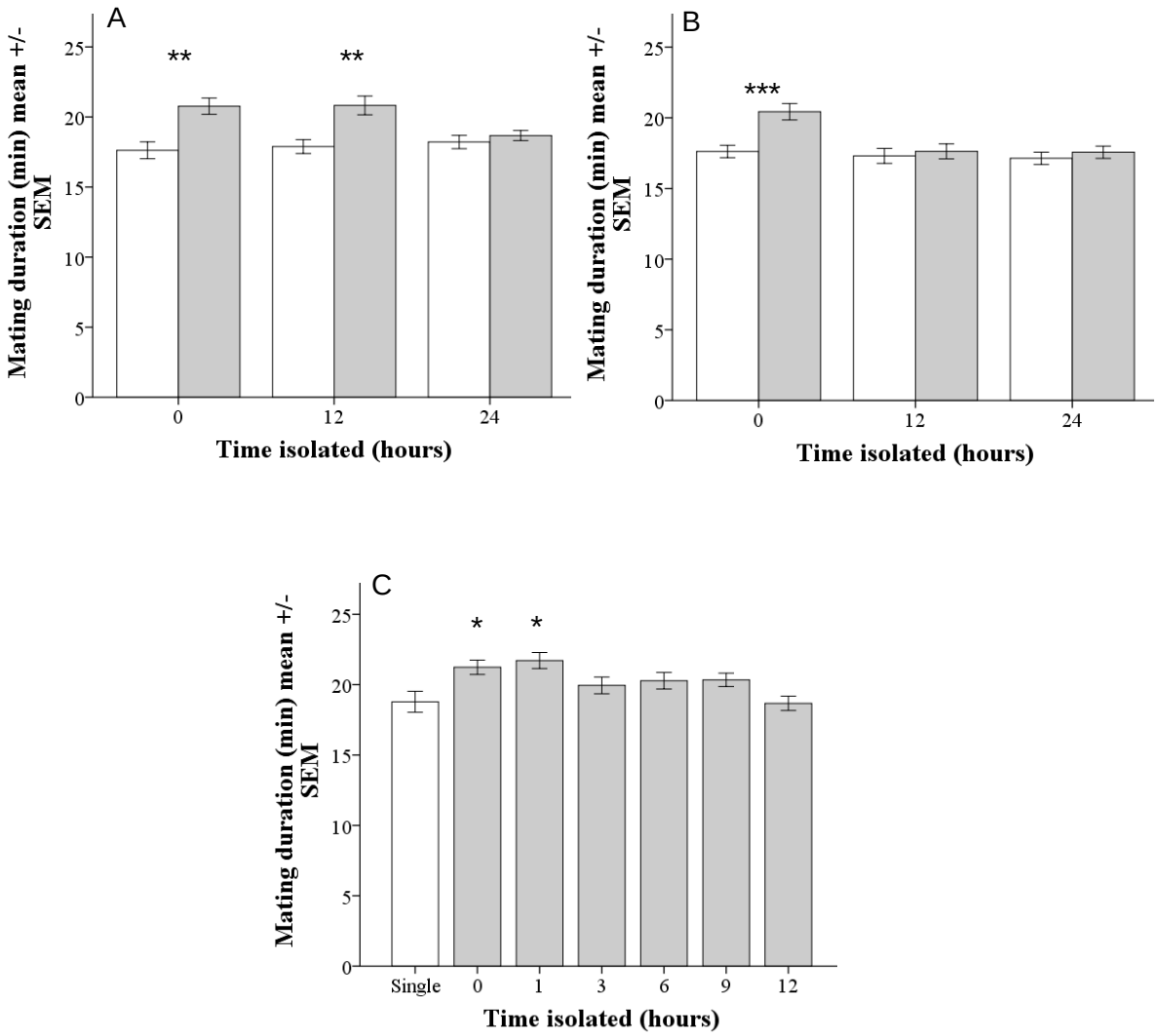

Fig 3: Effect of exposure time on maintenance time in response to a rival. Mating duration (mean +/- SEM) of males held singly or exposed to a rival for 36h (A) or 24h (B, C). A and B males were held singly (white bars) or exposed to a rival (grey bars) and separated for 0, 12 and 24h before mating. * indicates a significant difference between paired treatments (** < 0.01, *** < 0.001). C) Males were held singly or exposed to a rival then separated for 0-24h before mating. * indicates a significant difference compared to the single treatment, after Bonferroni correction.

2.5 Discussion

We show that the speed with which males can adjust their behaviour to a new sperm competitive environment is dictated by the length of time exposed to a rival. When exposure time was standardised to 72h, the increase in mating duration seen after exposure to a rival male was maintained for 12h after removal of that rival, in line with a previous report (Kim, Jan & Jan 2012). In addition, exposure time to a rival had a critical effect on the time the response was maintained. After 36h of exposure the behavioural response was maintained for at least 12h, similar to the pattern when males were exposed to a rival for 72h. However, after 24h exposure, a full response was realised but only persisted for 1h after removal of the rival. This suggests that whilst it is possible for males to alter behaviour shortly after a rival is removed, they do not if they have had at least 36h exposure.

Previously, we found it was necessary for males to be exposed to rivals for at least 24h before displaying an adaptive response, which could be considered a lag-time limit to plasticity. We suggested this time was required to enable a corresponding alteration of ejaculate components (Bretman *et al.* 2010), such as more seminal proteins (Wigby *et al.* 2009) or more/ better quality sperm (Garbaczewska, Billeter & Levine 2013; Moatt 2014). In *D. melanogaster*, the relationship between mating duration *per se* and ejaculate transfer is not straightforward (Gilchrist & Partridge 2000; Manier *et al.* 2010) but multiple studies have shown that its modulation in response to social contact does indeed affect fitness (Bretman, Fricke & Chapman 2009; Bretman *et al.* 2011; Bretman *et al.* 2012; Price *et al.* 2012; Bretman *et al.* 2013). Nevertheless, over successive matings, the duration response and fitness outcomes can become uncoupled (Bretman *et al.* 2012; Bretman *et al.* 2013), suggesting the behaviour alone does not alter fitness. This is the basis of our prediction that to build up the response requires time to produce more/ better quality ejaculate components. However, males could

reduce investment by transferring less ejaculate, so should immediately respond to the removal of competition, hence time to modulate ejaculate seems unlikely to explain why there is a time lag between removal of the rival and the decrease in mating duration. After 24h exposure, males do respond to rivals but reduce mating duration within 1h after the rival is removed. Males are therefore capable of making rapid adjustment to their behaviour, though here we did not measure whether there is a corresponding speedy adjustment to ejaculate transfer.

Theory generally predicts that males should invest more as sperm comeptition risk (probability of a female remating) increases. However, with respect to sperm competition intensity (number of competing ejaculates), investment should be maximised with one rival, as thereafter potential fitness returns diminish with each additional competitor (Parker *et al.* 1996; Parker *et al.* 1997). However there are many variations to these models incorporating factors such as the quality of information available to the male, female quality and male age, experience and condiditon (Parker & Pizzari 2010). In our *D. melanogaster* example, exposure time might give males information about both risk and intensity. However, previous work showed that males were not sensitive to the number or density of rivals (Bretman *et al.* 2010), suggesting that the critcal determinant of fitness is whether or not a male is in competition, rather than with how many other males. A further consideration is whether males respond to population mean rather than immediate threat. Longer exposure times might indicate that even though the immediate competitive threat is removed, the mean competition within the area or population is high and therefore greater investment should be maintained as insurance against sudden increases in local competition. Indeed, recent evidence suggests that males can be primed for the average levels of sperm competition within the population if they receive cues as larvae (Bretman *et al.* 2015), which might be 10 days before they become adult and are subject to that competition. Males raised in the presence of adult males or in

high larval densities developed larger accessory glands (Bretman *et al.* 2015) and the latter condition also increased their relative allocation of seminal fluid proteins when adult (Wigby *et al.* 2015). However, developmental environment was not found to affect adult behavioural strategies, suggesting that cues received as juveniles can accurately predict the average population level of sperm competition but are a relatively poor indicator of immediate competition at any particular mating (Bretman *et al.* 2015).

Our findings suggest that responding to the addition or removal of rivals immediately may not be the best strategy; hence we might question whether this time-lag is a true limit to plasticity or is actually adaptive. If the competitive environment can change rapidly, cues could be transient and therefore misleading to an individual about the level of competition in the environment. Males may therefore be better to wait and become certain of the level of competition before creating a potentially costly response. To disentangle whether the lag-time between environmental change and a change in behaviour is adaptive it would be informative to uncover the mechanism controlling the plastic behaviour. If the mechanism shows behavioural change could be accomplished without a time-lag, this suggests the time-lag is adaptive within the changing environment. For animals such as *D. melanogaster* that are difficult to observe in the wild, it is unlikely we could accurately measure the natural timescale of their social interactions. Nevertheless, it is likely that the environment varies, as without this variation the plasticity in responses should be maintained or initially evolve (Carroll & Corneli 1995). Given flies will aggregate around food sources, we can speculate that males could spend 72h in intense social contact (Stamps *et al.* 2005; Reaume & Sokolowski 2006). Conversely they could be socially isolated for 12h or longer, for example, when migrating between food patches or sheltering from adverse weather conditions, and in this context the ability to remember a previous social contact should be beneficial

(Stamps *et al.* 2005; Reaume & Sokolowski 2006). We employed manipulations where males were continuously with or without a rival for given periods, however natural fluctuations could occur at much shorter timescales than we tested.

In other contexts, differences in the time periods between presentations of cues can affect behavioural responses of flies. Associative learning involves training flies to associate a smell with a shock or reward and leads to different lengths of memory consolidation depending on the speed with which flies are presented with cues (Tully *et al.* 1994). When presented with cues multiple times in a spaced manner flies form long-term memory (LTM) and can remember an association for up to 7 days. Flies presented with cues on mass form protein synthesis independent memory, or anaesthesia resistant memory (ARM) and remember the same information for a shorter amount of time (Scheunemann *et al.* 2012). When viewed through our paradigm males are constantly exposed to a stimulus, even when not in physical contact, due to another male's smell (Gaudry, Nagel & Wilson 2012), and so are exposed to the equivalent of massed training. ARM builds up slowly, reaching asymptomatic levels after 2 hours and lasting up to 24 hours (Margulies, Tully & Dubnau 2005), a time period I have already discussed as potentially adaptive in terms of mating competition. In flies, ARM and LTM build up simultaneously but are mutually exclusive after multiple training trials (Placais *et al.* 2012). Training for both memory types is seemingly additive, in that increased training causes an increase in memory length (Margulies, Tully & Dubnau 2005), something that is also shown in the results here, though after an initial investment of 24 hours in a high sperm competition environment (Bretman *et al.* 2010). These multiple forms of memory are important when learning in a novel environment, as it allows organisms to create different temporal behavioural outputs from differing experiences. For example, parasitic wasps (*Cotesia glomerata* and *Trichogramma evanescens*) use differing memory types to remember the spatial distribution of

butterfly eggs depending on the species of butterfly. Butterflies that lay a greater number of eggs elicit longer term memory (controlled by LTM) in the wasp than single egg species where memory forms via ARM, allowing wasps to forget information at different speeds depending on the reward (Kruidhof *et al.* 2012). This is especially advantageous in extremely changeable environments where individuals can gain fitness from constantly updating learned information and changing their behaviour accordingly (Burns, Foucaud & Mery 2011).

Another learning and memory assay that parallels the paradigm I use here is that of courtship suppression, whereby male *D. melanogaster* exposed to unreceptive (recently mated) females learn to reduce courtship effort (Kamyshev, Iliadi & Bragina 1999). Similar to the response to rivals, exposure time to female cues is important, but interestingly, discrete training periods rather than constant contact is required for males to consolidate this from short term to long term memory (McBride *et al.* 1999). Although these behaviours show parallels, they may be quite cognitively different tasks: Courtship suppression is somewhat binary (i.e. learning a cue that the female is or is not receptive) whereas responding to rivals requires remembering an amount of time spent with a rival male as a proxy for the probability of future competition. As the pathways controlling courtship suppression and associative learning are well documented (Griffith & Ejima 2009), it will be fruitful to compare the learning and memory mechanisms involved.

Throughout this and much of our previous work, a single fly has been used as a competitor. Although in natural settings it might be expected that multiple males would simultaneously or successively interact, in a laboratory setting neither number nor density of rivals affects the magnitude of the response (Bretman *et al.* 2010). In addition, we used virgin flies throughout, and although the focal could be sexually experienced in the wild, this does not affect the mating duration response we report here (Bretman *et al.* 2012). Similarly, males can employ plastic sperm

competition strategies depending on female mating status, quality and age. For example, in *D. melanogaster* males can respond to female mating status by altering sperm number (Luepold *et al.* 2011) and seminal fluid composition (Sirot, Wolfner & Wigby 2011), though it should be noted that the direction of this response (i.e. more investment in mated or virgin females) is not consistent across studies (Friberg 2006). The mating status of the female has been shown not to affect the extended mating duration in response to rival-exposure (Bretman, Fricke & Chapman 2009), and females having little ability to control mating length once mating had begun (Bretman, Westmancoat & Chapman 2013). Nevertheless, future work could test whether male experience or age, or female mating status, alters the speed with which males respond to changes in competition cues or change ejaculate amount.

To further our understanding of the neuroecology and evolution of recognition systems, and plasticity in general, we need to examine these processes mechanistically, at neuronal, biochemical and genetic levels. Here *D. melanogaster* offers significant advantages, as it is a very well established model for exploring learning and memory mechanisms, in many ways already discussed (Margulies, Tully & Dubnau 2005; McGuire, Deshazer & Davis 2005; Ejima *et al.* 2007).

In conclusion, we have shown that in *D. melanogaster*, the speed of behavioural responses to sperm competition rivals is affected by prior exposure time and sensory cues. Behavioural plasticity is thought to be a cheap and fast way to cope with environmental change, yet we show that males do not always respond to changes in their competitive environment as quickly as they are capable. Our findings could be interpreted as limitations of plasticity, or alternatively that both the lag-time allows males to quantify sperm competition threat within a population. These findings could therefore have important implications for understanding context dependent decision making, especially as this *Drosophila* model will enable future studies to dissect these processes at many mechanistic levels.

Sensory limits affect temporal dynamics of a male's response to sperm competition

3.1 Summary

Organisms obtain information from the environment using a wide range of sensory cues. These cues can interact with each other to form multimodal sensory systems needed to distinguish specific individual types and so enable reactions to subtle modulations in an environment. The ability to respond quickly to changes in the environment is important, but is at risk when the sensory system employed is not optimal for the task. This may lead to information reliability limits to plasticity that could slow an individual's response to an environmental change. In *Drosophila melanogaster*, multiple senses interact to enable males to evaluate the make-up of the competitive environment, and so control any subsequent mating duration changes. However, if a single sense is removed it has no effect on the overall change in behavioural response to increases in sperm competition. In this chapter, I remove individual senses and measure a male's ability to respond to the addition of a rival over 24 hours. I also investigate the effect of sense removal on maintenance of the response to rivals over 24 hours. I reason that removal of single senses should not affect the speed of the build-up or maintenance of the behavioural response due to compensation from other senses enabling multimodal communication. However, removal of multiple senses should increase the amount of time males take to respond to changes in the competitive environment.

When single senses were removed males were slower to extend mating duration in response to an increase in sperm competition in comparison to normal males. However, single sense removal did not affect the rate at which males were able to match mating duration to a decrease in sperm competition. This suggests a more accurate sensory mechanism is needed when reacting to competitive cues than when reacting to a lack of competition. It may also show that reacting to

competition is more costly than failing to match a non-competitive environment so needs to be more tightly controlled. The change in temporal dynamics depending on sensory removal when reacting to an increase in sperm competition supports information-reliability as being a limit on plastic behaviour.

3.2 Introduction

Organisms extract information from the environment using a wide range of sensory cues (Munoz & Blumstein 2012). These can inform an individual about the location of food, mates and potential predators that allows the organism to respond to the environment in a suitable manner (Bro-Jorgensen 2010). Multiple sensory cues interact to form an accurate picture of the environment, i.e. are multimodal, and cross reference to maximise the accuracy of any sensory signal (Johnstone 1996). The need for one sense in preference to another is driven by an individual's ecology and the characteristics of the cue. For example, chemical cues are more important than visual cues when determining species recognition in shoaling fish due to impaired vision in murky water and the high dispersal characteristics of chemicals in this substrate (Ward, Axford & Krause 2002).

To be able to create an accurate environmental picture, information must be sensed and assimilated to identify change on a time scale similar to any variation occurring within an environment. Single cues that endure in the environment can give outdated information. For example, chemical cues can signal predator presence but may remain in the environment after the predator is gone (Ward & Mehner 2010). If organisms receive outdated information it could lead to a loss of fitness due to inappropriate actions leading an individual into danger, or away from opportunities. DeWitt and colleagues (DeWitt, Sih & Wilson 1998) defined this as information-reliability limits to plasticity, where limits to the ability to collect accurate sensory information, or pick-up misleading cues, causes an organism to mismatch

its phenotype to the environment. This leads to a subsequent loss of fitness compared with a less plastic individual and increases the need for a multimodal sensory system that can reduce inaccuracy in an individual's response to change, so reducing costs of mismatching to an environment.

The ability to respond to cues quickly and accurately is especially important when reacting to changes in the social environment due to quick fluctuations in potential disease transmission, predation pressure and intraspecific competition. Individuals in any group will release cues to inform other members of their dominance (Cornwallis & Birkhead 2008) and condition (Scheuber, Jacot & Brinkhof 2004). This can then be used by receivers to modulate their behaviour depending on the make-up of the social group. In *D. melanogaster*, male flies modulate their mating duration depending on the presence or absence of a competitor male, however, display either a 24 (Bretman *et al.* 2010) or 12 hour (Rouse & Bretman 2016) lag-time depending on whether they are reacting to the addition or removal of a rival (Rouse & Bretman 2016). In addition to this time lag, information-reliability limits should change how quickly males respond to changes in the environment. However, one sense may not have an effect if competition assessment relies on multimodal cues, such as *Drosophila*, whose senses show functional redundancy when reacting to mating rivals (Bretman *et al.* 2011). Therefore, loss of a competitive cue should be covered by the remaining senses, allowing males to respond to a change in the environment successfully and at the same rate as a male without sensory loss.

In this study, we use *Drosophila melanogaster* males to investigate how the loss of different sensory inputs affects the speed of behavioural plasticity induced by changes in the competitive environment. This gives us some understanding of the theorised sensory limits to plasticity and how multimodal sensory systems work to enable individuals to react quickly to their current environment.

3.3 Material and Methods

3.3.1 Fly stocks and husbandry

Experiments were conducted in a 25°C humidified room with a 12 hour light: 12 hour dark cycle (9 am to 9pm light cycle), using plastic vials (75x25mm) with 7 ml standard sugar-yeast-agar medium (Bass *et al.* 2007). For sensory experiments all wild type flies were the Dahomey strain as in our previous studies. Wildtype larvae were raised at a standard density of 100 per vial. At eclosion, flies were collected and sexed using ice anaesthesia, and stored 10 per vial. Females were supplemented with live yeast granules.

Where Canton-S strains were used the stocks were generously donated by Dr Tom Price. Flies were grown in vial by placing 5 males and 5 females in a vial and allowing them to mate. Again, at eclosion, flies were collected and sexed using ice anaesthesia, and stored 10 per vial.

Orco^{2} flies were generously donated by Tracey Chapman and were also grown in vial by placing 5 males and 5 females together and allowing them to mate. Offspring were collected at eclosion and sexed using ice anaesthesia. As all experiments were internally controlled (had a fly isolated and with rivals) strain differences were accounted for within any mating duration experiments.

3.3.2 Testing effect of vision on extended mating duration in two different strains of *Drosophila melanogaster*

There are previous conflicting reports about the role of vision in extended mating duration. One investigation concluded that any paired combination of auditory, olfactory and tactile cues were needed for males to recognise a rival presence (Bretman *et al.* 2011), whereas the other found that vision alone was enough for a male to extend mating duration (Kim, Jan & Jan 2012). To investigate the role

played by vision in controlling a male's ability to respond to a rival an experiment from Kim and colleagues (2012) was repeated using mirrors (12 mm diameter, 113.1 mm^2) to simulate the moving red eyes of a rival male *D. melanogaster*. Two strains of *D. melanogaster* were tested for their ability to extended mating duration using vision only, Dahomey, which has been used in Bretman et al (2011), and Canton-S, used in Kim et al (2012). In their respective studies both strains had shown extended mating duration in response to a rival presence. To simulate the presence of a rival through visual cues only mirrors were placed at the bottom of a vial with an otherwise single male. Two controls were used, focal males housed with a rival to control for day effects on mating duration, and a focal male placed with an upside-down mirror to control for any effect of mirror presence. Flies were kept in these environments for 3 days before being mated with a virgin female and the mating duration calculated. All manipulations were performed on both Dahomey and Canton-S strains. This give sample sizes of at least 30 individual male flies for all treatments across both strains of *D. melanogaster*.

3.3.3 Effect of sensory deprivation on speed of behavioural response

To investigate whether sensory deprivation would affect maintenance or build-up of extended mating duration, we manipulated olfactory and auditory cues as in our previous work (Table 1) (Bretman *et al.* 2011). To remove auditory signals rival male wings were removed under CO_2 anaesthesia (Figure 1). We removed olfaction by, using focal males mutants lacking *odorant receptor 83b (Orco^2)*, a co-receptor responsible for perceiving 80% of *D. melanogaster*'s odour range (Larsson *et al.* 2004). We also used wild type males with their 3rd segment of antennae removed under CO_2 anaesthesia (Figure 1), which removes sensillae required for males to respond to odour cues (van Naters & Carlson 2007) and also aristae which contribute to detection of sound (Gopfert & Robert 2002). All sensory manipulations

were performed before any behavioural experiments were undertaken. Flies were given at least 24 hours to recover. The first experiment focused on maintenance time of a response used focal males kept in the plus-rival treatment for 72h before isolating them for 0, 12 and 24h before mating. This gave sample sizes of 26 to 40 male flies for each group. To measure the effect of sensory manipulations on the build-up of the mating duration response over 29h males were collected singly before being exposed to rivals for 20, 24 and 29h prior to mating. This gave sample sizes of between 23 and 40 male flies for each group. Importantly in all experiments, comparisons were only made between males with the same sensory manipulation kept singly or with a rival, hence controlling for manipulation or genetic background effects

Table 1: Description of experiments 2 and 3 providing information about the exposure time, maintenance times and whether the dynamics of the behaviour changed compared to unmodulated males.

Experiment	Sense modulation	Exposure time (h)	Maintenance time (h)	Changed from unmodulated
2	Wing removal	72	0, 12, 24	No
	Orco^{2}	72	0, 12, 24	No
	Antennae removal	72	0, 12, 24	Yes
3	Wing removal	20, 24, 29	0	Yes
	Orco^{2}	20, 24, 29	0	Yes
	Antennae removal	20, 24, 29	0	Yes

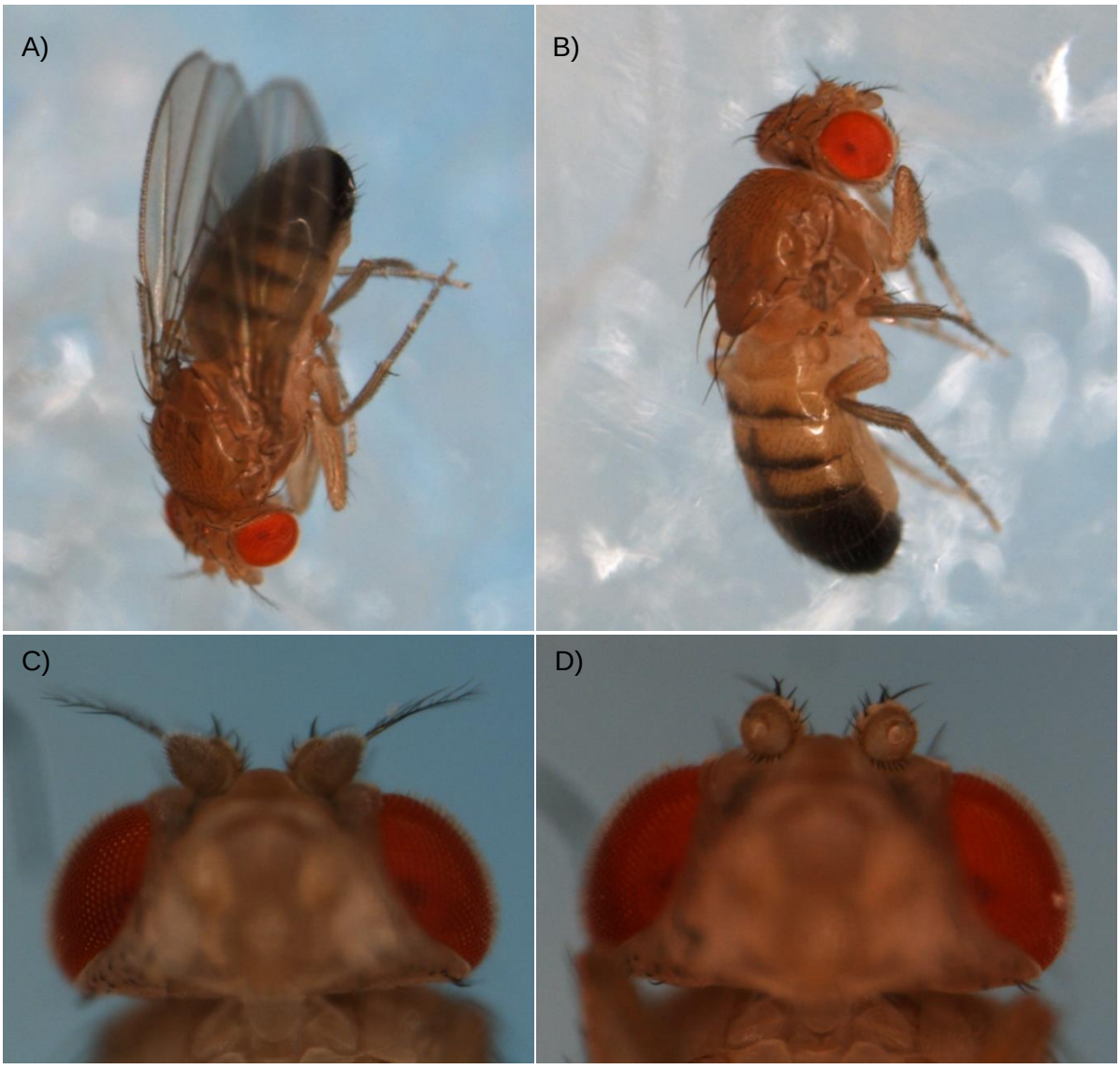

Figure 1: Physical sensory manipulations. A) No manipulation wildtype Dahomey. B) Wildtype Dahomey with wings removed (auditory cues) Magnification = 20 x C) 3rd segment of antennae intact. D) 3rd segment of antennae removed. Magnification = 100 x

3.3.4 Statistical analysis

Statistical analysis was performed using SPSSv14 and R v 3.3.1 (Ihaka & Gentleman 1996). To test the effect of vision on extended mating duration data was analysed using a GLM with quasi Poisson errors (accounting for under dispersion). Strain and rival treatment were fixed factors and Analysis of deviance was used to reduce from full to minimal model. Differences between the two strains were compared using a Mann-Whitney U test, with rival treatments then compared using *post hoc* Tukey pairwise comparisons (single vs upside-down mirror, single vs paired, upside-down mirror vs paired). Bonferroni correction was used for multiple comparisons. To test the effect of sensory deprivation on the time it took for males to build-up and maintain extended mating duration, comparisons between three or more treatments were made using ANOVA with Dunnetts post-hoc tests and pairs of treatments using t tests if the data was normal. If data did not meet the assumptions of these tests then Kruskall-Wallis (K-W) or Mann Whitney U tests (MWU) were used (as indicated in the Results section). To reiterate, where the design permitted, the key comparisons were between males kept singly or with a rival but treated the same in all other respects, as this controlled for any other manipulation effects.

3.4 Results

3.4.1 Testing the effect of vision on extended mating duration in two different strains of *Drosophila melanogaster*

I tested male reactions to visual cues achieved by placing a mirror with an otherwise single male. There was no significant interaction between strain and rival treatment, with both Dahomey and Canton-S strains responding in the same way to their pre-mating environment (AOD: $F_{1,203} = 0.629$, $p = 0.429$). There were significant effects

of both strain and rival treatment. When the effect of rival treatment was investigated further paired males were shown to mate significantly longer than both single males ($p = 0.006$) and males kept with an upside-down mirror ($p < 0.001$; Figure 2). Both single males and males previously housed with an upside-down mirror did not differ in their mating duration ($p = 0.964$). Overall, Dahomey flies mated for longer than Canton-S flies (Mann Whitney U: $Z = 6947.500$, $N = 207$, $p < 0.001$).

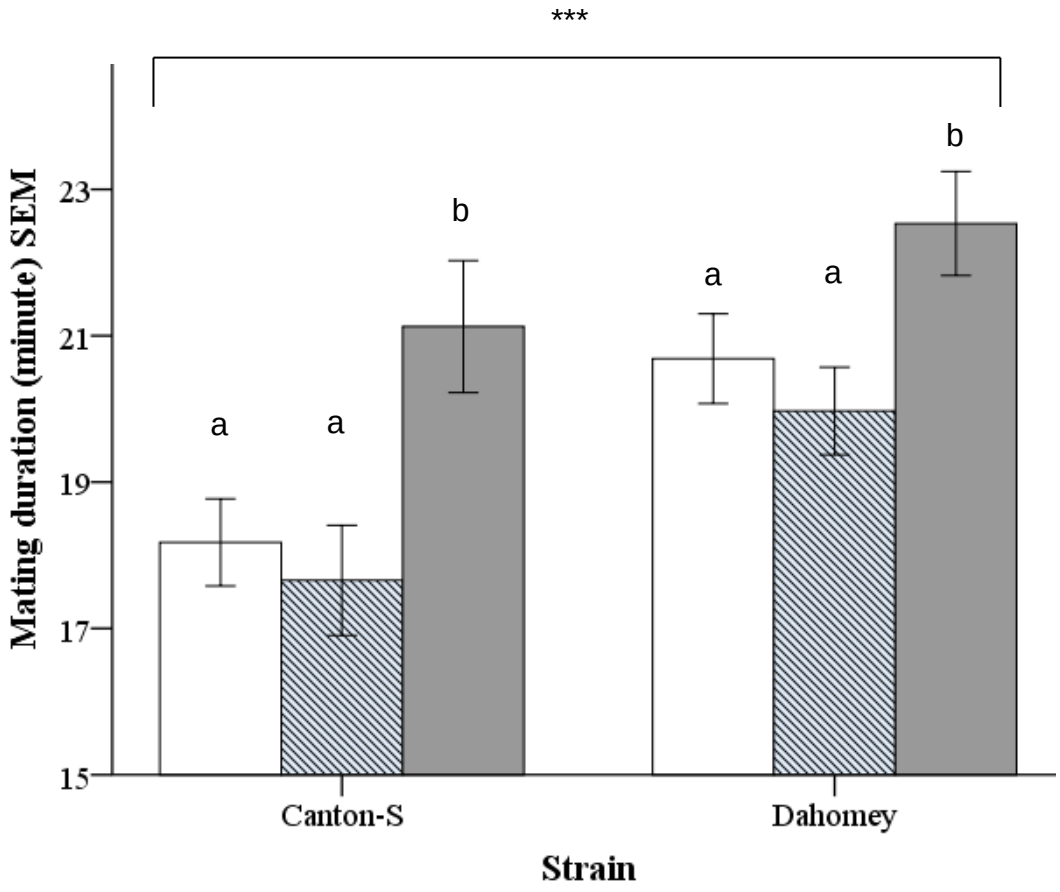

Figure 2: Singular visual cues are not enough to invoke extended mating duration. Mating duration of males held singly (white bars) with a mirror (hashed bars) or with rivals (dark grey bars). Significance between the two strains is represented by the overarching bar. Within strains, significance is represented by letters. Same letters represent no significance between two groups, different letters represent a significant difference between two groups. Error bars represent SEM. * indicates a significant difference between paired treatments (* $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$).

3.4.2 Effect of sensory deprivation on the build-up of response to rivals

I investigated how sensory manipulations affect the speed with which males built up a response to rivals. Previous work has shown that males respond to a rival male presence after 24h exposure time (Bretman *et al.* 2010), confirmed in Chapter 2, Fig 3c. However, in each of our sensory manipulations we found no significant increase in mating duration even after 29h exposure to a rival (Wing removal: K-W X^2_3 = 7.774, p = 0.500; Fig 3*a*. *Orco*^{2}: ANOVA $F_{3, 80}$ = 1.302, p = 0.280; Fig 3*b*. 3rd segment: ANOVA $F_{3, 94}$ = 1.589, p = 0.197; Fig 3*c*). This suggests that sensory deprivation increases the lag-time between environmental change and behavioural response.

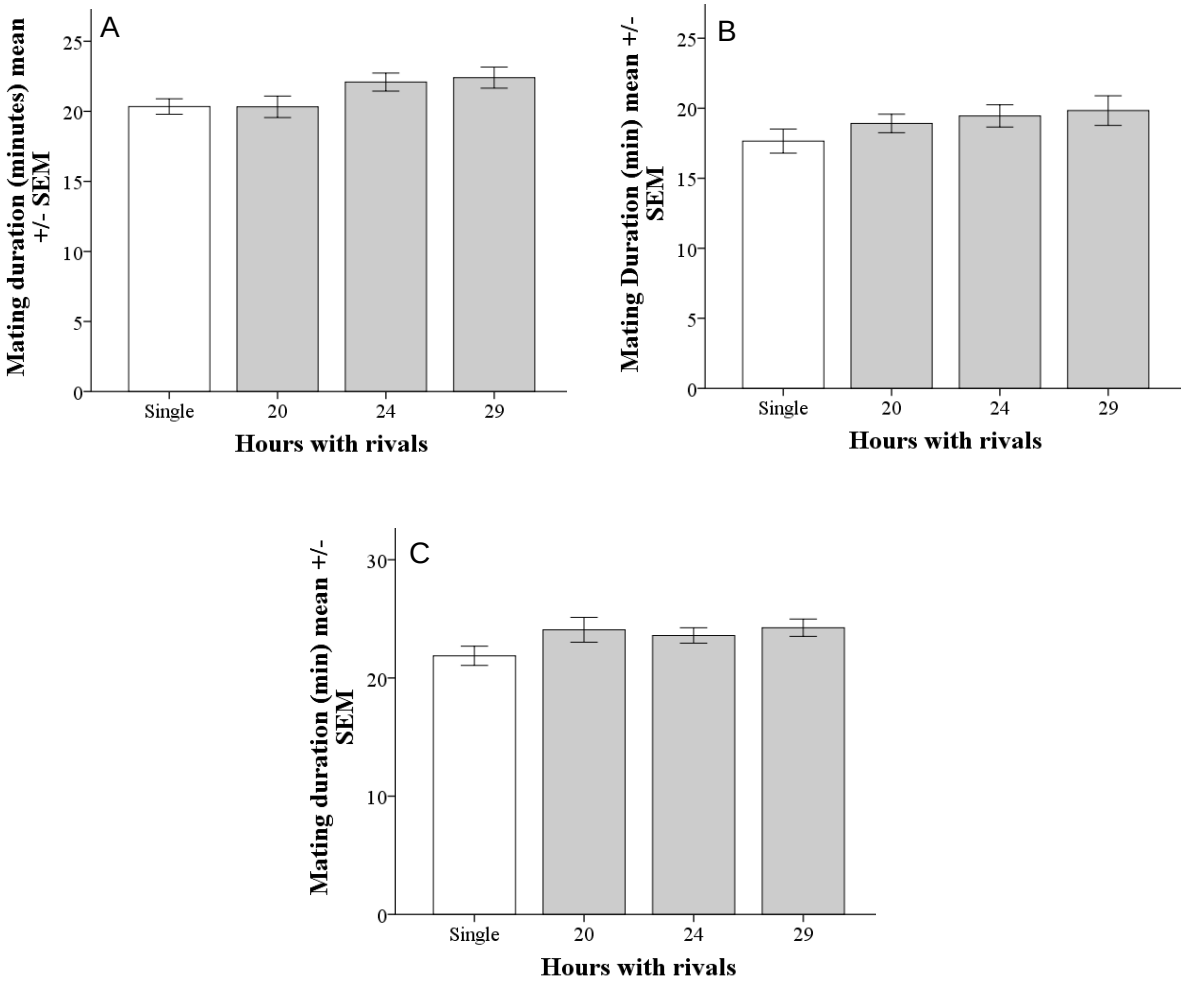

Figure 3: Sensory deprivation affects build-up of sperm competition response. Mating duration of males held singly (white bars) or with rivals (grey bars) for 20, 24 and 29 h before immediate mating to females. A) Males maintained with wingless rivals. B) *Orco*^{2} focal males lacking odorant co-receptor. C) Wild type focal males with the 3rd segment of their antennae removed. Error bars represent standard errors. There was no significant difference between any of the paired treatments.

3.4.3 Effect of sensory deprivation on the maintenance of response to rivals

I also tested how sensory manipulations modulated the maintenance time of extended mating duration. Males exposed to rivals but not receiving auditory (wing-removed rivals) or olfactory cues (use of *Orco*^{2} mutants) showed a pattern similar to unmanipulated wild type flies (Chapter 2, Fig 3a). These males increased their mating duration for 12h after removal of the rival (Wing removal: MWU Z = -2.812, N = 73, p = 0.005; Fig 4*a*. *Orco*^{2}: MWU Z = 2.388, N = 58, p = 0.017; Fig 3*b*), but not after 24h isolation (Wing removal: t_{73} = -0.659, p = 0.512; Fig 4*a. Orco*^{2}: t_{51} = -1.124, p = 0.266; Fig 4*b*). In contrast, when the 3rd antennal segment was removed, males continued to extended mating duration for 24h (MWU Z = -2.891, N = 66, p = 0.004; Fig 4*c*), ~10h longer than unmanipulated wild type males.

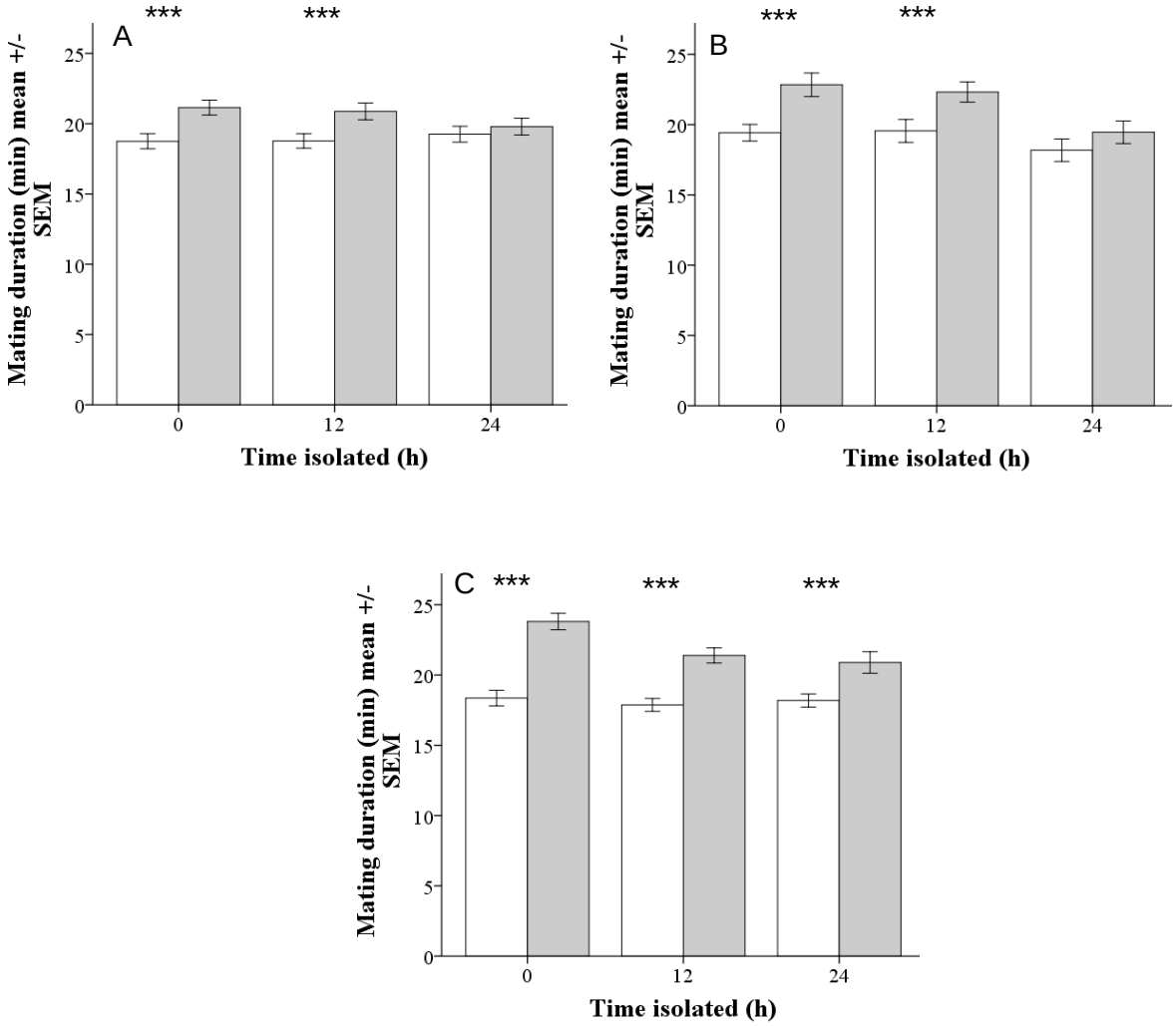

Figure 4: Sensory deprivation effects on maintenance of extended mating duration. Mating duration of males held singly (white bars) or with rivals (grey bars) for 72h before being isolated for 0, 12 or 24 h. A) Males maintained with wingless rivals. B) *Orco*^{2} focal males lacking odorant co-receptor. C) Wild type focal males with the 3rd segment of their antennae removed. Error bars represent standard errors * indicates a significant difference between paired treatments (** $p < 0.01$, *** $p < 0.001$).

3.5 Discussion

In this chapter I show that vision as a single sense has no effect on a male's ability to respond to a rival male, supporting previous work (Bretman *et al.* 2011), but bringing into doubt other findings from a separate lab (Kim, Jan & Jan 2012). In addition, we have also shown that the speed of a male's response to fluctuations in the socio-sexual environment changes depending on the cue received from that environment and whether males are responding to the presence or absence of said cue. Removal of a sensory cue had no effect on a male's ability to reduce his mating duration when removed from a high sperm competition environment. However, it did reduce the ability of a male to respond as rapidly to the introduction of a rival male than if left with a whole suite of cues about the environment. This suggests sensory cues received from the environment are not totally redundant, as previously suggested (Bretman *et al.* 2011), and that it is more costly to incorrectly respond to the presence of rival males than it is to decrease mating duration when sperm competition may still be high.

3.5.1 Testing effect of vision on extended mating duration in two different strains of *Drosophila melanogaster*

There have been conflicting reports on the importance of vision in promoting male extended mating behaviour in response to increased sperm competition. Previously, it had been suggested that males reacted to visual cues, specifically moving red eyes, when extending mating duration (Kim, Jan & Jan 2012). My data show that the image of a rival male was not enough to induce either a Dahomey or Canton-S male to significantly extend mating duration. If a male did indeed respond to moving red eyes then an increase in mating duration would be seen whenever a male interacted with any species of *Drosophila*, not just their own. This is in contrast to reports showing that *D. melanogaster* males do not extend mating duration in

response to a *D. simulans* presence (Price *et al.* 2012), it also makes little evolutionary sense, as males of a species under no competition would incur lifetime fitness costs due to the presence of a non-competing species male (Bretman *et al.* 2013). The weight of evidence therefore suggests that previous work to look at the sensory signals underlying a male's ability to respond to rivals (Bretman, Gage & Chapman 2011) is a good place to start when investigating sensory limits to plasticity in this model. Other authors have explained the discrepancy between the two studies discussed above by referencing differences in the two strains used (Maguire, Lize & Price 2015); however, this does not seem to act as a full explanation as each strain has been kept under the same evolutionary pressures (very high mating in a cage) where benefits of one specific sensory cue over all others (as shown for the Canton-S strain) would be unusual. Our results show the same pattern of mating duration for both strains, which does not support the idea that the two strains have evolved different sensory systems to recognise rival males. However, it does open up the possibility the differences between the two studies may be a result of different lab environments or viewers. In particular, there are discrepancies between sample sizes and methods across the two studies. Bretman and colleagues (2011) measured mating duration to the nearest minute for a sample size of at least 30. In comparison, Kim and colleagues (2012) measured mating duration every 10 seconds but used sample sizes as small as 14. In addition, to create a high sperm competition environment Kim and colleagues placed four males within the same environment, potentially creating pseudo-replication and reducing sample sizes by a factor of 4. Another area where the two studies used different methods is in the transfer of males between environments, specifically in the use of CO_2 anaesthesia, an anaesthetic proven to affect *D. melanogaster* behaviour for up to 24 hours (Barron 2000; Bartholomew *et al.* 2015). Bretman and colleagues (2011) aspirated males from housing to mating chambers whereas Kim et al (2012) anaesthetised males using CO_2. Overall, considering the weight of evidence

suggesting vision is unimportant for males to extend mating duration in response to increased sperm competition (Bretman *et al.* 2011; Maguire, Lize & Price 2015) the genetic dissection of extended mating duration carried out by Kim and colleagues (2012) will be treated with some caution within this thesis.

3.5.2 Effect of sensory deprivation on the maintenance and build-up of response to rivals

A hypothesised limit to plasticity is that of information-reliability (Auld, Agrawal & Relyea 2010), specifically that plastic responses require sensory recognition systems that accurately perceive and process environmental information. In our paradigm this means sensing that another individual poses a sperm competition threat, with senses possibly incorporating information about whether the rival is conspecific, male and sexually mature. Senses may also have to include information about the time a rival has been present within an environment and incorporate this into a measure of rival persistence. It has previously been suggested that because males lacking one sense are still able to respond to increases in sperm competition, this shows sensory redundancy (Bretman *et al.* 2011). Which would be in line with the redundant signal hypothesis for sexual ornamentation (Zuk, Ligon & Thornhill 1992). In the current study, males could still respond to a lack (i.e. reduce mating duration) or increase of competition (i.e. increased mating duration) when single senses were manipulated (either 80% olfaction through use of *Orco*^{2} or auditory through removal of rivals wings). However, the speed of behavioural response was reduced compared to unmanipulated males (Bretman *et al.* 2010; Rouse & Bretman 2016) suggesting senses are not fully redundant and there is an element of information-reliability relied upon to achieve extended mating duration that matches the timescale of environmental change. In a simple odour associative learning task in *D.*

melanogaster, odour detection was not the rate limiting step in decision making, but when faced with more difficult tasks (distinguishing between low-contrast stimuli), flies took longer to gather information before making a choice (DasGupta, Ferreira & Miesenboeck 2014). Arguably integrating information from multiple cues to detect the presence or absence of a rival is rather more cognitively challenging than such single–odour tests. Nevertheless, this might suggest that in responding to rival males, the rate at which sensory information can be gathered does not impose a limit on plasticity, but the task becomes more difficult (though not impossible) when senses are removed.

Another point of interest is the time difference between the build-up of the behavioural response and the maintenance of the same behavioural response when senses were removed. The speed of response patterns are not fully symmetrical; whilst removal of one sense affected the build-up of the response when exposed to a rival, single-sense manipulations had no effect on the decline of the response when the rival was removed. However, removal of the 3rd antennal segment, which inhibits both olfaction and hearing (Gopfert & Robert 2002), affected both build-up and decline of mating duration. This raises the question of whether the build-up of the response is more sensitive to information-reliability limits, perhaps because it is likely to be more costly to make the wrong decision and build-up a response when competition is low than to maintain it once competition has been removed (Bretman *et al.* 2010). As a caveat to this idea, it is important to note that the build-up of mating duration was tested more frequently in the same time period than the return of mating duration to pre-exposure levels. Whereas the build-up of extended mating duration was tested every 3 hours, the loss of the same behaviour was tested on a 12 hours period. Therefore, it is possible that the return of mating duration to pre-exposure levels occurs at the same or similar rate to the build-up of the response but is hidden by the lack of resolution in the experimental set-up.

One possible way for there to be differences between the senses required in the build-up versus loss of the extended mating response may come from the different spatial quality needed for each cue to be processed. Cues work on different spatial and temporal scales, meaning in a multimodal sensory system cues are processed in an additive fashion. A long-range cue can alert an individual to the potential for a secondary cue, thereby enhancing the detectability of the second cue (Rowe & Guilford 1996; Rowe 1999). In fish chemical cues that provide information about predator risk act to 'prime' other senses that work on a closer spatial scale (mosquitofish, *Gambusia holbrooki* Ward & Mehner 2010; Guppies, *Poecilia reticulata* Stephenson 2016). Chemical cues are inaccurate and may provide outdated information about the presence of a predator (Ward & Mehner 2010) and so individually do not elicit a behavioural response. In this way fish show no behavioural change to an olfactory predator cue but react rapidly when other cues concur with this first warning. Similarly, male wolf-spiders (Family Lycosidae) use a form of multimodal communication to attract females. It has been suggested that these different modes are important to the female at different stages of pre-copular due to differences in distances females are able to detect and recognise different cues (Barth 1993; Uetz & Roberts 2002). Within this model long distance cues are used by multiple species, making them an important but inaccurate way to initially recognise a potential partner (Barth 1993). Cues that work on a closer spatial scale are then used to differentiate between potential partners and individuals of a different species.

In *D. melanogaster*, long range cues are typically thought of as vision or smell (Gaudry, Nagel & Wilson 2012), with smell the only cue out of the two currently known to affect other fly behaviour (Farine, Ferveur & Everaerts 2012). When reacting to a rival, the removal of a male ability to smell through the use of *Orco*^{2} mutants may therefore have the effect of dampening other cues that act on a

closer spatial scale e.g. mechanosensory stimuli, by failing to 'prime' other senses to a potential rival presence. In comparison, when rivals are removed, focal males immediately lose all cues that work on a close spatial scale. This leaves only long-range cues that act to 'prime' behaviours, but these do not elicit a behavioural response by themselves. As wildtype males do not respond to these cues individually (Bretman *et al.* 2011), probably due to the potential inaccuracy of long-range cues, this could explain why the removal of smell in manipulated males does not create a different temporal dynamic in extended mating behaviour when compared to wildtype males.

In parallel to the senses involved in extended mating duration, courtship suppression, as discussed in chapter 2, uses a similar sensory repertoire. In brief, courtship suppression exposes males to a mated trainer female, causing males to reduce courtship towards any subsequent tester females regardless of their mating status (Griffith & Ejima 2009). Within this paradigm, males use visual, olfactory and auditory cues to find and court potential mates (Ejima & Griffith 2008). Similarly to extended mating duration, the removal of any one sense will not stop courtship suppression; suggesting behaviour is controlled by redundant signalling (Ejima *et al.* 2005; Krstic, Boll & Noll 2009). Although changes in the speed of behavioural plasticity have not been explicitly investigated in this model, differences in how the test is carried out give us some idea of how senses interact to efficiently suppress courtship. When carried out in a larger chamber, male ability to characterise the tester female is reduced and training has to last for longer to create the same effect as training in a smaller chamber (Griffith & Ejima 2009). This mirrors the reduction of a male's ability to process long-distance communication, slowing down the speed in which plastic behaviour can change, and supports the ideas in our study.

In contrast to the single-sense manipulations, removal of the 3rd antennal segment, affected both build-up and decline of the response. This manipulation

likely inhibits both olfaction and hearing (Gopfert & Robert 2002), but probably does not fully remove either sense, as for example *Orco* is also expressed in the maxillary palps (Larsson *et al.* 2004). It is thought that competitor recognition in general (e.g. direct aggressive conflict over territories) requires multiple cues across different sensory modalities (Grether 2011). In the few examples where cues of sperm competition rivals have been explored, most require only a single auditory (Bailey, Gray & Zuk 2010) or chemical cue (delBarco-Trillo & Ferkin 2004; Carazo, Font & Alfthan 2007; Aragon 2009; Larsdotter-Mellstrom *et al.* 2016). The only other study so far to report a requirement for multiple cues showed that the fruit fly *D. pseudoobscura* requires both odour and tactile cues (Maguire, Lize & Price 2015), similar but not identical to *D. melanogaster*. As yet we cannot explain these differences, especially as speed of response has not been considered in these other animals, but this variation shows the evolutionary variability of cue recognition systems (Maguire, Lize & Price 2015). Multimodal communication is thought to increase reaction times (Rowe 1999), but this idea relates to reactions on a timescale of seconds (Zeyl & Laberge 2011) rather than hours as we describe. Whether the multiple cues males use in this context convey different information (e.g. sex or species), or contribute similar information but achieve a response threshold faster, remains to be investigated.

In conclusion, vision is not responsible for an increase in mating duration when male *Drosophila melanogaster* are exposed to an increased sperm competition threat in either Dahomey or Canton-S strains. We have also shown that the speed of behavioural responses to changes in the sperm competition threat is affected by the availability of sensory cues. This could be seen as a limitation to behavioural plasticity in line with those suggested by Auld and colleagues (2010) and shows that a full sensory repertoire is needed for males to quantify the sperm competition threat within a population.

Anaesthesia sensitive memory controls male *D. melanogaster* reactions to the sperm competitive environment

4.1 Summary

Plastic behaviour requires individuals to learn and memorise cues associated with environmental change before using this experience to subsequently modify behaviour. In *D. melanogaster* male extended mating duration is an important plastic behaviour allowing males to gain fitness benefits from a constantly fluctuating social environment. Previous work has shown expression changes of genes associated with learning and memory after exposure to a rival male. Coupled with the importance of olfaction on a male's ability to extend mating, I hypothesised that an olfactory learning and memory pathway may play an important role in controlling male's reaction to increases in sperm competition. To test this hypothesis I use multiple mutant stocks to target genes that play a well-known role in learning and memory. In addition, I investigate the brain structures important for extended mating duration to answer whether particular brain structures play a role in controlling extended mating duration. I specifically focus on the MBs as the structure most associated with olfactory learning and memory.

I show that extended mating duration depends on learning and memory genes *dunce*, *rutabaga* and *amnesiac* involved in anaesthesia sensitive memory, and the behaviour is therefore dependent on protein synthesis. I also show that the MBs, as the centre for olfactory memory, play an integral part in controlling the plastic behaviour. These results reveal the properties and temporal dynamics of acquisition, consolidation and retrieval of memory specific to extended mating duration. This suggests that the type of memory used to control extended mating duration aligns with the frequency of environmental fluctuation to which the behaviour responds. The mechanism also supports ideas in Chapter 2 that the lag

time seen between receiving environmental cues and expression of plastic behaviour is adaptive.

4.2 Introduction

Plastic behaviour requires individuals to learn and memorise cues associated with environmental change before using this experience to subsequently modify behaviour (Mery & Burns 2010). There are many types of memory defined by the length of time they allow individuals to retain information, however, little is known about how these different memory types may act to control the dynamics or extent of plasticity in individual behaviours (Smid & Vet 2016). *Drosophila melanogaster* males respond to the presence of rivals by increasing their mating duration (Bretman, Fricke & Chapman 2009), facilitating the release of a greater amount of sperm and seminal fluid into the female reproductive tract (Wigby *et al.* 2009). This leads to fitness benefits for the responding male, who gains paternity and reduces competition for his own sperm (Bretman, Fricke & Chapman 2009).

Previous work has suggested that expression changes in learning and memory genes underlie some of the behavioural patterning seen in extended mating duration (Mohorianu *et al.* in prep). Coupled with the importance of olfaction on mediating responses to rivals (Bretman *et al.* 2011) this points to a specific learning and memory pathway that may be involved in extended mating duration, namely the 3'-5'-cyclic adenosine monophosphate (cAMP) pathway. The cAMP second messenger system is linked to olfactory ability (Restrepo, Teeter & Schild 1996) and has traditionally been investigated by training a fly to associate an odour with reward or punishment (Tully *et al.* 1990). Genes involved in this pathway include *dunce (dnc)*, *rutabaga (rut)*, *amnesiac (amn)* and *neurofibromin* (*nf1)* (McGuire, Deshazer & Davis 2005) that are associated with the control of different

memory phases and may be genetically knocked down to investigate memory dynamics. Typically, these genes show enriched expression in the MBs (Han *et al.* 1992; Gervasi, Tchenio & Preat 2010), the olfactory centre of the brain, or in the case of *amn* are expressed in DPM neurons closely associated with the MBs (Waddell *et al.* 2000). In *D. melanogaster*, MBs are comprised of bilateral clusters of approximately 2,500 Kenyon cells and are classified into α/β, α'/β' and γ subdivisions (Davis 2005). The MB neurons act as olfactory coincidence detectors, allowing synchronised coactivity of stimuli to create associative memory (Stopfer 2014).

There are two distinct memory pathways utilised when training a fly to associate a smell with a shock. Anaesthesia sensitive memory (ASM) relies on the cAMP dependent pathway and can be split into short-term memory (STM), medium-term memory (MTM) and Long-lasting long term memory (LLTM) depending on training (Quinn & Dudai 1976; Margulies, Tully & Dubnau 2005). Short-term and MTM appear after only one training cycle that can create memory for up to 4 hours, however, LLTM only appears after flies are exposed to spaced training, and is a factor of reinforcing MTM (Yu, Akalal & Davis 2006). LLTM relies on cAMP-dependent protein kinase activation of CREB, a cellular transcription factor, and subsequent protein synthesis (Davis 2011) causing memory that lasts up to a week in flies that undergo multiple training periods spaced at 15 minute intervals. In addition to ASM, anaesthesia resistant memory (ARM) also forms with associative training but is distinguishable from ASM by its resistance to post-training anaesthesia and its ability to last up to 24 hours with only one training cycle (Isabel, Pascual & Preat 2004). It is a form of long-lasting memory that can be consolidated to last multiple days through massed training (multiple training trials with not breaks) and is dependent on *radish* (*rsh*) expression in the MBs (Folkers, Waddell & Quinn 2006).

The response to potential sperm competitors by male *D. melanogaster,* referred to as extended mating duration, can be initiated by exposing males to rivals for 3 days. Males then continue to respond to rivals for 12 hours, suggesting the behaviour is a function longer term memory, either LTM or ARM (Rouse & Bretman 2016). In addition, rival exposure would seem to parallel continual conditioning cycles in that males are continually challenged with rival stimuli over 3 days. Therefore I predicted extended mating duration to be under the control of ARM. Previously, this idea has drawn some attention (Kim, Jan & Jan 2012), however, inconsistencies have arisen in the identification of cues involved in controlling extended mating duration, a factor directly affecting any memory processes that may be involved. In light of this and to test the prediction outlined above that extended mating duration is controlled by long lasting ARM I first established whether extended mating duration was abolished by application of anaesthesia, before testing ability to increase mating duration in *dnc, rut, amn, nf1* and *rsh* mutants. These mutants have previously been used to isolate different memory phases (Margulies, Tully & Dubnau 2005) and therefore provide information about which memory phases are most important for controlling extended mating duration. I also investigated parts of the neural circuitry that may underlie extended mating behaviour, focusing on the MBs essential to controlling olfactory memories.

4.3 Materials and Methods

4.3.1 Flies

Experiments were conducted in a 25°C humidified room with a 12 hours light: 12 hours dark cycle (9 am to 9pm light cycle), using plastic vials (75x25mm) with 7 ml standard sugar-yeast-agar (SYA) medium (Bass *et al.* 2007). All wild type flies used in extended mating duration experiments were the Dahomey strain as in our previous studies. Larvae for this strain were raised at a standard density of 100 per

vial. At eclosion, flies were collected and sexed using ice anaesthesia, and stored 10 per vial. Females were supplemented with live yeast granules.

All non Dahomey stocks were raised 'in vial', with crosses using 5 females and 5 males to create progeny. Mutant flies were first tested in an associative learning paradigm to check for successful abolishment of learning and/or memory ability. Flies were then tested for extended mating duration (described below). w^{1118} males were crossed to drivers and used as a control in any associative learning experiments where mutants came from the same white background. Canton-S stocks donated by Tom Price were used as control males for comparison of learning and memory scores in associative learning trials. All stocks were purchased from Bloomington Stock centre except where specified.

4.3.2 Cold shock to knock-out ASM

Previously it has been shown that application of cold anaesthesia effectively knocks out ASM but leaves ARM intact (Folkers, Drain & Quinn 1993). To test the need of ARM when reacting to rival males, focal males were transferred to a vial in ice for 2 minutes after being exposed to a rival for 3 days. Flies were then allowed 30 minutes to acclimatise to room temperature before being isolated and placed with a female and the latency to mating and duration of mating scored. All experimental procedures were also performed on flies kept singly. This gave sample sizes of 38 to 40 individual males for each group.

4.3.3 Associative learning

I employed a bioassay to confirm the expected phenotype of transgenic flies. Following the experimental procedure from Mery and Kawecki (2005; 2007) conditioning and tests were performed on 40-70 flies in male only groups. 4-

methylcyclohexanol (MCH) and 3-octanol (OCT) were diluted in light oil (OCT: 1.85 µl/ml MCH: 2 µl/ml) so that naïve flies distributed evenly between the 2 odours over 2 minutes. Odours were delivered into vials containing flies via air drawn through a vacuum pump.

Flies were conditioned by delivering one odorant for 1 minute accompanied by mechanical shock administered by a vortex for 2 seconds every 5 seconds. This was followed by 30 seconds of air before delivering the second, distinct odour for 1 minute with no mechanical shock. To test learning or memory ability flies were presented with both odorants simultaneously for 2 minutes, in which time flies could choose the odorant they preferred (Figure 1) (Mery & Kawecki 2005; Mery *et al.* 2007). A learning index (LI) was calculated by subtracting the flies making the 'wrong' choice (segregating towards the odour they had received while undergoing mechanical shock) from flies making the 'correct' choice before dividing this number by the total flies tested. To generate one data point two groups of independent flies were trained reciprocally i.e. one group was trained against MCH and the other trained against OCT, before the LI for the two groups were averaged. This was to account for any skew in the distribution of flies between the two odours.

To test different types of memory all flies underwent one training trial, but differed in their post-training handling, details below.

4.3.3.1 Early memory or learning

To test learning flies were conditioned once before being immediately tested between the two odours.

4.3.3.2 STM

To test STM flies were conditioned once before being removed from the apparatus for 30 minutes. Flies were then re-introduced with 30 seconds of air before being transferred to the choice point and presented with both odours.

4.3.3.3 ARM

To test ARM flies were conditioned once before being removed from the apparatus for 2 hours. Flies were then cold-shocked for 2 minutes before being given 30 minutes to recover. Flies were then re-introduced with 30 seconds of air before being transferred to the choice point and presented with both odours. This followed protocol from Folkers et al (2006).

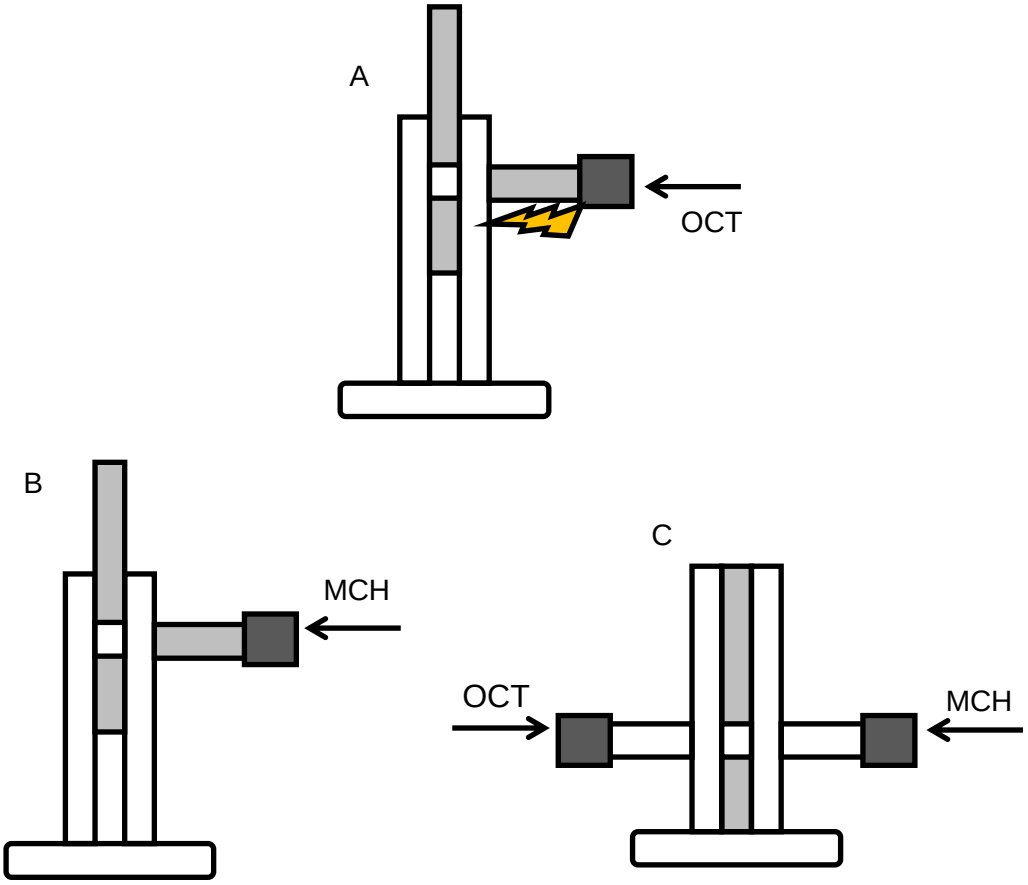

Figure 1: Basic associative learning for early term memory. Flies were given a mechanical shock while receiving one of either 3-octanol or 4-methylcyclohexanol (A). They were then left for 30 seconds before being given the reciprocal smell without any shock (B). They were then transferred into a lift and given a choice of both smells simultaneously for 2 minutes before being collected and counted (C).

4.3.4 Extended mating duration

For all extended mating duration tests each mutant had a focal male kept singly and with a rival. Each experimental period was run in addition to wild-type Dahomey controls to check that the extended mating duration phenotype was successfully represented on a particular day. In all experiments males were exposed to rivals for 3 days, or kept singly. The latency to mate and mating duration were recorded for all mating's within the first 3 hours. Each extended mating duration test was performed with 20 to 40 individual male flies per treatment.

4.3.5 Stocks

Each stock is treated separately within this section. Number in parenthesis after the stock title names are the numbers used to define the stock on Flybase. Each mutant relates to a different learning and memory phase tested for its effect on extended mating duration. The related learning or memory phase of each stock is represented in Figure 2a with the prediction for each mutants effect on extended mating duration represented in Table 1.

4.3.5.1 w^{1118} 3605

White-eye flies were generously donated by Dr Liz Duncan. When crossed to any Gal-4 drivers used to knock-down learning and/or memory in associative learning and memory trials this controls for the effect of white eye for any mutant that also incorporates w^{1118} mutation into its genotype. This is a common occurrence as a way to signal the successful insertion of a mutant gene.

4.3.5.2 Elav $_{8765}$

P{GAL4-elav.L}2/CyO is a commonly used Gal-4 enhancer trap driving expression in the nervous system. *Elav* drivers were heterozygous for *elav*, a neuro specific protein expressed throughout the nervous system. To test spatial expression in the Gal-4 line P{GAL4-elav.L}2/CyO was crossed to GFP and imaged (Figure 2b).

4.3.5.3 OK107 $_{106098}$

w^{*}; P{GawB}ey^{OK107} is a commonly used Gal-4 enhancer trap expressed in all three subclasses of mushroom body neuron throughout development (Aso *et al.* 2009). To test spatial expression in the Gal4 line w^{*}; P{GawB}ey^{OK107} was crossed to GF and imaged (Figure 2c) The stock was purchased from Kyoto stock centre.

4.3.5.4 NP3061 $_{104360}$

w^{*}; P{GawB}CG8379^{NP3061} is a Gal-4 enhancer trap driving expression in the α/β lobes of the mushroom body (Aso *et al.* 2009). To test spatial expression in the Gal4 line w^{*}; P{GawB}CG8379^{NP3061} was crossed to GFP and imaged (Figure 2d). The stock was purchased from Kyoto stock centre.

4.3.5.5 Dunce $_{6020}$

Dnc knockout flies were homozygous for dnc (1). To measure learning and memory was successfully reduced flies underwent testing for early memory in the above associative memory paradigm (Figure 1). To measure how knocking out dunce affected extended mating duration, males were exposed to rivals for 3 days before being removed and mated to females immediately. To test whether dunce males

were able to increase mating duration through a short term memory period males were isolated for 30 minutes before being mating to females. Wildtype Dahomey females were used for calculation of mating duration.

4.3.5.6 Neurofibromin$_{10201}$

NF1 knock-down flies were created by crossing w^{1118}; PBac{PB}$Nf1^{c00617}$ males to P{GAL4-elav.L}2/CyO to create focal males of the genotype w^{1118}; PBac{PB}^{Nf1c00617} ; P{GAL4-elav.L}2 which were selected by picking flies with straight wings. To test associative learning males underwent testing for early memory. Control males for associative learning tests were w^{1118} males crossed to P{GAL4-elav.L}2/CyO females to create w^{1118}; P{GAL4-elav.L}2 males thereby controlling for both elav and white-eyed phenotypes. To test whether males required NF1 to control mating duration, males were exposed to rivals for 3 days before immediate exposure to females.

4.3.5.7 Amnesiac $_{10150}$

Amnesiac knock-down mutants were created by crossing w^{1118}; P{EP}amn^{EP346} males to virgin P{GAL4-elav.L}2/CyO females to drive the mutation in the CNS. This created focal males of the genotype w^{1118}; P{EP}amn^{EP346}; P{GAL4-elav.L}2 as males were selected against the curly wing phenotype. *Amnesiac* mutants lacking amnesiac in the MBs were created by crossing w^{1118}; P{EP}amn^{EP346} males to virgin w^{*}; P{GawB}ey^{OK107} females. This created focal males of the genotype w^{1118}; P{EP}amn^{EP346}; P{GawB}ey^{OK107}. To test associative memory males were trained to the STM protocol. Control males for these tests using w^{1118}; P{EP}amn^{EP346}; P{GAL4-elav.L}2 focal males were w^{1118} males crossed to P{GAL4-elav.L}2/CyO to create w^{1118}; P{GAL4-elav.L}2 males therefore controlling for both elav and white-

eye phenotypes. Control males for w^{1118}; P{EP}amn^{EP346}; P{GawB}ey^{OK107} males were w^{1118} males crossed to w^{*}; P{GawB}ey^{OK107} females to create w; P{GawB}ey^{OK107} males controlling for white eye and OK107 expression. To test extended mating duration males were placed with rival males for 3 days before being immediately mated to females.

4.3.5.8 Rutabaga $_{9405}$

Rut knock-down flies were homozygous for rut P{lArB}rut^{2080}; P{UAS-rut.Z}2. This genotype was mated to multiple Gal4 drivers to rescue rut spatially in the brain and investigate the brain structures needed for extended mating duration. To rescue rut in the MB lobes, male P{lArB}rut^{2080}; P{UAS-rut.Z}2 were crossed with virgin w^{*}; P{GawB}ey^{OK107} females to create focal males of the genotype P{lArB}rut^{2080}; P{UAS-rut.Z}2; P{GawB}ey^{OK107}. To rescue rut in α/β MB neurons male P{lArB}rut^{2080}; P{UAS-rut.Z}2 were crossed with w^{*}; P{GawB}$CG8379^{NP3061}$ females to create focal males of the genotype P{lArB}rut^{2080}; P{UAS-rut.Z}2; P{GawB}$CG8379^{NP3061}$. To test associative learning males were tested with immediate memory protocol, control males for these tests were Canton-S males. To test extended mating duration males were placed with rival males for 3 days before being immediately mated to females.

4.3.5.9 Radish $_{V39931}$

Radish knock-downs in the CNS were created by crossing w^{1118}; P{GD8769}v39931 males to virgin P{GAL4-elav.L}2/CyO females to create focal males of the genotype w^{1118}; P{GAL4-elav.L}2; P{GD8769}v39931. To test associative memory knockdowns underwent the ARM protocol, control males for these tests were w^{1118} males crossed to P{GAL4-elav.L}2/CyO females to create w^{1118}; P{GAL4-elav.L}2

males and thereby control for both elav and white-eye phentoypes. To test extended mating duration males were exposed to rivals for 3 days before being immediately mating to a female, or isolated for 6 or 12 hours before female mating. The stock was purchased from Vienna stock centre.

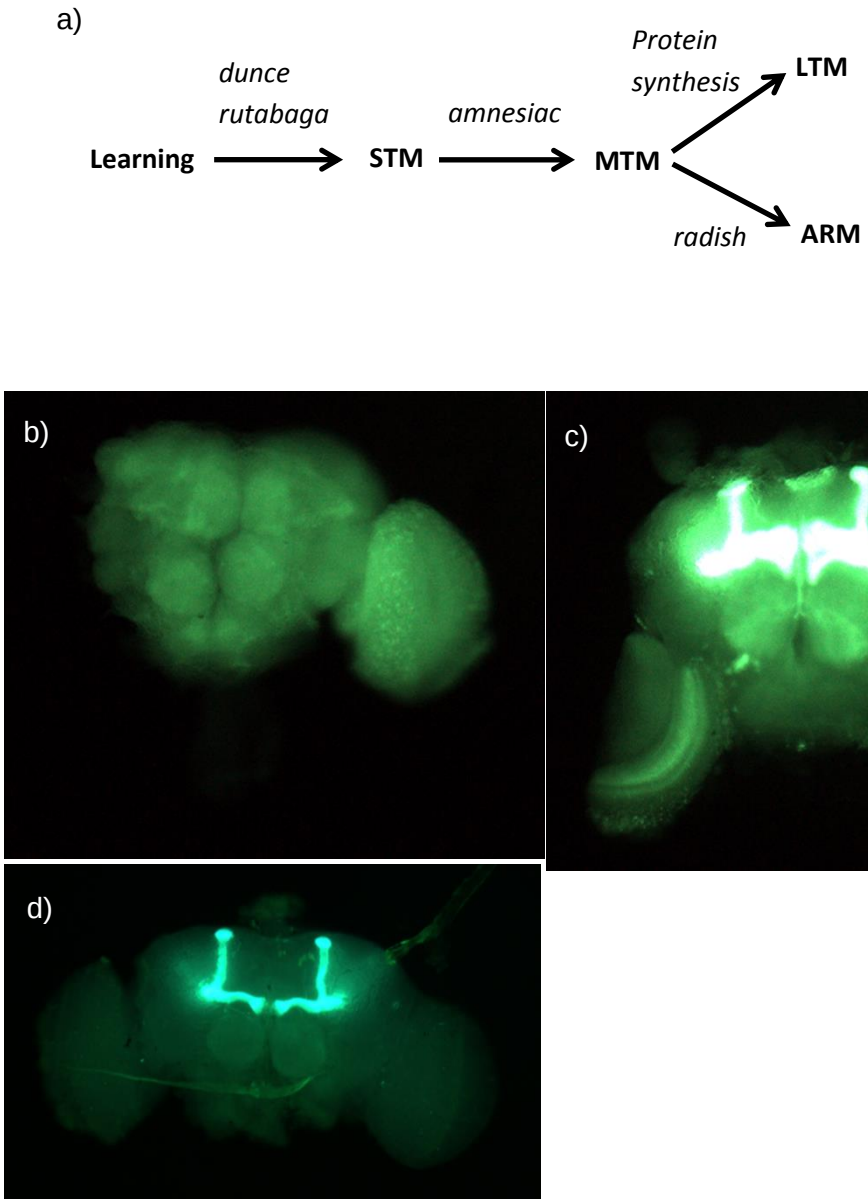

Figure 2: cAMP genes controlling *D. melanogaster* memory phases and test of Gal-40 drivers a) Genes responsible for controlling areas of memory formation in *Drosophila melanogaster.* Reproduced with modifications from (McGuire, Deshazer & Davis 2005) b) elav expressing GFP in all the neurons of the brain. Gain in the picture was reduced to 0.5 x normal to allow for effective image capture c) OK107 expressing GFP in the MB lobes of the *D. melanogaster* brain d) NP3061 expressing GFP in the α/β mushroom body lobes. Magnification X 120

4.3.6 Confirmation of driver tissue specificity through fluorescence

In order to confirm driver lines drove expression of mutants in expected tissues, expression patterns were determined by crossing GFP males to driver females of the lines P{GAL4-elav.L}2/CyO, w^{*}; P{GawB}CG8379^{NP3061} or w^{*}; P{GawB}ey^{OK107} before progeny male brains were dissected and mounted in 1 x Phosphate Buffered Saline. Pictures were taken on a Leica M165 FC fluorescent stereo microscope at 120 x magnification with pE-300 CoolLED illumination system providing illumination, and using a QIClick^{TM} CCD camera. Images were processed using Q capture Pro 7 software. Gain allows the brightness of an image to be modulated before taking a picture, by increasing or reducing the output of incident light. Gain was reduced to allow for effective imaging in both pictures, figure 2a had a gain 0.5 x the gain in figure 2b.

4.3.7 Statistical analysis

Analysis was carried out in SPSS. The results of cold shocking males were normally distributed and analysed using a Linear Model with rival exposure and anaesthesia as fixed factors. Differences in mating duration between males kept singly or with rivals were compared using T-tests for both males that had undergone anaesthesia and males that had not. Associative learning tests were analysed with simple pairwise comparisons between control males and males of a mutant genotype. Extended mating duration assays were also analysed by pairwise comparisons of flies of the same genotype either kept single or with rivals. As the key comparisons are always within genotype, this gives an internal control for genetic background and off target effects. Depending on the normality of each comparison either a Mann-Whitney U test or a t-test was used.

Table 1: Detailed function of important genes used in this chapter, what their mutants were used to test in relation to extended mating duration, and predictions of how these mutants may affect extended mating behaviour.

Gene	Function	Testing	Prediction
Dunce	Learning	Whether males can learn and memorise their sperm competition environment for 30 minutes	Extended mating duration to be abolished when tested immediately after isolation
NF1	Rut associated learning in the α/β MB neurons	Whether males use their α/β MB neurons to learn and/or remember their sperm competition environment	Extended mating duration to be abolished when tested immediately after isolation
Amn	MTM	Whether males remember an increase in sperm competition after learning	Males to extend mating duration but for this plastic behaviour to cease after 2 to 3 hours in isolation
Rut	Learning and STM	Whether males can learn and/or memorise an increase in sperm competition	Extended mating duration to be abolished when tested after isolation
Radish	ARM	Whether males use ARM to memorise an increase in the sperm competition environment after learning	Males to extend mating duration after exposure to a high sperm competition environment. However, after isolation males to fail to continue to extend mating duration for a full 12 hour response (shown in Chapter 2)

4.4 Results

4.4.1 Cold shock

Mating duration was significantly affected by both rival exposure (LM: $F_{1,145}$ = 10.683, p = 0.001) and anaesthesia (LM: $F_{1,145}$ = 7.739, p = 0.006), but these two factors did not interact (LM: $F_{1, 290}$ = 0.910, p = 0.342). Males were then spilt into anaesthesia treatments and compared pairwise depending on social environment. Males that had not undergone anaesthesia significantly increased mating duration when kept with a rival for 3 days (F_{74} = 1.033, p = 0.002), however, this response was abolished in males that had undergone cold-shock (F_{67} = 0.135, p = 0.135) (Figure 3). To check for latent effects of anaesthesia males kept singly were compared between anaesthesia treatments, and showed no effect of the cold shock (F_{77} = 0.330, p = 0.146).

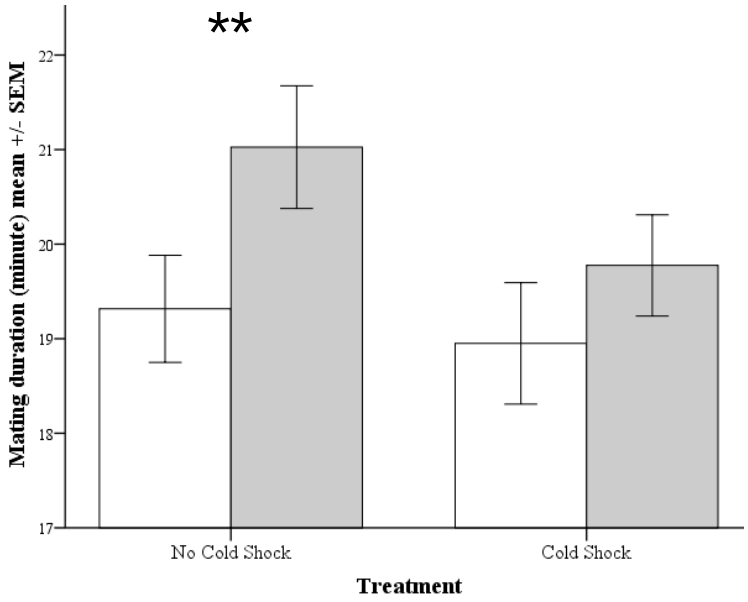

Figure 3: Effect of cold shock. Mating duration for males kept singly (white bars) and males kept with rivals (grey bars). Males were either kept as a control or underwent cold shock where males were placed on ice for 2 minutes half an hour prior to calculation of mating duration. Error bars represent standard error. * indicates a significant difference between paired treatments (* $p < 0.05$ ** $p < 0.01$).

4.4.2 *Dunce*

Males mutant for *dnc* were less able to learn and memorise an association than wild-type flies after 30 minutes (t-test: $t_{15} = 2.930$, $p = 0.010$: Figure 4A) as reported in previous work (Dudai *et al.* 1976). As mutant males were unable to memorise an association their reaction to increased sperm competition levels was then tested. When immediately moved from the presence of a rival to a female, *dnc* males significantly increased mating duration when housed with a rival when compared to a single male (t-test: $t_{41} = -2.565$, $p = 0.014$). When tested for effects of rival housing after isolation for 30 minutes wild-type males performed as expected (Mann-Whitney: $Z_{70} = -2.473$, $p = 0.013$), however, *dnc* males kept with rivals failed to increase their mating duration compared to single males (T-test: $t_{41} = -1.679$, $p = 0.101$: Figure 4B). *dnc* males were therefore able to learn, but not memorise the presence of a rival.

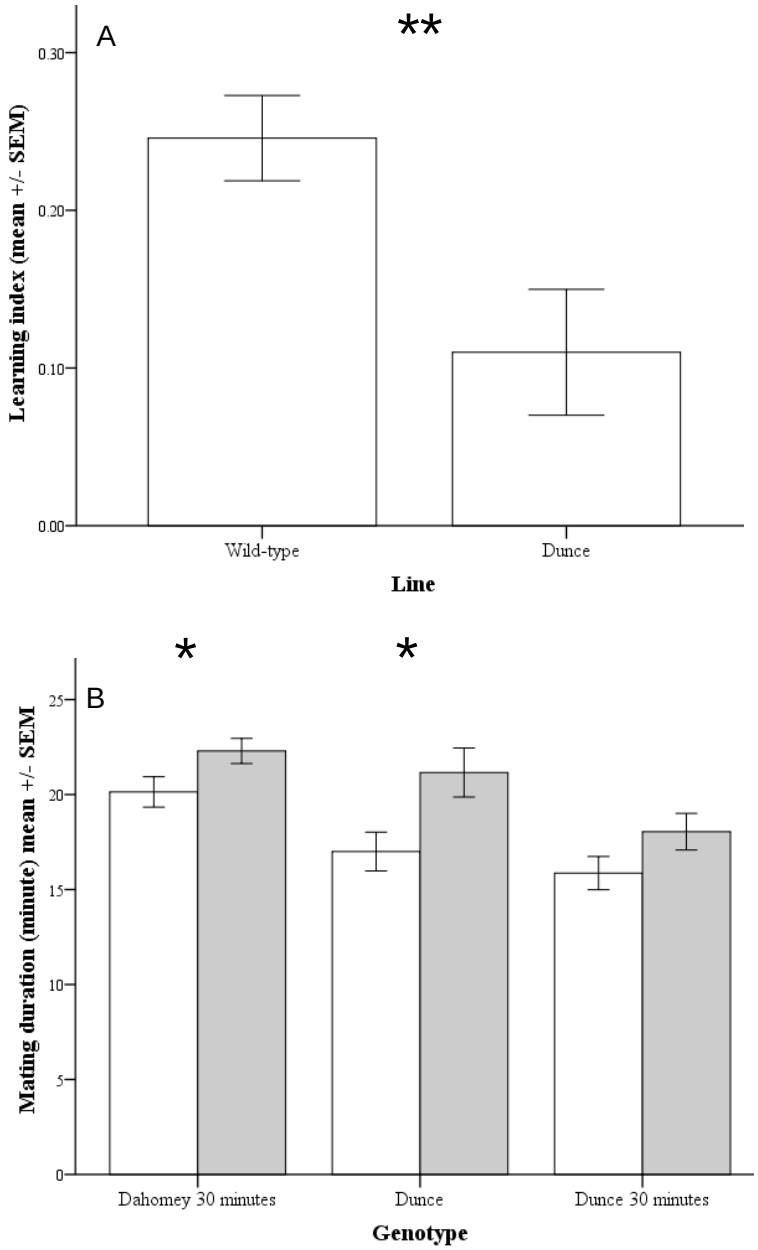

Figure 4: *dnc* 30-minute memory ability and effect on extended mating duration. A) *dnc* and wild-type learning indices B) Mating duration of single males (white bars) and males kept with rivals (grey bars) of wildtype and *dnc* males. Error bars represent SEM. * indicates a significant difference between paired treatments (* $p < 0.05$ ** $p < 0.01$).

4.4.3 *Neurofibromin*

NF1 mutant males showed significantly reduced learning ability in a associative learning protocol (t-test: $t_8 = 2.535$, $p = 0.035$: Figure 5A) in line with previous reports (Guo *et al.* 2000). Mutant males were then tested for their ability to extend mating duration. *NF1* mutant males were able to significantly extend mating duration (Mann-Whitney: $Z_{56} = -2.449$, $p = 0.014$) in a similar manner as wild-type males (Mann-Whitney: $Z_{152} = -2.889$, $p = 0.004$: Figure 5B). Therefore *NF1* has no role to play in controlling extended mating duration in males.

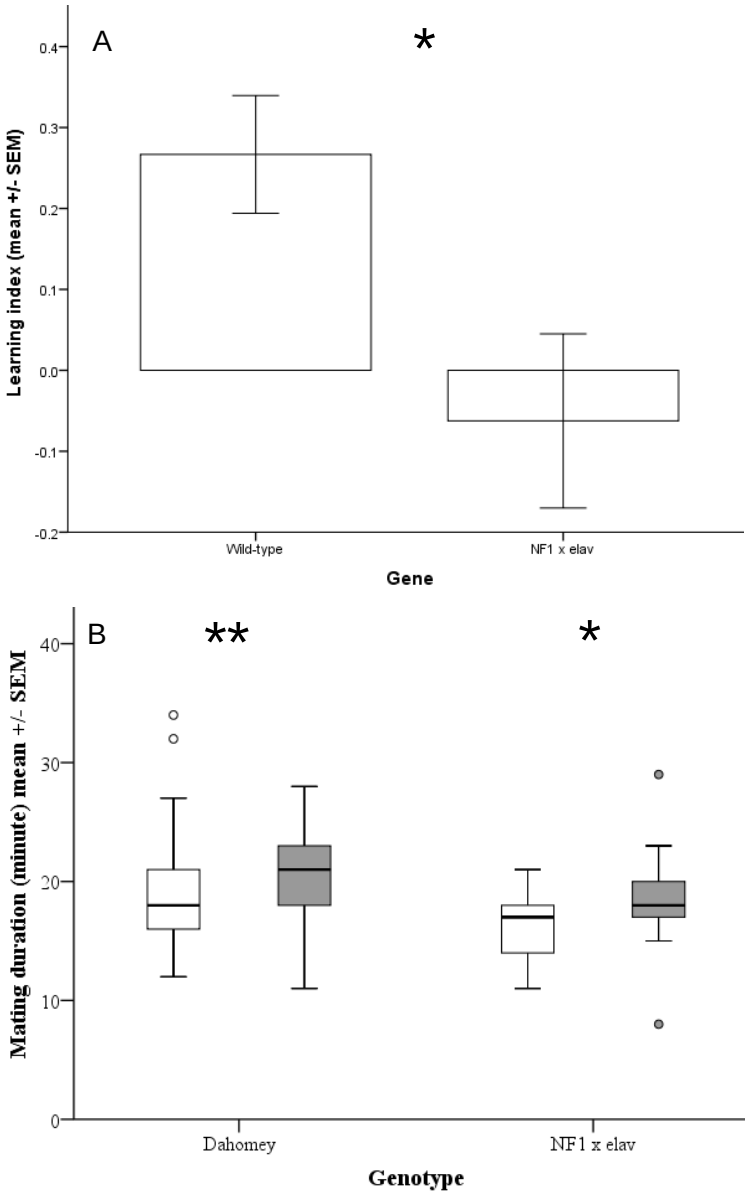

Figure 5: Ability of *NF1* to learn an association, and effect of *NF1* mutation on extended mating duration. A) learning index of *NF1* compared to wild type learning. B) Mating duration of single males (white bars) compared to males kept with rivals (grey bars) for Dahomey and mutant *NF1* males. Error bars represent SEM. * indicates a significant difference between paired treatments (* $p < 0.05$ ** $p < 0.01$).

4.4.4 *Amnesiac*

Amn male learning was significantly reduced when mutations were driven in the central nervous system (T-test: $t_8 = 2.940$, $p = 0.019$), however, when driven in the MBs mutant males did not show any difference in learning ability compared to wild-type flies (T-test: $t_8 = 0.821$, $p = 0.435$: Figure 6A). This was expected as *amn* is vital in DPM neurons that synapse onto the MBs but do not need to be expressed in the MBs themselves. When tested for extended mating duration ability males mutant for *amn* driven in the MBs significantly extended mating duration when housed with rivals and compared to single males (Mann-Whitney: $Z_{52} = -2.439$, $p = 0.015$), similar to wild-type male reactions (Mann-Whitney: $Z_{65} = -4.600$, $p < 0.001$). However, males with mutant *amn* driven in the CNS failed to respond to rivals in the same way (T-test: $t_{53} = -0.883$, $p = 0.381$: Figure 6B), showing that *amn* is needed for controlling extended mating duration.

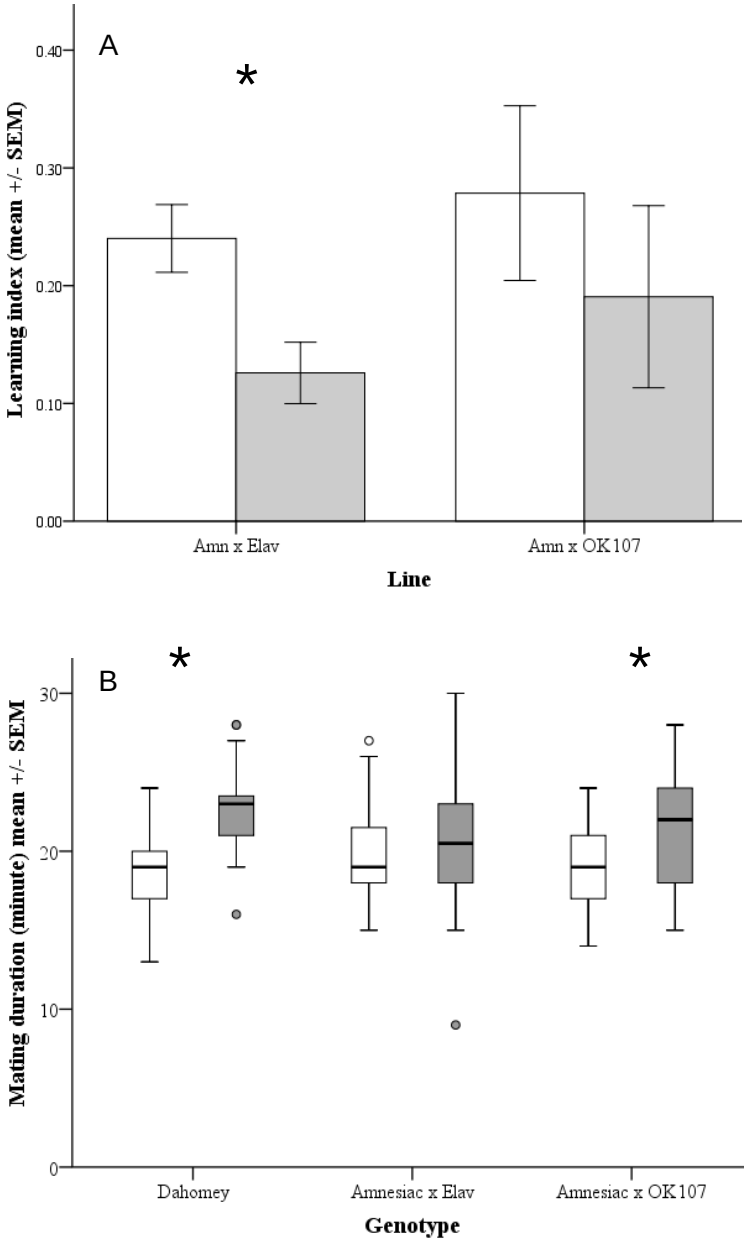

Figure 6: *Amn* male performance in an associative learning task, and effect of amnesiac mutation on extended mating duration. A) wild type learning (white bars) compared to *amn* male learning (grey bars) driven separately in either the general CNS (elav) or MBs (OK107). B) Mating duration of single males (white bars) compared to males kept with rivals (grey bars) for wildtype and mutant *amn* males. Error bars represent SEM. * indicates a significant difference between paired treatments (* $p < 0.05$).

4.4.5 *Rutabaga*

Learning was significantly compromised in male *rut* mutants (t_6 = 3.103, p = 0.021), before being rescued by expression of wild-type *rut* in the MBs (T-test: t_6 = -0.203, p = 0.846: Figure 7A) (Livingstone, Sziber & Quinn 1984). Mutant males were tested for their ability to extend mating duration when housed with a male rival. *Rut* mutants were unable to respond to a rival presence by extending mating duration (Mann-Whitney: Z = -0.960, N = 44, p = 0.337), however rescue of *rut* within all the lobes of the MBs enabled a return of the behaviour (T-test: t_{54} = -2.580, p = 0.013) to the same level as Dahomey wild-type controls (Mann-Whitney: Z = -4.332, N = 71, p < 0.001). Rescue of *rut* within the α/β MB neurons was not sufficient to rescue behaviour (Mann-Whitney: Z = -0.309, N = 55, p = 0.757: Figure 7B). Therefore, *rut* is needed in the MBs for males to be able to respond to rivals.

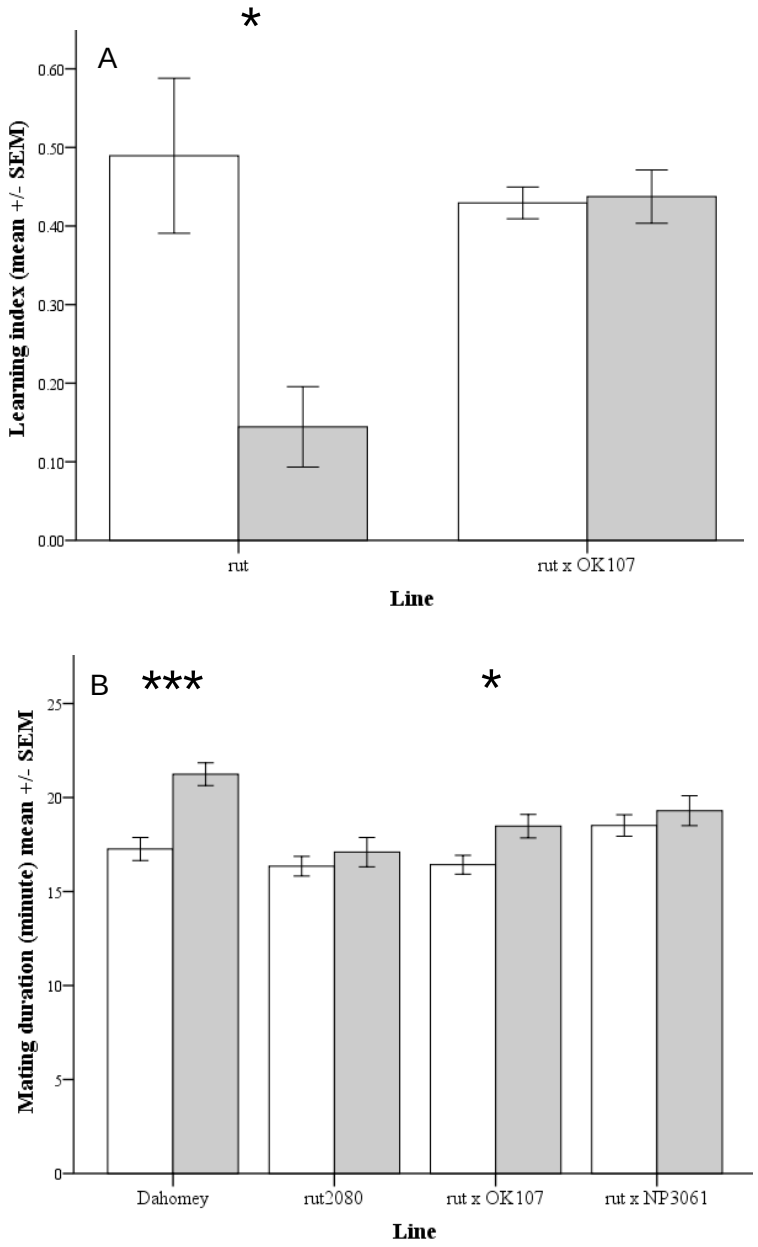

Figure 7: *rut* mutant male performance in an associative learning task, and effect of *rut* mutation on extended mating duration. A) wild type learning (white bars) compared to *rut* male learning (grey bars). *rut* was either mutant or rescued by driving wild-type rut in the MBs (OK107) or α/β neurons of the MBs (NP3061) B) Mating duration of single males (white bars) compared to males kept with rivals (grey bars) for wildtype and mutant *rut* males. Error bars represent SEM. * indicates a significant difference between paired treatments (* $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$).

4.4.6 *Radish*

ARM was significantly down-regulated in *rsh* RNAi knockdowns driven in the CNS compared to controls (T-test: $t_{12} = 2.198$, $p = 0.048$: Figure 8A). Knock-down of the *rsh* gene did not reduce a male's reaction to a rival male at 0 (Mann-Whitney: $Z = -2.259$, $N = 49$, $p = 0.024$), 6 (Mann-Whitney: $Z = -3.998$, $N = 68$ $p < 0.001$) or 12 (Mann-Whitney: $Z = -3.526$, $N = 63$ $p < 0.001$: Figure 8B) hours. Indeed, it seems that *rsh* may extend the time period of a male reaction to increased sperm competition when compared with a wild-type male with a rival, where extended mating duration had started to reduce to pre-exposure levels (Mann-Whitney: $Z = -2.151$, $N = 71$ $p = 0.031$). Results for all genes, and the effects of their mutants on extended mating duration are summarised in Table 2.

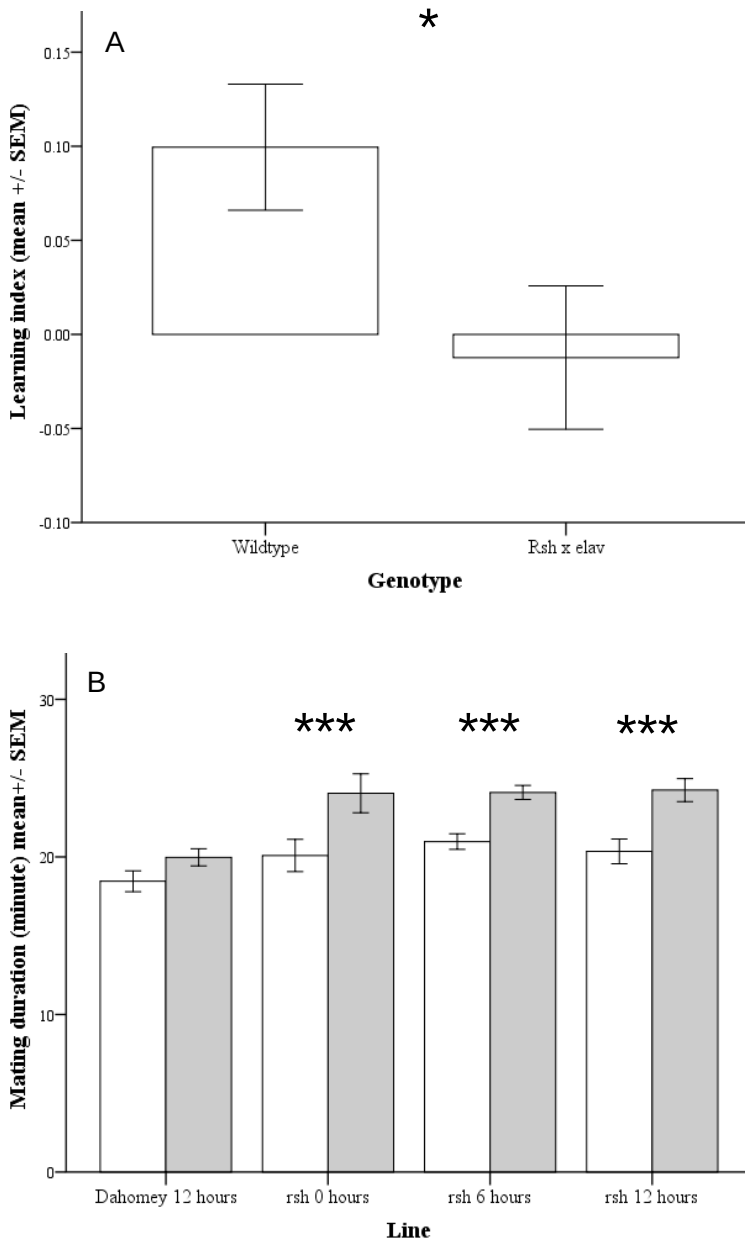

Figure 8: *rsh* knockdown male performance in an associative learning task (A), and effect of *rsh* knockdown on extended mating duration (B). Mating duration of single males (white bars) compared to males kept with rivals (grey bars) for wildtype and *rsh* knockdown males driven in the CNS. *rsh* knockdown males were immediately mated after 3 days with a rival, isolated for 6 hours after rival exposure, or isolated for 12 hours after rival exposure before mating. Error bars represent SEM. * indicates a significant difference between paired treatments (* $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$).

Table 2: Detailed function of important genes used in this chapter, predictions of how these genes mutants may affect extended mating behaviour and results for these predictions.

Gene	Function	Prediction	Result
Dunce	Learning	Extended mating duration to be abolished when tested immediately after isolation	Extended mating duration still functioning when tested immediately after isolation of a male previously exposed to a rival, but abolished if male isolated for 30 minutes before testing
NF1	Rut associated learning in the α/β MB neurons	Extended mating duration to be abolished when tested immediately after isolation	Extended mating not abolished
Amn	MTM	Males to extend mating duration but for this plastic behaviour to cease after 2 to 3 hours in isolation	Extended mating duration abolished immediately after focal male removal from a rival male
Rut	Learning and STM	Extended mating duration to be abolished when tested after isolation	Extended mating duration abolished immediately
Radish	ARM	Males to extend mating duration after exposure to a high sperm competition environment. However, after isolation males to fail to continue to extend mating duration for a full 12 hour response (shown in Chapter 2)	Males continue to extend mating duration for the full length of the response i.e. 12 hours (calculated in Chapter 2)

4.5 Discussion

Within this chapter I provide evidence that males control extended mating duration through ASM, a genetically distinct learning and memory mechanism. Mutations in three of the genes most commonly associated with the learning and memory cAMP cascade (*dnc*, *rutabaga* and *amnesiac*) lead to abolishment of extended mating behaviour. This provides unequivocal evidence that extended mating behaviour is at least in part controlled by ASM, but not ARM, and mirrors some of the results of a previous report (Kim, Jan & Jan 2012).

4.5.1 *Amnesiac*

amn encodes a preproneuropeptide released from the DPM neurons to initiate cAMP synthesis via stimulation of *rut* (Waddell *et al.* 2000). In addition, *amn* acts to initiate a excitatory feedback loop to the MBs to consolidate STM into MTM (Yu *et al.* 2005; Keene & Waddell 2007). In associative learning and courtship suppression *amn* flies learn to associate two stimuli, but subsequently forget any association after 30 to 90 minutes (Quinn, Sziber & Booker 1979; Ejima *et al.* 2005). Within this investigation, after exposure to an increased sperm competition environment, *amn* mutant males fail to extend mating duration immediately after removal from a rival presence. This result places *amn* as an important gene in regulating extended mating duration. Within the extended mating duration assay males have 2 hours to mate with females, within the time period amn mutants return to baseline memory (Waddell *et al.* 2000; Ejima *et al.* 2005). Therefore, my result shows that *amn* is needed by males to effectively extend mating duration, however, tells us very little about whether *amn* is required to learn about an environmental increase in sperm competition, or whether *amn* mutants learn this increase then forget competitive changes. Considering *amn* has never been described as a learning mutant, the

more likely scenario is that *amn* mutants successfully learn an increase in the sperm competition environment, but forget within the time period males take to mate. Given *amn* creates an excitatory feedback with the MBs during consolidation of memory (Keene & Waddell 2007; Guven-Ozkan & Davis 2014), this would suggest that MB neurons are also needed for the effective control of extended mating duration.

4.5.2 *Dunce*

Dnc is a cAMP phosphodiesterase responsible for degrading cAMP released into a neuron and is expressed at high levels in the MBs (Nighorn, Healy & Davis 1991). Similar to *amn*, *dnc* mutants display a temporal anomaly when reacting to increased sperm competition that differs from associative learning. *Dnc* mutants cannot learn to associate a smell with a shock when trained to associatively learn (Dudai *et al.* 1976), but here continue to extend mating duration beyond immediate recall i.e. 0-2 hours after isolation from another male. However, once isolated for 30 minutes, *dnc* mutants stop responding to increased environmental sperm competition, this highlights *dnc* as an important gene needed to control extended mating duration.

The temporal discrepancy in *dnc* mutants between associative learning and extended mating duration is puzzling. One explanation could be to do with other factors of fly's physiology affected when mutating *dnc*. *Dnc* mutants show a loss of pheromone specificity (Devaud, Keane & Ferrus 2003), that can affect their ability to identify individuals with different hydrocarbon profiles in other learning and memory assays (Ejima *et al.* 2005). Previously, olfaction has been shown to be one of the main drivers behind extended mating duration (Bretman *et al.* 2011). This could mean *dnc* mutants fail to differentiate between olfactory signals when switched from rival male to female housing and therefore still perceive a sperm competition threat when housed with females. When isolated, *dnc* males do not receive any signal and

therefore may ‘forget’ previous housing, and abolish extended mating duration. Conversely, my results may show that *dnc* is not needed for initial assessment and memory of the competitive environment, but is responsible for behavioural consolidation when removed from an environment with high sperm competition. This could occur through a feedback loop when *dnc*-mediated cAMP signals are important for memory consolidation, such as the MB-antennal lobe feedback loop controlling the STM precursor to ARM, where there is some evidence *dnc* expression is needed for this feedback loop to occur (Hu, Zhang & Wang 2010; Scheunemann *et al.* 2012).

4.5.3 *Rutabaga* and *neurofibromin*

Rutabaga is an adenyl cyclase responsive to G-proteins and Ca^{2+} and is highly expressed in the MBs (McGuire, Deshazer & Davis 2005). During associative learning it is thought to act as a coincidence detector and facilitate association of the conditioned and unconditioned stimuli (Tomchik & Davis 2009). Mutants for *rut* therefore show no learning or memory ability in associative learning assays (Livingstone, Sziber & Quinn 1984). Similarly, extended mating behaviour is totally abolished in *rut* mutant males. Interestingly, however, wildtype *rut* expression in the MBs can rescue extended mating duration, but not when *rut* expression is only rescued in the α/β MB neurons. This suggests that expression of *rut* is important for extended mating behaviour only when expressed in either the α’/β’ or γ lobes of the MBs. Due to the close association between *rut* and *NF1* (Guo *et al.* 2000), *NF1* was therefore also tested in relation to extended mating duration. *NF1* encodes a ras GTPase activating protein that is required for *rut* activation in the α/β neurons and subsequent development of memory, but is not required for *rut* activation in α’/β’ or γ lobes (Buchanan & Davis 2010). *NF1* is not needed by males to facilitate

extended mating duration, further supporting the α'/β' and γ neurons as responsible for controlling extended mating duration.

Functionally, neurons within the MBs can be separated by their participation in different aspects of memory, though relationships between the neurons and memory are complex. The γ neurons are thought to process and express early memories by detecting the coincidence of conditioned and unconditioned stimuli (Blum *et al.* 2009). These early memories are thought to consolidate in the α'/β' MB neurons, which hold early memories for up to 3 hours post-conditioning after one training cycle (Krashes *et al.* 2007; Cervantes-Sandoval *et al.* 2013). Finally, the α/β neurons are thought to retrieve and express memory after training is complete (Guven-Ozkan & Davis 2014), but also act as a secondary channel for memory retention in addition to γ neurons (Blum & Dubnau 2010). The γ and α/β neuron dual memory pathways build-up in parallel to express memory between 9 and 24 hours, however reduce dependence on γ neurons over time, until LLTM relies totally on α/β neurons for behavioural expression (Cervantes-Sandoval *et al.* 2013). LLTM is independent of any activity in the α'/β' neurons and does not build up through cross-referencing STM between γ and α/β lobes (Trannoy *et al.* 2011), functionally separating memory consolidation into α/β neuron dependent or independent (Guven-Ozkan & Davis 2014). Focusing on γ neurons, functional cellular imaging has shown the memory trace in the γ lobes to take up to 18 hours to build, before persisting for up to 48 hours (Akalal, Yu & Davis 2010). These time periods are remarkably similar to the time taken to build-up extended mating duration (Bretman *et al.* 2010) and reduce the behaviour to pre-exposure levels after 36 hours in the competitive environment (Rouse & Bretman 2016). Control of extended mating duration through γ lobes therefore allows for a short-term response to relatively short-term increases in the sperm competition environment and a longer response after greater exposure to competitive rivals.

Why extended mating duration seems to use γ and α'/β' neurons to consolidate memory of increased sperm competition, but not α/β neurons remains unclear, but the answer may be rooted in the ecology of extended mating behaviour. Tailor made memory refers to the properties and temporal dynamics of acquisition, consolidation and retrieval of memory after learning and is specific for each ecological context (Smid & Vet 2006; Smid & Vet 2016). For example, parasitoid wasps differ in the spatial memory pathway (ARM or ASM) used to remember different species of host depending on the oviposition reward the host is associated with (Kruidhof *et al.* 2012). As ARM is less costly that ASM (Mery & Kawecki 2005) this means the wasp only invest in LTM when the reward is large. Recently, it has been suggested that the 24 hour lag time seen before *D. melanogaster* males extend mating duration only occurs so males can confirm that a competitive threat is sustained (Rouse & Bretman 2016). After this initial investment the maintenance time of this behaviour relies on exposure time. This mirrors memory development in the wasp in that long lasting behavioural change is only initiated if a threat (Rouse & Bretman 2016) or reward (Kruidhof *et al.* 2012) is substantial.

In *D. melanogaster*, memory developed by the γ neurons is a more malleable form of memory than that which is developed in the α/β neurons (Trannoy *et al.* 2011) after training in that it controls both STM and LTM periods (Guven-Ozkan & Davis 2014). This should increase the ability of a male to react to quick changes in the sperm competition environment through STM and also guard against reduction of behaviour when sperm competition threat within a locality is still high but the immediate cue of rival presence has been removed. In comparison, memory developed through α/β neurons is 'all or nothing' LTM that may become mal-adaptive in a quick changing environment. The specific neuronal structure relied upon for extended mating duration, specifically γ neurons, can therefore create

behaviour that can act on a transient or sustained timescale depending on the sperm competition environment in an ecologically relevant way.

In conclusion, males require *NF1* independent *rut* expression in the γ and α'/β' neurons of the MBs to facilitate extended mating duration in response to increased sperm competition. However, to explicitly show this, further work would have to be performed on extended mating duration focussing specifically on different MB neuron subsets and component interactions in the ASM pathway.

4.5.4 *Radish*

In addition to ASM, *D. melanogaster* possess a separate genetically and functionally distinct consolidated memory phase called ARM. ARM requires expression of a gene called rsh in the mushrooms bodies, thought to encode a protein which binds to Rac-1 to control synaptic morphology (Folkers, Waddell & Quinn 2006). Males that underwent retrograde amnesia or knockdown of *rsh* still responded to rival males by extending mating duration, showing ARM is not needed for an increase in mating duration due to a rival presence. However, knockdown of *rsh* did have an interesting effect on the magnitude of the behavioural response when focal males had been isolated. Previous reports have defined extended mating duration as lasting 12 hours after isolation of rivals, before mating duration returns to pre-rival exposure levels (Rouse & Bretman 2016). Here, *rsh* knockdown males seem to continue to strongly respond to rivals at 12 hours, in comparison to a wild-type male's whose response to rivals started to reduce at 12 hours (as expected). This could signal *rsh* knockdown males differ in their temporal reaction to isolation, or that ARM is needed to respond to an absence of a rival. However, further work would be needed to determine is this is indeed the case as effect size is not an ideal indicator of future behaviour in extended mating duration (Bretman, Fricke & Chapman 2009).

4.5.5 Ecologically relevant memory phases

The main conclusion to draw from my results in this chapter is after 3 days males require ASM, but not ARM to successfully extend mating duration in response to an increased sperm competition threat. This is unexpected as male-male interactions within the sperm competition environment seem to mirror massed training in associative learning (discussed in chapter 2) that would promote ARM. In addition, ARM is less stable than ASM (Tully *et al.* 1994) and has a lower fitness cost (Mery & Kawecki 2005), both seemingly advantageous points when reacting to quick changing environments.

Reasons males may rely on ASM over ARM when reacting to changes in the sperm competition environment are two-fold. I have previously argued (chapter 2) that males must receive continual stimuli from a rival in the form of olfactory stimulus due to the far reaching effects of olfaction in the environment (Gaudry, Nagel & Wilson 2012). However, multiple sensory stimuli are needed for males to increase mating duration (Bretman *et al.* 2011), meaning whenever a rival male is not physically present males may only be exposed to one stimulus. The physical movement of rivals within a focal male's sensory range may therefore act to promote extended mating duration only at spaced intervals, effectively bringing about ASM. Firstly, little is known about natural populations of *D. melanogaster*, so it is difficult to assess whether the response in the wild requires continuous stimulation from rival males. Tentative measurements of *D. melanogaster* dispersal in the wild measuring density of flies showed 2-3 flies per $100m^2$ after release, but with a tendency to aggregate in shady areas (McInnis, Schaffer & Mettler 1982). Although no time period was specified for individuals to move between aggregations individual dispersal between points of intense social interactions would fit with a spaced training cycle.

Secondly, it may not be advantageous to use the most malleable memory system available when addressing the changing competitive environment. Compared to ARM, ASM takes longer to build to LTM (one memory trial initiates 4-hour ASM memory, but 24-hour ARM memory), is longer lived and more stable (Tully et al 1994). ASM could therefore function as an initial way to 'gate' extended mating duration through the need for multiple exposures to rival males to confirm a sperm competition threat is sustained. ASM could then increase the time period extended mating duration lasts to guard against reducing extended mating duration when environmental sperm competition threat is still high. In comparison to ASM, ARM would create and reduce a behavioural response more quickly. This is especially important considering the costs of extending mating duration are also due to increased sperm and seminal fluid proteins production, and hints that any additional costs accrued by using ASM over ARM (Mery & Kawecki 2005) are outweighed by the benefits of extended mating duration. Similar ecological memory can be seen in the parasitic wasp genus *Cotesia*, where different species use separate mechanisms to control plastic behaviour dependent on the spatial distribution of host opportunities. Here, species that form LTM but not ARM parasitize hosts with wide distributions that supply spaced stimuli, and take a long time to build up memory (Smid *et al.* 2007). This supports the above assertion that males may only receive competitive stimuli at spaced intervals, and ideas from chapter 2 that suggest males rarely come into contact with male-male competition.

Alternatively to the two points outlined above, reliance on ASM could be due to the temporal foundations of extended mating duration assays. Males are left with rivals for 3 days, 2 days more than the time period needed to create an extended mating response. ASM has been shown to remove ARM with multiple spaced training (Placais *et al.* 2012), raising the possibility initial reactions to a rival presence after 24 hours are due to ARM, but with increased exposure ASM

becomes more relevant. Explicit experiments to look for this switch would therefore be very useful to see whether these two memory types interact in any way to control extended mating duration.

Overall, learning from experience is crucial for animals to reach their peak fitness. In addition, dealing with multiple cues within an environment means an animal must prioritise those cues which are most reliable. Here, I find *D. melanogaster* use a memory process that requires multiple spaced cues to create behaviour, effectively minimising the risk of responding to a transient environmental change. I also find this memory dynamic is kept malleable to the social environment by only employing a subset of the nervous architecture available. How memory is employed to control mating duration in this model raises questions about how memory types may control timescales of plastic behaviour in other systems. In addition to examples used above, other research also show that the type of memory induced by environmental cues links to the length of the plastic behaviour in *Nasonia* parasitoid wasps (Hoedjes *et al.* 2011; Hoedjes *et al.* 2012; Hoedjes & Smid 2014), though this research is limited to the difference between ASM and ARM. I would therefore expect that memory type used to control a plastic behaviour parallels the timescale at which the behaviour works. Considering this, a plastic behaviour which is fully malleable but occurred on a seasonal basis may therefore use a memory type that is extremely long-lasting to prevent accidental behavioural switches not consistent to the current season. However, this prediction would need to be tested and such long-lasting changes in behaviour may be better controlled by other mechanisms, such as genomic change (Cardoso, Teles & Oliveira 2015).

Expression of memory-associated genes in response to sperm competition cues

5.1 Summary

To understand how behavioural change is controlled it is necessary to understand the underlying physiological, neural and genomic proximate mechanisms that interface the genotype and the environment. Specific types of behavioural plasticity are thought to use these mechanisms in different ways to control the speed at which a plastic behaviour can act. Recent theory has postulated that short term flexible behaviour should be controlled by a switch in neural circuitry and show transient changes in the neurogenomic state of the brain. Our model of male behavioural responses to the sperm competition environment could be categorised as this type of plasticity as male *D. melanogaster* respond to an increase in the competitive environment by modulating mating duration, a fully flexible behaviour. Previous work has suggested that extended mating duration is paralleled by transcriptomic change over 50 hours, but does not consider how this change relates to the time period of the behavioural switch.

In this chapter, I investigate whether changes in expression levels of genes highly expressed in the brain control extended mating duration in *D. melanogaster.* Four different learning and memory genes either known to respond to the presence of a rival or be important in controlling extended mating duration were tested for expression differences. I predicted that expression of neuronal genes should show transient changes paralleling behavioural change and so measured males kept with or without a rival male across six time periods. These time periods spanned the build-up of extended mating duration over 72 hours, and the return to pre-rival exposure levels. Ultimately, there was no clear pattern in gene expression levels for any of the genes investigated when comparing between males kept singly or with a rival. This suggests that changes in transcription levels do not underpin changes

seen in male behaviour due to increased sperm competition threat. Instead it could be that neural mechanisms are more important than neurogenomic changes when males respond to rivals, or that post-transcriptional modifications control the ability of a male to respond to a rival male.

5.2 Introduction

To understand behavioural change to fluctuations in the environment, it is necessary to understand the underlying physiological, neural and genomic proximate mechanisms that interface the genotype and the environment (Aubin-Horth & Renn 2009). Behavioural change due to social plasticity can be viewed as a consistent expression of set behaviour in response to relevant social information (Cardoso, Teles & Oliveira 2015). Different types of social plasticity are predicted to rely on different combinations of physiological, neural and genomic change to control behaviour. Physiological changes can occur early in development to influence behaviour throughout a lifetime, or can act on a short-term 'activational' basis where release of a hormone pre-empts the switch between behaviours (Moore, Hews & Knapp 1998; Hau & Goymann 2015). Neural control of behavioural plasticity is thought to be controlled by structural plasticity that promotes the development of new neural circuits, or by biochemical switching that occurs between established neural networks (Oliveira 2009). Finally, socially regulated neurogenomic states are thought to correspond to different behavioural states and so control social plasticity. For example, neurogenomic expression allows researchers to predict individual switches in behaviour between hive work and foraging in the honey bee *Apis mellifera* (Whitfield, Cziko & Robinson 2003). How neurogenomic change leads to social plasticity is complex, but may be through the activation of immediate early genes that then act as neuromolecular switches to

induce plastic behaviour (Perez-Cadahia, Drobic & Davie 2011; Cardoso, Teles & Oliveira 2015; Teles, Cardoso & Oliveira 2016).

Drosophila melanogaster male extended mating behaviour is short-lived (Rouse & Bretman 2016) and reversible within a lifetime of an individual (Bretman *et al.* 2012). This suggests that it is a fully flexible plastic behaviour associated with transient changes in gene expression between behavioural states (Cardaso et al, 2015, table 1). Genomic changes between behavioural states can be investigated in two ways. Firstly, by the comparison of two reaction norms measuring genotype pre- and post- environmental change. Or secondly, via a molecular time series investigating the transitional period between reaction norms where genomic change is initiated before genotypes are stabilised at the end of the plastic behaviour (Aubin-Horth & Renn 2009). Previously, Mohorianu, Bretman and colleagues (2016, in review) have shown a marked early upregulation in sensory genes expressed in the head/thorax after 2 hours in a rival presence. When tested at later time periods (26 and 50 hours) expression of the same genes were not upregulated. Additionally, a number of neuronal genes were differentially expressed in males after 2, 26 or 50 hours with a rival, timescales that mirror changing behavioural reaction norms, and hint at neurogenomic change controlling behaviour. Here, I address the transitional period where males build up the extended mating behaviour response, using the temporal dynamics of the behaviour (Rouse & Bretman 2016) as a template for the time periods investigated.

Similar studies have shown that transcriptional change can be induced by social or sexual environmental fluctuations that drive physiological changes on a short and reversible timescale (Lopez-Maury, Marguerat & Bahler 2008; Robinson, Fernald & Clayton 2008), in line with the theory laid out above. In flies, rival exposure is already known to cause differential expression of two seminal fluid genes (Fedorka, Winterhalter & Ware 2011) and short term social exposure can

change transcription levels of genes associated with perception and spermatogenesis (Carney 2007). Generally, environmental enrichment, including the addition of other individuals to increase social complexity, can cause mass transcriptional change in the brain. In rodents, addition of novel objects into an environment promoted the expression of genes linked to neuronal structure, synaptic plasticity and transmission (Rampon *et al.* 2000). In fish, change in social dominance behaviour correlates with changes in expression of immediate early genes thought to promote brain activation wherever they are expressed (Fernald & Maruska 2012). From these examples, and considering the theory discussed above, I therefore predict genes involved in learning and memory show differential expression on the same time scale as changes into control mating duration.

To establish whether extended mating duration requires transient changes in gene expression, I target four genes involved in the *D. melanogaster* learning and memory pathway. Of these four genes, *rut* and *dnc* are already known as vital to controlling extended mating duration (Chapter 4), while *dnc* also shows a change in expression depending on exposure to rivals (Mohorianu *et al.* in prep). Considering the important of *dnc* and *rut* on controlling extended mating duration I hypothesise that both genes will be upregulated in males kept with rivals at time points soon after rival males were introduced to the environment (Table 1). The two other genes tested have not been shown to have a role in extended mating duration, but are involved in olfactory learning and memory. *Neurexin* (*Nrx-1*) is involved in learning and is crucial for the development and function of synapses (Li *et al.* 2007; Zeng *et al.* 2007). Along with *dnc* it is differentially expressed after males have been exposed to rivals for two or 50 hours (Mohorianu *et al.* in prep) and functions in similar areas of the brain to *dnc* and *rut*, namely the MBs (Sun, Zeng & Xie 2016). It therefore should be upregulated in males exposed to rivals at all time points (Table 1). *Notch* is involved in long-term memory, but not learning or short-term memory

(Ge *et al.* 2004) and therefore I hypothesise should be upregulated in males kept with rivals after a rival male has been removed (Table 1).

Table 1: Detailed function of important genes used in this chapter and predictions of how the initiation of extended mating duration may affect expression of these genes.

Gene	**Function**	**Prediction**
Dunce	Learning	*Dunce* to be upregulated within the first hours of rival introduction into the environment or soon after rival removal
Nrx-1	Synapse development and function	*Neurexin* to be upregulated at all times points
Rut	Learning and STM	*Rut* to be upregulated within the first 6 hours of rival introduction into the environment or soon after rival removal
Notch	LTM	*Notch* to be upregulated after rival removal between 63 and 72 hours after original exposure

5.3 Materials and methods

5.3.1 Fly husbandry and experimental set up

Experiments were conducted in a 25°C humidified room with a 12 hour light: 12 hour dark cycle (9 am to 9pm light cycle), using plastic vials (75x25mm) with 7 ml standard sugar-yeast-agar medium (Bass *et al.* 2007). Wildtype larvae were raised at a standard density of 100 per vial. At eclosion, flies were collected and sexed using ice anaesthesia and randomly assigned to a social environment, either kept single or kept with rivals. Focal males from both social environments were snap frozen in liquid nitrogen 5, 24 or 48 after induction of a rival to measure how gene expressin changed with the build-up of the extended mating response (Bretman *et al.* 2010). To measure how gene expression changed with the reduction of mating duration when males were isolated (shown in Chapter 2) males were left with rivals for 48 hours before being isolated in separate vials. The males were isolated for 12, 15 and 24 hours before being snap frozen in liquid nitrogen to cover the time males reduced mating duration back to pre-exposure levels (Rouse & Bretman 2016). Control males underwent the same transfer of vials but were kept isolated for the whole process. Flies were stored at -80°C and the head and thorax dissected from the abdomen on dry ice.

5.3.2 Assessment of gene expression through qPCR

RNA was extracted from between 17 and 30 pooled dissected male thorax and heads per sample using Direct-zol™ RNA miniprep columns following the manufacturer's protocol. This was checked for concentration and purity on a nanodrop before being run on a gel to check for RNA degradation (Figure 1). cDNA synthesis was carried out using the Life Technologies First strand cDNA kit following the manufacturer's protocol. Synthesis output underwent PCR with Act5c

primers and was then run on a 1% agarose gel to check for efficient synthesis. The corresponding no reverse transcriptase control was also run to check for contaminants (Figure 2).

All qPCR primers were designed with a melting temperature of 60°C +/- 1°C with CG content between 20-80%. These primer pairs were tested for efficiency using a 10 times dilution series on whole body RNA and accepted if the efficiency fell between 90 and 110% for dilutions that mirrored similar expression levels within samples. R^2, a measure for pipetting accuracy of standards was accepted if above 0.99 (Table 2). Genes of interest were selected from a list of genes that had previously shown differences in expression due to rival exposure (Mohorianu *et al.* in prep) and known learning and memory genes (McGuire, Deshazer & Davis 2005). Housekeeping genes Actin 5c, Rap21 and EF1 were selected for their stability of expression under different social environments (Ling & Salvaterra 2011).

All time points listed were originally tested for changes in gene expression in three replicated pooled samples. Subsequently, males kept with rivals for 5 and 24 hours, and males isolated from rivals for 12 and 24 hours were again tested for changes in gene expression using another three independent pooled samples. These time points were investigated in more detail as they were deemed the most likely to show potential differences in gene expression between social environments from previous behavioural (Rouse & Bretman 2016) and molecular data (Carney 2007; Mohorianu *et al.* in prep). This gave an N of 6 for the time points highlighted above and an N of 3 for males kept with rivals for 48 hours, and males isolated from rivals for 15 hours.

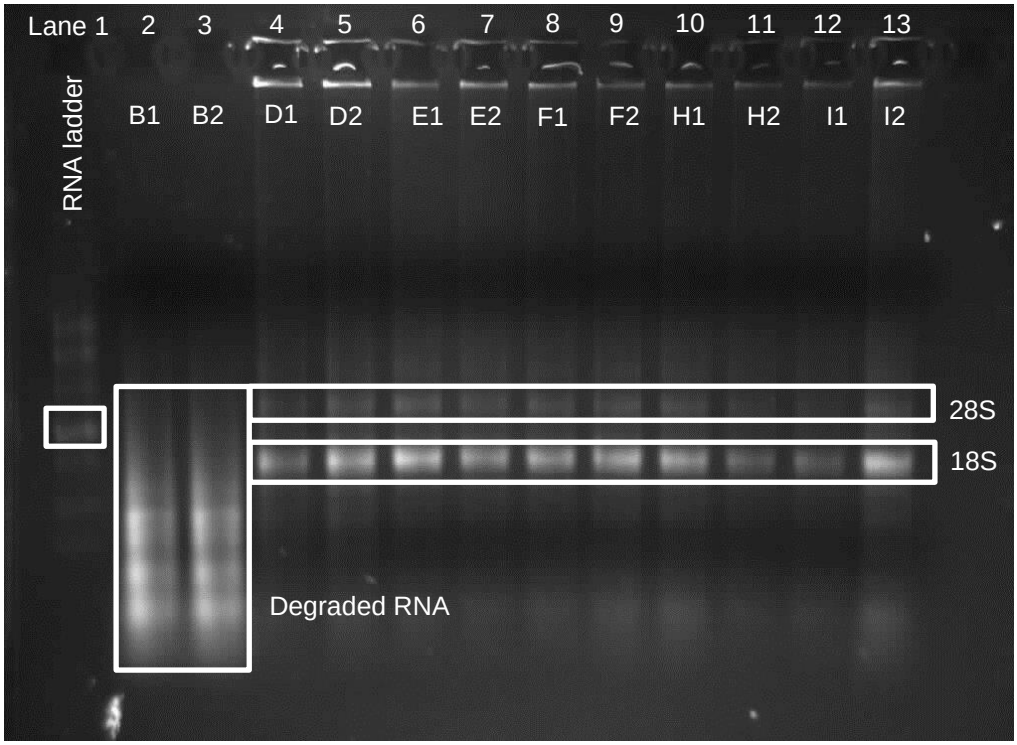

Figure 1: Assessment of RNA quality for qPCR via gel electrophoresis a) RNA extraction on a 1% agarose gel. Lane 1 = ssRNA ladder with major band marked at 3000 bp. Lane 2 and 3 are examples of RNA degradation (samples B1 and B2); other samples have not degraded and are useable in cDNA synthesis. Insect rRNA looks less clean than mammalian rRNA as there is a endogenous hidden break within the RNA that causes two similar sized fragments to 18S RNA to be created (Winnebeck, Millar & Warman 2010).

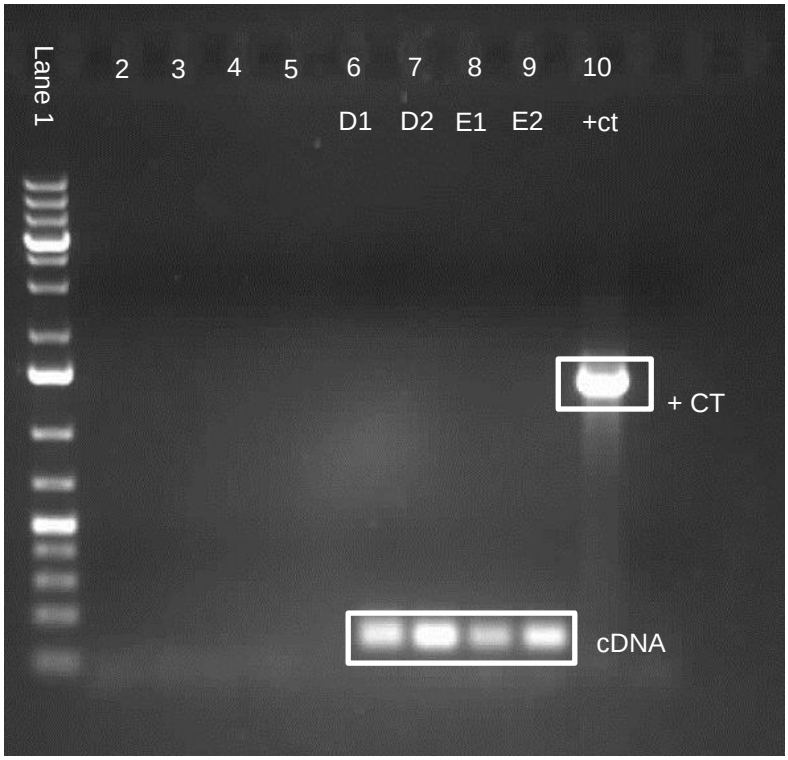

Figure 2: Assessment of cDNA quality for qPCR via gel electrophoresis. Check for efficient synthesis of DNA from previously shown RNA products. Lane 1 shows 100 bp DNA ladder with major band sizes marked in base pairs. Lanes 2-5 show no-reverse-transcription control (NRT) samples. Lanes 6-9 show successful cDNA synthesis. Lane 10 shows an *E.coli* positive control to confirm successful PCR. Once all RNA is confirmed as being converted into cDNA with no signal in NRT samples qPCR can continue.

Table 2: Forward and reverse oligonucleotides for individual genes with efficiency. Primers were designed with a melting temperature of 60°C +/- 1°C and with a CG content between 20-80%.

Gene	**Forward**	**Reverse**	**Efficiency %**	**R^2**
Act5c	GTGGATACTCCTCCCGACAC	GCAGCAACTTCTTCGTCACA	91.3	0.999
Rut	AGAATGTGAGCATCCTTTTC	TACGCAAACAGTGATTATCG	101.6	0.997
Ef1	GTCTGGAGGCAATGTGCTTT	AATATGATGTCGCCCTGGTT	106.6	0.999
Rap21	TTCACTTACGAACCATCAAACATT	GCTGGCTGACTTCCTTTCAC	107.4	0.999
Notch	GCACCAAACACTTGGATTTGT	GGTTTTGCCATTGAGTTGTG	92	0.999
Dunce	TGTGGCATACACCATATTTCAG	GAAACGGATTGTCTTTGACG	97.9	0.998
Nrx-1	GACAACAACTGGCACACGAT	TACTGTGGCGACCCAGAAT	98.8	0.996

Quantification of transcript levels in flies relative to controls (EF1, Act5c and Rap21) were performed using the ΔΔCT method (Hellemans *et al.* 2007). For all genes, Ct values for samples were quantified against the lowest value across biological replicates, taking into account the efficiency of the primer used. For housekeeping genes this number was then used to calculate a geometric mean of all the housekeepers in each sample. Each gene of interest was then divided by the geometric mean of the housekeepers for each sample before being averaged across biological replicates to get a relative expression for each gene when males were both single and with rivals.

5.3.3 Statistical analysis

Statistical analysis was performed using SPSSv14 and R 3.3.1 (Ihaka & Gentleman 1996). Before any analysis was undertaken, relative expression scores from samples with unusual qPCR curves were removed. This was due to multiple samples being retested (through experimenter error) and in the case of *Nrx-1* expression qPCR plates being run one year after RNA was extracted. Relative expression was non-normal and was fitted to a generalised linear model with quasi-Poisson errors (to account for underdispersion) using gene, time and social environment as fixed factors, and using Analysis of Deviance (AOD) to reduce from the full to minimal model. To elucidate any differences between social environments within each gene, *dunce*, *rut, Nrx-1* and *Notch* expression were explored individually. For *dunce, rut and Nrx-1* expression data was non-normal and therefore a generalised linear model with quasi-Poisson distribution (to account for underdispersion) was fitted using time and exposure to rivals as fixed factors. Relative expression for *Notch* was normal and therefore a linear model was fitted using time and rival exposure as fixed factors. After models, post-hoc tests were

performed (Mann-Whitney or T-tests) to investigate whether males kept singly or with rivals differed at discrete time periods.

5.4 Results

Considering all data together expression levels did not differ due to gene (AOD: $X^2_{216} = 15.724$, $p = 0.463$) or social housing (AOD: $X^2_{217} = 15.736$, $p = 0.690$) but did significantly differ depending on the experimental time period (AOD: $X^2_{218} = -0.631$, $p = 0.004$). This difference is almost definitely driven by the time each group was frozen for RNA extraction and represents how gene expression may change with circadian rhythm (Claridge-Chang *et al.* 2001). However, as there were no significant interactions between these factors, analysis was then carried out for each gene and for each time point.

5.4.1 By gene

To investigate whether there were differences in relative expression for individual genes depending on time and sociality, models were run by individual genes with sociality and time as fixed factors. Relative expression levels differed significantly depending on the time males from both groups were snap frozen in liquid nitrogen for *rut* and *Nrx*, however, a pattern to how gene levels changed over time was not easy to distinguish (Table 3). Again, differences in expression compared across time periods is a function of how expression may change with circadian rhythm (Claridge-Chang *et al.* 2001). In addition, there was no change in relative gene expression for any gene when comparing across social environments (Figure 3-6).

5.4.2 By time period

As the key question in the study was how social interactions may have affected relative gene expression depending on the time a male was exposed to a rival, and the subsequent time without a rival presence, pairwise comparisons were made for each gene at each time point between rival-exposed or non-exposed treatments. Here, there was no interest in comparing gene expression between time points. Relative expression levels of *dunce*, *rut*, *notch* or *Nrx-1* did not significantly change depending on the social environment they had been previously exposed regardless of the time period investigated (Table 3). This was different to my predictions that a change in gene expression would control the behavioural dynamics of extended mating duration (Table 4).

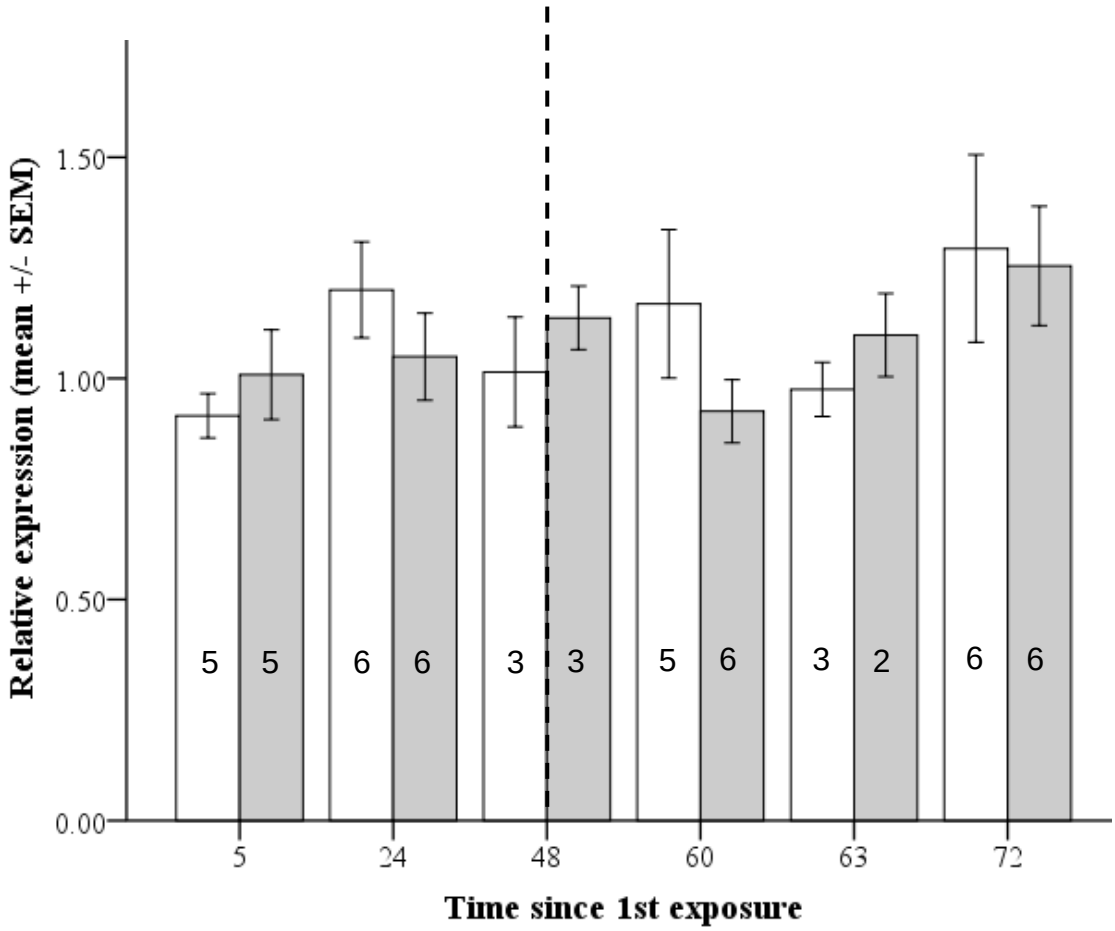

Figure 3: Relative expression of *dunce* when kept singly (white bars) and with a rival (grey bars) for distinct periods. The dotted line represents when males were removed from a rival presence, 48 hours after originally being exposed to a rival. Previous to this, males were kept with rivals for 5 or 24 hours. Error bars represent 1 SEM, numbers within the bars show the number of pooled samples tested.

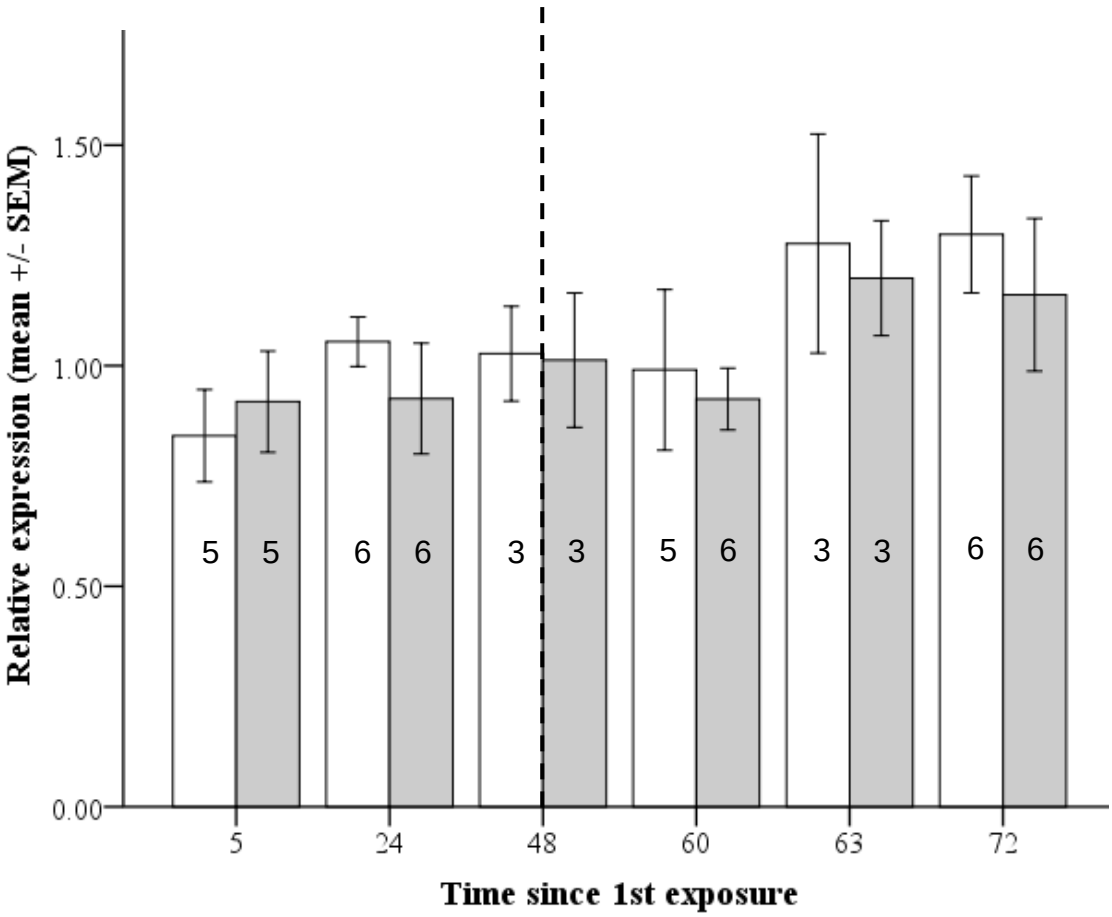

Figure 4: Relative expression of *rut* when kept singly (white bars) and with a rival (grey bars) for distinct periods. The dotted line represents when males were removed from a rival presence, 48 hours after originally being exposed to a rival. Previous to this, males were kept with rivals for 5 or 24 hours. Error bars represent 1 SEM, numbers within the bars show the number of pooled samples tested.

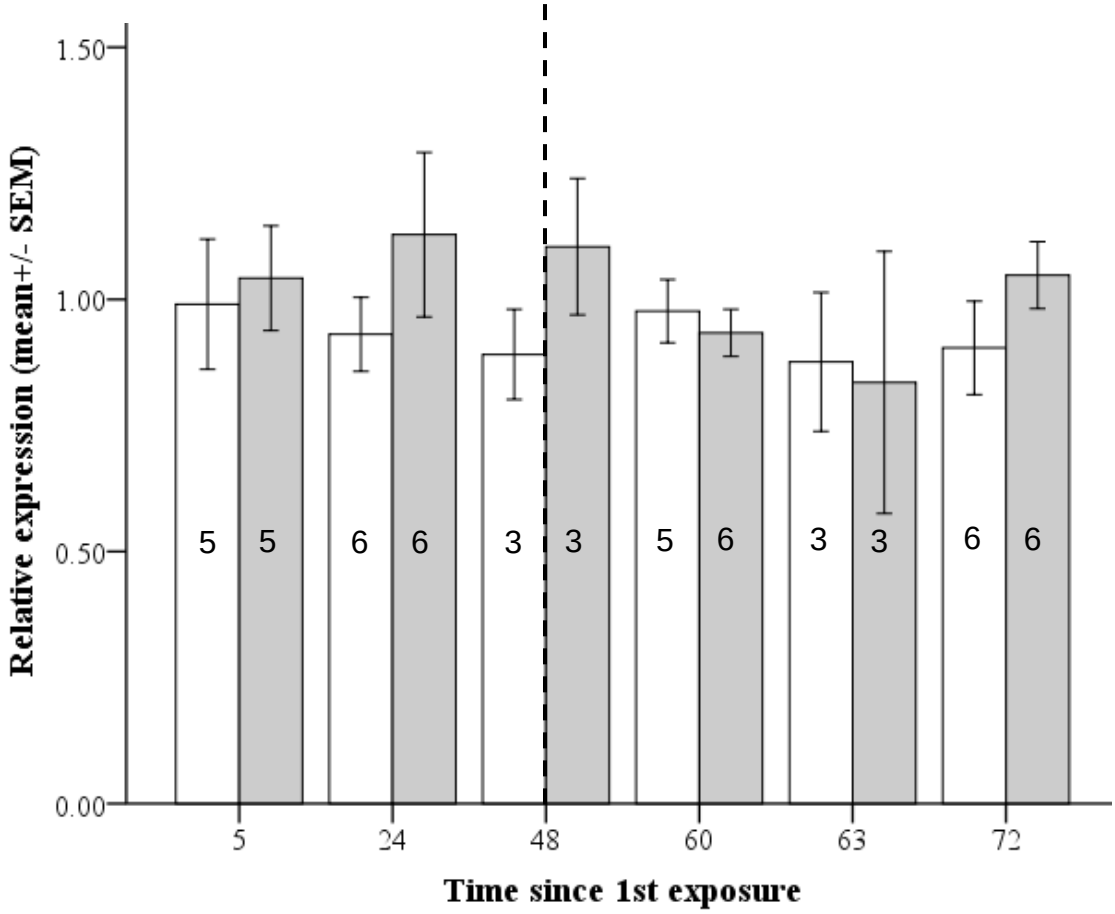

Figure 5: Relative expression of *Notch* when kept singly (white bars) and with a rival (grey bars) for distinct periods. The dotted line represents when males were removed from a rival presence, 48 hours after originally being exposed to a rival. Previous to this, males were kept with rivals for 5 or 24 hours. Error bars represent 1 SEM, numbers within the bars show the number of pooled samples tested.

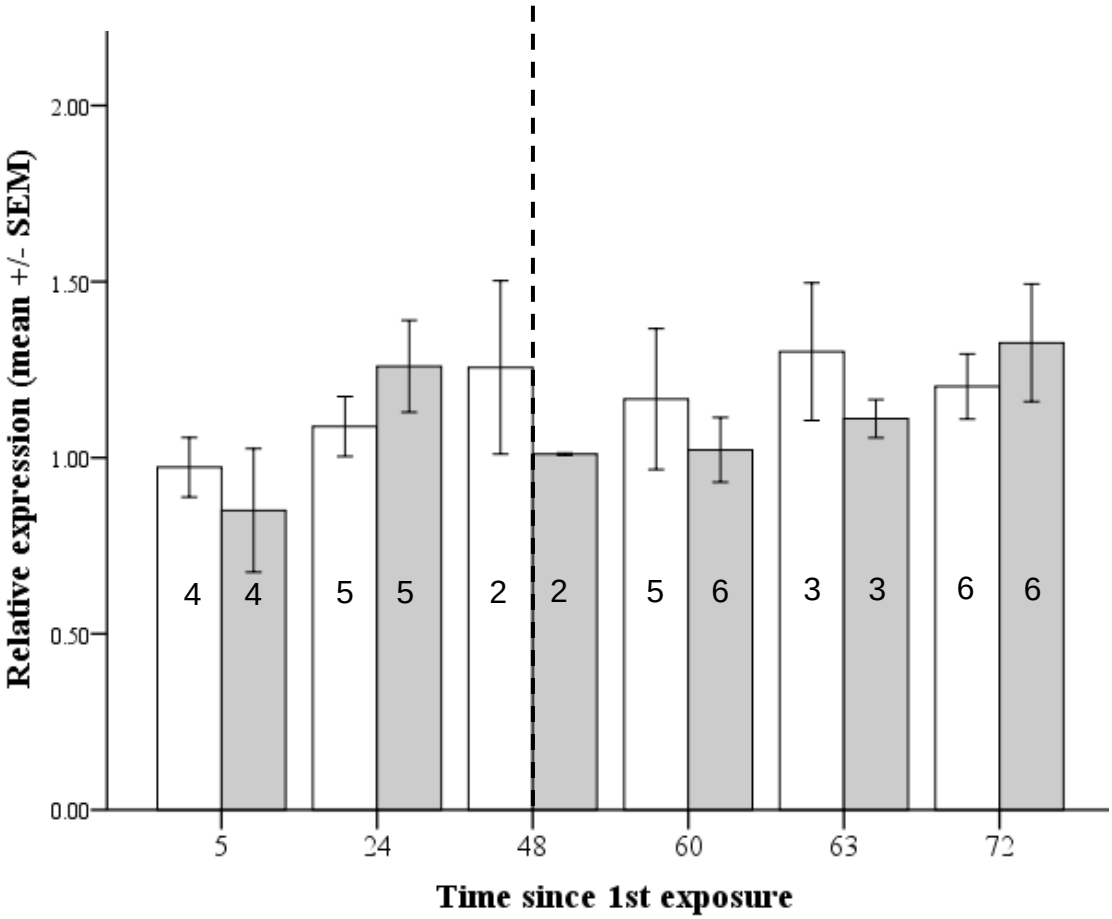

Figure 6: Relative expression of *Nrx-1* when kept singly (white bars) and with a rival (grey bars) for distinct periods. The dotted line represents when males were removed from a rival presence, 48 hours after originally being exposed to a rival. Previous to this, males were kept with rivals for 5 or 24 hours. Error bars represent 1 SEM, numbers within the bars show the number of pooled samples tested.

Table 3: Test used, test statistic and significance value (p) for tests performed on relative expression differences across four learning and memory genes.

Gene	Test	Test stat	*N*	*p*
Dunce	Social*Time	AOD: $X^2_{52} = 3.566$		0.676
	Social	AOD: $X^2_{53} = 3.603$		0.477
	Time	AOD: $X^2_{53} = 3.787$		0.082
Rut	Social*Time	AOD: $X^2_{54} = 4.134$		0.563
	Social	AOD: $X^2_{55} = 4.202$		0.349
	Time	AOD: $X^2_{55} = 4.7320$		0.005 **
Notch	Social*Time	$F_{45} = 0.477$		0.791
	Social	$F_{45} = 1.640$		0.207
	Time	$F_{45} = 0.477$		0.791
Nrx-1	Social*Time	AOD: $X^2_{47} = 3.481$		0.976
	Social	AOD: $X^2_{48} = 3.494$		0.676
	Time	AOD: $X^2_{48} = 3.806$		0.037 *
Dunce	5	$Z = -0.940$	10	0.347
	24	$Z = -0.641$	12	0.522
	0	$Z = -1.091$	6	0.275
	12	$Z = -1.643$	11	0.100
	15	$Z = -0.775$	4	0.439
	24	$Z = -0.160$	12	0.879
Rut	5	$Z = -0.104$	10	0.917
	24	$Z = -1.441$	12	0.150
	0	$Z = -0.218$	6	0.827
	12	$Z = -0.548$	11	0.584
	15	$Z = -0.218$	6	0.827
	24	$Z = -0.801$	12	0.423
Notch	5	$t_8 = -0.312$		0.763
	24	$t_{10} = -1.104$		0.306
	0	$t_4 = -1.320$		0.257
	12	$t_9 = 0.563$		0.587
	15	$t_4 = 0.138$		0.897
	24	$t_{10} = -1.266$		0.234
Nrx-1	5	$Z = -0.289$	8	0.773
	24	$Z = -0.732$	10	0.465
	0	$Z = -0.775$	4	0.439
	12	$Z = -0.183$	11	0.855
	15	$Z = -0.218$	6	0.827
	24	$Z = -0.183$	11	0.855

Asterisks represent the level of significance. * < 0.05, ** < 0.01, *** < 0.001.

Table 4: Detailed function of important genes used in this chapter, predictions of how the expression of the genes may differ when focal males are exposed to rival males at different time points and the actual results for these predictions.

Gene	Function	Prediction	Result
Dunce	Learning	*Dunce* to be upregulated within the first hours of rival introduction into the environment or soon after rival removal	No difference in expression between male kept singly or with a rival at any time period
Nrx-1	Synapse development and function	*Neurexin* to be upregulated at all times points	No difference in expression between male kept singly or with a rival at any time period
Rut	Learning and STM	*Rut* to be upregulated within the first 6 hours of rival introduction into the environment or soon after rival removal	No difference in expression between male kept singly or with a rival at any time period
Notch	LTM	*Notch* to be upregulated after rival removal between 63 and 72 hours after original exposure	No difference in expression between male kept singly or with a rival at any time period

5.5 Discussion

In this chapter I show that rival induced extended mating duration in male *D. melanogaster* does not require expression changes in a subset of learning and memory genes. The genes investigated have previously been shown to be involved in the control of extended mating duration (Chapter 4 (Kim, Jan & Jan 2012)) or be responsible for synaptic remodelling associated with plastic development in the brain (Knight, Xie & Boulianne 2011). If these genes acted to control extended mating duration via transcription change it was expected that they would fluctuate depending on the social environment. The lack of response suggests that although learning and memory plays a role in controlling extended mating duration, it does so through mechanisms other than change in the transcriptomic state of the brain.

As discussed in previous chapters, extended mating duration bares a close resemblance to courtship suppression, both in the length of time needed to create a memory phase and the sensory modalities needed to induce plastic behaviour. In addition, learning and memory genes controlling behaviour seem to be conserved between these two slightly different physiological processes (Chapter 4). In long-term courtship suppression, the memory phase suggested to be most analogous to extended mating behaviour (Rouse & Bretman 2016), genes associated with muscular cytoskeletal dynamics were significantly upregulated in flies that had undergone suppression training compared to naïve flies (Winbush *et al.* 2012). These genes act to alter neuronal architecture and reorganise axonal connections critical for cognition (Dent & Gertler 2003) and therefore upregulation appears to be a possible function whereby learning and memory is controlled. The gene investigated within this chapter associated with a similar process is *Neurexin* (*Nrx-1)* which acts to modulate interactions between pre and post synaptic compartments at septate junctions (Baumgartner *et al.* 1996) and so structure synaptic architecture.

Indeed, when males were trained to supress courtship this increased *Nrx-1* isoform abundance; though did not change total gene expression (Winbush *et al.* 2012).

In comparison, a study that measured genomic change after males had been exposed to rivals for 20 minutes found that any gene involved in learning or axon development was downregulated. This included *Fasciclin II*, a cell adhesion protein found at septate junctions that works in a comparable manner to *Nrx-1*. Similar genes that work in comparable pathways are therefore regulated very differently between the two studies (Ellis & Carney 2011; Winbush *et al.* 2012) depending on whether learning and memory or competition is induced. The change in gene expression between the two studies is potentially a factor of the interacting partner's sex. Genomic changes created by courting or competing depending on the sex of a rival are well established (Certel *et al.* 2007; Certel *et al.* 2010). However, the genes discussed in this chapter do not show differential expression depending on the sex of the interacting partner in other studies (Winbush *et al.* 2012). Assuming an increase in learning and memory plays a role in controlling reactions to a rival male presence (Chapter 4) both studies should therefore show agreement on the direction of expression in a similar suite of genes. The fact they do not may be due to two possibilities. Firstly, that courtship suppression or extended mating duration are in conflict and therefore any shared molecular mechanisms would not agree on the direction of gene expression. Any shared molecular mechanisms controlling conflicting behaviours are thought to be modulated by context specific transcription factors (Chandrasekaran *et al.* 2011) that upregulate or downregulate gene expression by taking into account the organisms internal environment (Sanogo & Bell 2016). An example of such a transcription factor in bee (*Apis mellifera*) behaviour is *Creb* that plays a role in multiple behavioural changes (Chandrasekaran *et al.* 2011). The presence of a rival and learning and memory may therefore be deemed conflicting behaviours and rely on internal environment to

establish the neurogenomic change that may control behaviour and so show different expression depending on experimental process (Ellis & Carney 2011; Winbush *et al.* 2012).

Conversely, reaction to a rival presence and learning may not share a molecular mechanism. This is supported by work that examined how gene expression could change with olfactory associative learning, a learning paradigm using stimuli directly controlled by the researcher. Here, differentially expressed genes did not mirror any of the genes shown to vary with rival male presence (Dubnau *et al.* 2003). Males may therefore rely on a neural or post-transcriptional mechanism to control extended mating duration. Indeed, learning and memory via olfactory association reveals small changes in expression levels for proteins with genes tested in this study. All three of *dnc*, *rut* and *Notch* change protein expression after learning, suggesting some role for post-translational control in memory formation (Zhang *et al.* 2014). Post-translational expression differences can occur in multiple ways, for example, through phosphorylation or acetylation. A previous study has shown that loss of histone acetylase function significantly decreased *rut* and *Fas II* expression within the adult head, which also impacted on courtship suppression ability (Xu *et al.* 2014). In order to conclude whether control of extended mating duration is due to post-transcriptional protein content/levels the groups discussed here would have to be compared. Different protein levels depending on sperm competition, coupled with the result shown in this chapter, would support post-transcriptional change as a controller of complex physiological plasticity.

Overall, my results point to a need for post-transcriptional change to control extended mating duration. However, previous research has shown social challenge leads to gene expression changes involved with neuronal architecture in multiple species (Rampon *et al.* 2000; Rittschof *et al.* 2014; Greenwood & Peichel 2015). I

was therefore expecting expression change in at least one of the genes tested here. In addition to the need for post-transcriptional controls a possible explanation for the lack of gene expression change stems from the tested genes' importance in other molecular pathways. *dunce* and *rut* are both significant players in the cAMP-learning and memory pathway. *dunce* has a role in controlling visual attention (van Swinderen *et al.* 2009) and both genes regulate cAMP levels in the embryo, helping to define normal development (Whitehousehills, Bellen & Kiger 1992). Considering *dunce* is involved in visual attention, I would not expect an increase in expression levels as there is no evidence for vision bringing about extended mating duration (Bretman et al 2011 and Chapter 3). In addition, visual attention is linked to contextual learning (Brembs & Wiener 2006; Zhang *et al.* 2007), meaning normal *dunce* expression is needed in areas outside of simple learning and memory. Another gene I tested in this chapter is well known for controlling a wide range of physiological processes. *Notch* signalling controls cell communication, cell fate and genesis of new tissue in development (Lai 2004). In addition it also controls plasticity of multiple neuronal systems in adult *D. melanogaster* (Ables *et al.* 2011; Kidd, Struhl & Lieber 2015) and the possible maintenance of pluripotent stems cells (Guo & Ohlstein 2015). It is therefore not surprising that a small change in sociality would not have a large effect on the expression of this gene as this would then go on to have significant effects on other physiological processes, potentially not involved in rival induced extended mating duration.

The evidence presented above, that genes involved in controlling extended mating duration are also involved in a multitude of other pathways, supports a previous suggestion in Chapter 4. That extended mating duration has co-opted learning and memory circuitry to ensure behavioural change works on accurate timescales. Extended mating duration relies on mushroom body (MB) circuits that

also modulate a wide range of behaviours such as temperature preference (Hong *et al.* 2008), learning, memory (Stopfer 2014) and sleep (Joiner *et al.* 2006).

Considering the lack of neurogenomic change seen in this chapter after the introduction of rivals for 48 hours this would support MB circuitry as a potential controller for extended mating duration. If indeed extended mating duration is controlled by neural circuitry the most parsimonious explanation would involve biochemical switching between neuronal networks modulated by the release of neuromodulatory hormones with the addition of a rival. In *D. melanogaster* these hormones are known to act on G-protein coupled receptors that interact with adenylate cyclase to regulate production of 2nd messengers (Nassel 2002). Previously extended mating duration has been abolished when the gene for a specific neuromodulator, amnesiac, is knocked out (Chapter 4). This would further support the need for a biochemical switch between neurons to enable extended mating duration. To test this idea neuromodulators would need to be measured within the brain of males exposed to rivals at time periods that correlate to a build-up of behavioural plasticity.

Considering extended mating duration seems to have co-opted at least some mechanisms involved in learning and memory it may also act as another pressure allowing learning and memory to evolve. Males able to learn and memorise gain a fitness advantage when in mating competition through a suite of behaviours (Dukas, Clark & Abbott 2006; Saleem *et al.* 2014), to which extended mating duration can now be added (Bretman, Fricke & Chapman 2009). Overall, when mating competition is removed and these behaviours are not needed anymore learning and memory ability is reduced (Hollis & Kawecki 2014). This highlights reactions to social rivalry as a key component in the need to evolve effective cognitive ability.

The main limitation of the study is the ability to look at expression differences for only four genes. These genes were carefully picked as the most likely to show differential expression after exposure to rivals and also have a role to play in learning and memory. However, as seen in previous investigations, the use of transcriptomics would be more applicable to questions I am trying to answer. Although qPCR is a very powerful tool to investigate gene expression differences a whole genome approach as seen in studies by Carney (2007) Winbrush and colleagues (2012) and Smith and colleagues (2013) would be able to highlight gene expression differences in the brain that would not have occurred to me to target. This would also allow me to establish whether differences in gene expression, though not statistically different, are biologically meaningful (Bickel 2004). To do this, each gene could be analysed as part of a module of other genes with similar biological properties to establish whether a set of genes generally showed a change in expression (Cho, Wang & Galas 2011). In addition to the limits discussed above, qPCR does not allow for comparisons of transcript abundance between conditions unless looked for specifically. However, the drawback to high-through-put techniques such as RNAseq would have been the reduction of different time periods I would have been able to use when exposing males to rivals as the cost would be prohibitive.

In conclusion, genes typically thought of as controlling physiological processes through learning and memory do not use differential expression as a mechanism of control in extended mating duration. Of the genes involved in learning and memory that do undergo differential expression none change expression levels in a constant way depending on whether learning and memory or male-male competition has been induced. This raises the possibility that learning and rival exposure are not transcriptionally linked when controlling extended mating duration.

Post-transcriptional changes would therefore be the front runner for a molecular mechanism underlying extended mating duration.

Effect of social contact and sperm competition on cognition in male *Drosophila melanogaster*

Thanks to Laurin McDowall for all the help collecting data for this chapter

6.1 Summary

It has been hypothesised that increases in cognition, the ability to sense and react to environmental stimuli, are due to an increase in sociality, specifically the development of complex relationships. However, the interaction between sociality and the evolution of cognition is controversial, and in particular the role in sexually selected behaviour. Recent evidence shows that competition for matings seem to have driven an increase in male cognitive ability in *D. melanogaster*. There is also evidence that sperm competition induces at least one plastic behaviour known to be controlled by learning and memory. Despite this, it is still not known whether individual males show within generation cognitive plasticity to changes in sperm competition.

Here, I show an increase in sperm competition from the presence of conspecific rivals increases cognitive ability in male *Drosophila melanogaster*. I also show an increase is general competition from heterospecific rivals does not lead to a comparable increase in cognition. Furthermore, an increase in cognitive ability was paired with an increase in the expression of a subset of genes associated with increased neural complexity and synapse control. The results indicate that male-male competition is important for *D. melanogaster* males to develop cognitive ability, and may be involved in the evolution of increased cognition in insects.

6.2 Introduction

Cognition is defined as the neural processes needed to acquire, process, retain and use information (Dukas 2004a). It includes but is not limited to perception, learning, memory and decision making (Shettleworth 2010). However, whilst the processes by which brains have evolved, and how the environment affects the cognition of animals has been widely researched, a consensus about the critical drivers of cognitive evolution has not yet emerged. Previously it has been suggested that a more complex social environment has led to an increase in brain size and cognition in social mammals. The 'social brain hypothesis' was originally put forward to explain increased cognitive ability in primates, and postulates that an increase in relative brain size evolved due to increased social complexity (Dunbar 1998; Dunbar 2009). Since then, evidence has been presented from outside the primate system including ungulates (Shultz & Dunbar 2006), carnivores (Perez-Barberia, Shultz & Dunbar 2007) and bats (Pitnick, Jones & Wilkinson 2006), all in support of the idea that sociality increases brain complexity. As a critical facet of social complexity, mating systems have been scrutinised to help pinpoint parts of the social environment responsible for impacting the evolution of the brain. Monogamy, especially pairbonding, was found to lead to an increase in neocortex size and so functioned to increase cognition (Dunbar & Shultz 2007). It was reasoned that the ability of an animal to form a complex social relationship, such as pairbonding, was cognitively more challenging than any social relationship developed in polygamous mating systems. However in insects, recent theory suggests that male-male competition may be important for the evolution of quantity assessment (Shifferman 2012), suggesting the role of sexually selected reproductive behaviours is cognitively challenging and therefore requires further investigation.

Although insects possess relatively small brains, they exhibit sophisticated social behaviours and relatively advanced cognitive abilities such as associative

learning (McGuire, Deshazer & Davis 2005), spatial memory (Menzel *et al.* 2000; Menzel *et al.* 2005; Collett, Chittka & Collett 2013; Collett 2014), anticipatory memory (Greenspan & van Swinderen 2004) and second order conditioning (Brembs & Heisenberg 2001; Menzel 2001). In addition, their generation time allows for experimental evolution to directly answer questions that are left to theory in larger, longer lived animals. It may be therefore surprising that in comparison to mammalian mating systems, there is a paucity of evidence linking social interactions and cognition (Lihoreau, Latty & Chittka 2012). Those studies that have examined the link between social interactions and cognition in insects have based their conclusions around the size and complexity of the insect MBs. These are involved in many of the cognitive tasks insects are able to perform and parallel the cortex in mammals as a centre of higher-order learning (Heisenberg 1998; Devaud *et al.* 2015). In addition, they react structurally to changes within an insect's lifetime, with both the honey bee *Apis mellifera* (Ismail, Robinson & Fahrbach 2006) and the ant *Camponotus floridanus* (Gronenberg, Heeren & Holldobler 1996) increasing MB complexity with increased foraging experience, and *D. melanogaster* decreasing synapse number (Donlea, Ramanan & Shaw 2009) when socially isolated. To accrue these relatively long-term structural changes there is usually a parallel change in the neurogenomic state of the brain (Whitfield, Cziko & Robinson 2003; Lutz *et al.* 2012). When comparing solitary and eusocial insect species, MB complexity is reduced with increased sociality in direct opposition to results seen in mammals (Farris & Schulmeister 2011). However, as previously discussed (Chapter 1) these two social environments do not just entail a comparative increase in social interactions but also the emergence of behaviourally distinct clones in eusocial insects (Gronenberg & Riveros 2009). It is therefore likely that studies of non-eusocial insects will generate more generally applicable understanding of the implications of insect social interactions on brain evolution.

One study to investigate the link between sociality and cognitive ability in a small individualised society found that removal of male-male competition for 100 generations significantly decreased a *D. melanogaster* male's ability to learn (Hollis & Kawecki 2014). This was measured by evaluation of a male's capacity to perform a complex social learning task and a simple Pavlovian conditioning assay. As it is now known that males find responding to rivals cognitively challenging (Chapter 4) and considering the ability for the insect brain to plastically respond to environmental change (Donlea & Shaw 2009), this study would suggest that removal of male-male competition may affect individual cognitive ability. I would therefore predict that individuals receiving cues from an environment with male-male competition would show greater cognitive ability within life when compared to socially isolated individuals.

To assess whether individual cognition was affected by social environment individual flies were socially isolated, then exposed to a conspecific or heterospecific male partner for 10 days. This enabled us to distinguish between whether general social interactions such as competition for food and space may be influencing cognitive ability, or whether male-male (sperm) competition specifically affected cognition. Following the methods in Hollis and Kawecki (2014) cognition was measured via two learning and memory paradigms, associative learning, and virgin finding (learning in a complex environment). Associative learning was measured using a T-maze and examined a male's ability to form a simple association between a smell and shock. Virgin finding is an ethologically relevant complex association task that requires a male to learn and memorise information about a female's mating status over 20 minutes, and apply that information to court the female most likely to mate out of a choice of five. In addition, gene expression for a subset of synaptic genes was quantified to measure potential changes in the neurogenomic state of the brain as a proxy for potential structural plasticity.

6.3 Materials and Methods

6.3.1 Fly stocks

Drosophila melanogaster fly rearing and virgin finding (an assay to assess the cognitive ability of a male to learn and remember the identity of a virgin female in a group of five females) were performed in a 25°C humidified room, with a 12:12 light dark cycle (9 am to 9pm light cycle). Flies were maintained in plastic vials containing 7ml sugar-yeast-agar medium (100 g brewer's yeast, 100 g sugar, 20 g agar, 30 ml Nipagin (10% solution) and 3ml propionic acid per litre of medium; (Bass *et al.* 2007). Wild-type flies are originally from a large laboratory stock population collected in Dahomey (Benin) and are the same as used in previous studies from our associated lab (Bretman *et al.* 2012; Bretman, Westmancoat & Chapman 2013; Bretman *et al.* 2013) etc. Larvae for all experiments were raised 100 per vial and supplemented with live yeast. Upon eclosion sexes were separated on ice anaesthesia with females supplemented with live yeast. As *Drosophila virilis* have a slightly lower thermal preference than *D. melanogaster* (Sayeed & Benzer 1996), *D. virilis* (Yamamoto 1994) rivals were grown in vials at 20°C before being collected and sexed on ice.

6.3.2 Social treatments

6.3.2.1 Conspecific competition

To test the effects of intraspecific male-male competition on cognition focal wild-type males were randomly assigned to one of two treatments, either single or exposed to rival males identified by a wing clip, for 10 or 50 days. They were then assigned to one of three experiments, virgin finding, associative learning, or gene expression studies. Males used in virgin finding or associative learning tests were taken directly from their social environments and used in their respective assays. Males that

underwent qPCR were snap frozen in liquid nitrogen, head and thorax dissected away from the abdomen on dry ice and frozen at -80°C before extraction.

6.3.2.2 Heterospecific competition

Previously, *D. virilis* rival males (Figure 1A) have been shown to have no effect on the mating duration of a *D. melanogaster* focal male (Bretman *et al. in prep*). To test the effects of interspecific male-male competition and therefore distinguish the effects of sperm competition (shown with *D. melanogaster* rivals) and general competition focal wild-type males were randomly assigned to one of two treatments, either single or exposed to rival *Drosophila virilis* male (Figure 1A), for 10 days. The social environment was kept at 20°C to keep the *D*. *virilis* alive and active (Dillon *et al.* 2009). After exposure all focal flies were treated in the same way as previously detailed (Figure 1B). Virgin finding and expression experiments were later repeated at 25°C to investigate whether the temperature affected any patterns found.

6.3.2.3 Variable environment

To test the effects of a plastic environment on cognitive ability and how timing of sociality can influence cognitive development, males were randomly assigned to one of two changeable social environments. Males were either exposed to another *D. melanogaster* rival for 10 days before being allowed 10 days in isolation. Over the same time period males were kept singly for 10 days before being exposed to a *D. melanogaster* rivals for 10 days. After exposure all males underwent virgin finding as a proxy for cognitive ability (Figure 2).

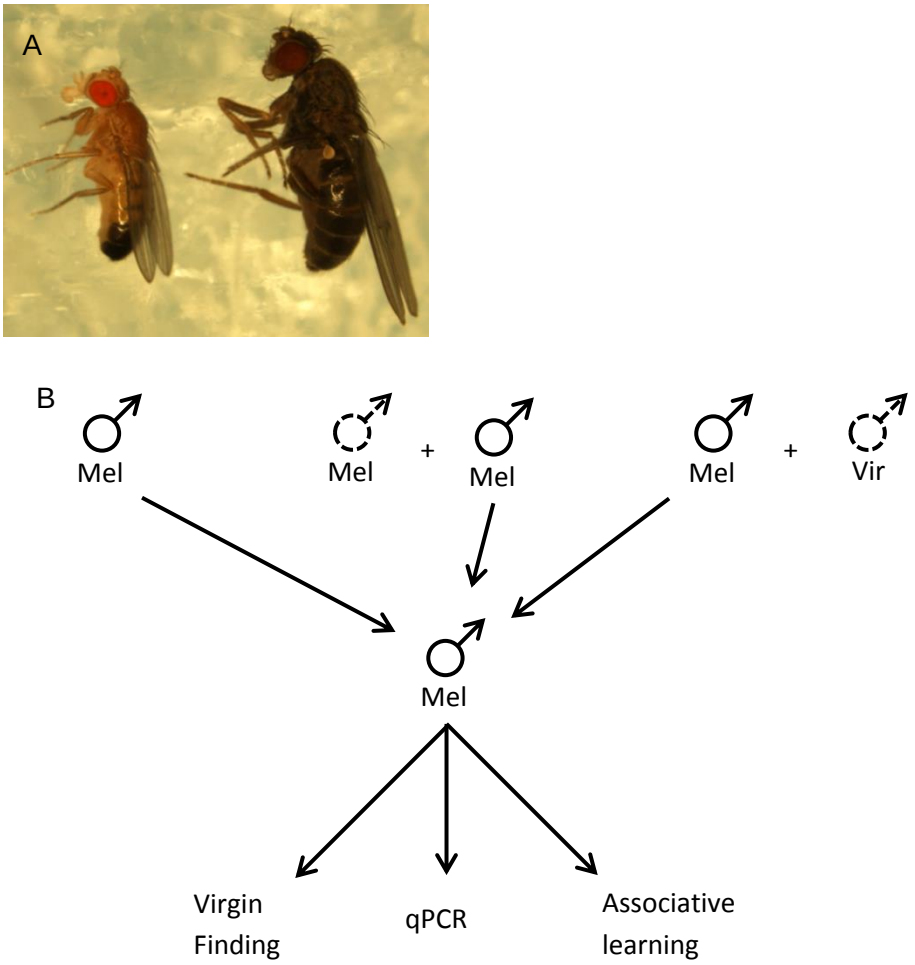

Figure 1: *Drosophila melanogaster* focal individuals and experimental set-up. A) Focal male *D. melanogaster* (left) compared to a rival *D. virilis* male. Magnification = x 7.5 B) B) Experimental set-up and data collection. *D. melanogaster* males (Mel) were removed from their individual environments, either single, with a *D. melanogaster* rival or with a *D. virilis* (Vir) rival, and data collected through virgin finding, associative learning or analysis of gene expression. Each test was independent of all others. Uninterrupted male signs represent males taken forward for testing, dotted male signs represent rival males.

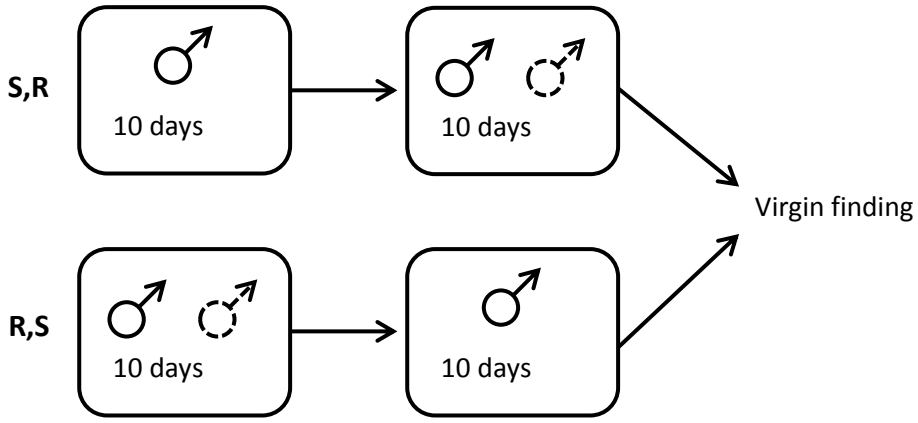

Figure 2: *D. melanogaster* males in a variable environment. S,R describes males kept singly for 10 days and then exposed to a *D. melanogaster* rival for 10 days before undergoing the virgin finding assay. R,S describes males kept with a *D. melanogaster* rival for 10 days and then being moved to an isolated environment for 10 days before undergoing virgin finding. For both B) and C) solid male signs represent males taken forward for testing, dotted male signs represent rival males

6.3.3 Mating duration response to *D. virilis* rivals

Data had been previously collected by Amanda Bretman, James Westmancoat and Tracey Chapman and comes from a larger body of work investigating male *D. melanogaster* reactions to heterospecific rivals (Bretman *et al. in prep*). This provides evidence that rival *D. virilis* do not provide a sperm competition challenge to focal *D. melanogaster* allowing me to use this species as a general competitor and so distinguish the effects of sperm competition on cognitive ability later in the Chapter. Male mating duration responses to either a *D. melanogaster* or *D. virilis* rival after 3 days were measured over three independent experiments. Data non-normal and were examined with a generalised linear model with Poisson errors, using Analysis of Deviance to reduce from the full to minimal model. Differences between treatments were then compared using Mann-Whitney U tests and significance values corrected using Bonferroni correction. There was no effect of experimental repeat on mating duration (AOD: $X^2_{1,324} = -0.243$, $p = 0.622$) but there was a significant effect of treatment on mating duration (AOD: $X^2_{1,324} = -3.951$, $p = 0.047$). When these differences were examined in more detail males kept with *D. melanogaster* rivals significantly increased mating duration compared to both single males (Mann-Whitney U: $Z = -7.875$, $N = 218$, $p < 0.001$) and males kept with *D. virilis* rivals (Mann-Whitney U: $Z = -7.414$, $N = 218$, $p < 0.001$). Single males and males kept with *D. virilis* rivals did not differ in their mating duration (Mann-Whitney U: $Z = -1.143$, $N = 216$, $p = 0.759$; Figure 3).

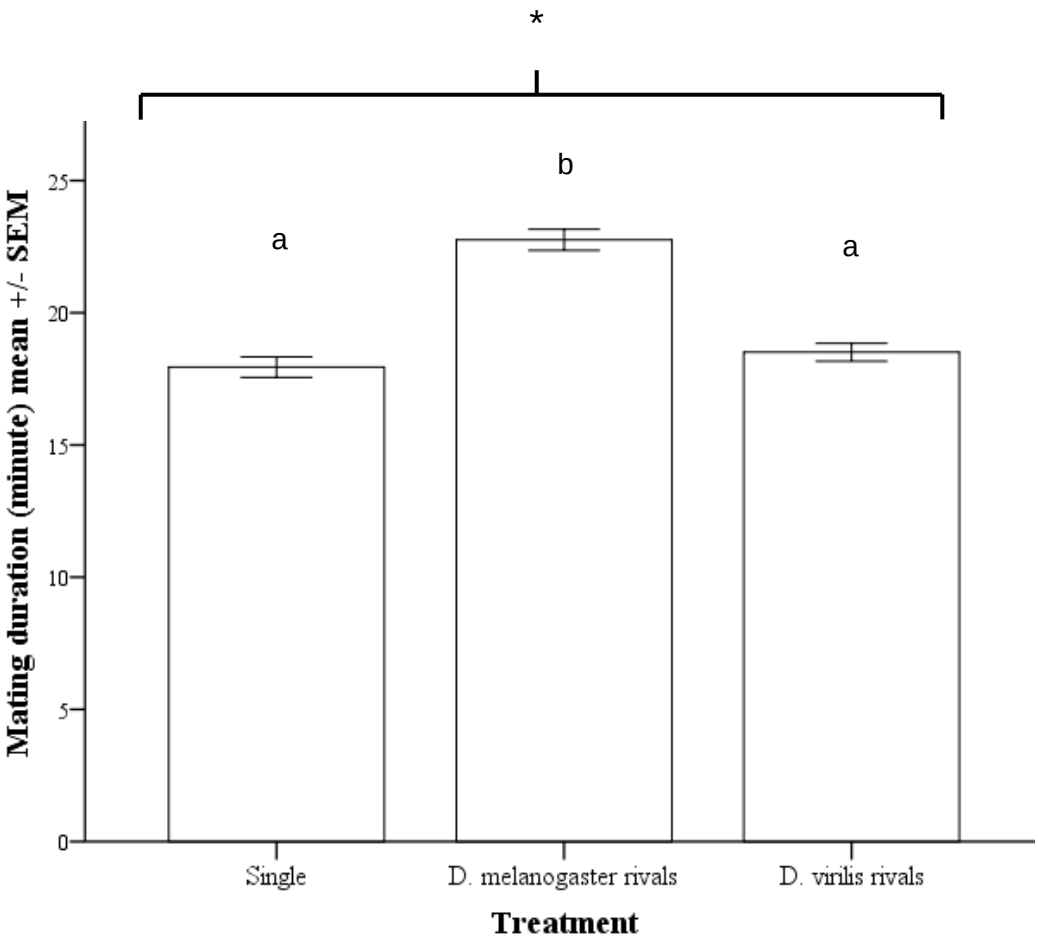

Figure 3: Response to conspecific and heterospecific rival males. Mating duration of males maintained singly, with a rival *D. melanogaster* male or with a *D. virilis* male for 3 days. Error bars represent SEM. Overall significance between treatments is shown above bar. Treatments that do not share a letter were significantly different (*post hoc* Mann-Whitney tests with Bonferroni adjustment).

6.3.4 Virgin Finding

To measure how efficiently males could find a virgin female in a complex social environment an individual male was placed in a vial with four previously mated females and one virgin. This meant to gain the maximum fitness by finding and mating the virgin female (Dukas 2005; Hollis & Kawecki 2014) males had to integrate information about mating status from all females over 20 minutes while using this information to identify the virgin. Vials were scored every minute for 20 minutes to see whether the male was courting, and if so, whether the courted female was mated or virgin. The viewer was able to distinguish the type of female by a small wing clip carried by either the virgin female or mated females depending on the test number. This was to ensure wing clipping did not affect the ability of females to reject male's advances. For males who were successful in mating, data were only counted up until and including the minute of the successful mating. This gave between 78 and 88 individual males per group tested for their ability to find a virgin spread over three repeats.

6.3.5 Aversive associative conditioning

Aversive associative testing was performed on individual flies similar to Claridge-Chang and colleagues (2009) and tests the ability to associate between a smell and shock. All experiments were performed at 22-25°C in a dark room under red light. We used 3-octanol (2.7µL/mL) and 4-methylcyclohexanol (1µL/mL) diluted in light mineral oil as odorants. The odours were drawn through the T-maze and delivered to the flies with a vacuum pump. Individual flies were trained in the T-maze by first calculating the innate preference of the fly for either odour over 2 minutes. The fly was then exposed to the preferred odour accompanied by mechanical shock for 1 second every 5 seconds for 1 minute. This period was followed by a 30 second rest period where the fly was exposed to air. The next minute flies were exposed to the

other odour without shock. This training protocol was repeated once more. To test 2 minute memory flies were immediately moved to a choice point and given both smells simultaneously. The time an individual spent showing preference for either smell was recorded over 2 minutes. This was subsequently calculated as a percentage of the total time an individual made a decision (moved away from the choice point). If an individual had shown preference for both smells in the original test, the percentage of time spent in the smell later not associated with the shock was subtracted from the learning score.

6.3.6 Measuring gene expression via qPCR

RNA was extracted from a pooled sample of 10 to 25 dissected male thorax and heads using Direct-zol^{TM} RNA miniprep columns following the manufacturers protocol. This was checked for concentration and purity on a nanodrop before being run on an agarose gel to assess purity (Figure 4a). cDNA synthesis was carried out using the Life Technologies First strand cDNA kit following the manufacturers protocol and checked against the corresponding no reverse transcriptase controls to check efficient synthesis (Figure 4b).

Genes were chosen from a previous subset of genes shown to be involved with controlling extended mating behaviour (Chapter 4) or as being differentially expressed in the presence of rivals (Mohorianu *et al.* in prep). Housekeeper genes *EF1* and *Rap21* were chosen as the least likely to change with rival presence from multiple genes tested in a previous report (Ling & Salvaterra 2011). All qPCR primers were designed with a melting temperature of 60°C +/- 1°C with CG content between 20-80%. These primer pairs were tested for efficiency using a 10 times dilution series on whole body RNA and accepted if the efficiency fell between 90 and 110% with an R^2 greater than 0.98 (Table 1).

Table 1: qPCR Primer sequence and efficiency. Showing forward and reverse nucleotide sequence, efficiency, and R^2 value for each primer investigated. Efficiency was deemed acceptable between 90 % and 110 %, R^2 values show the pipetting accuracy of standards and should be above 0.98.

Gene	**Forward**	**Reverse**	**Efficiency**	**R^2**
Ef1	GTCTGGAGGCAATGTGCTTT	AATATGATGTCGCCCTGGTT	106.4	0.999
Rap21	TTCACTTACGAACCATCAAACATT	GCTGGCTGACTTCCTTTCAC	107.4	0.999
Brp	GACATCAAGGACCGCAAGAT	GCCATATCCACCTGGTTGTC	95.2	0.999
Dunce	TGTGGCATACACCATATTTCAG	GAAACGGATTGTCTTTGACG	97.8	0.998
Futsch	ACGTTTCCGATTGTCACGTC	GCTGCTACCTCCTCATCGTC	99.6	0.992
Dikar	CATCTATAAAATCCCGCAGAGG	CGGTATCTCCCACCATGATT	99.8	0.998
Neurexin	GACAACAACTGGCACACGAT	TACTGTGGCGACCCAGAAT	98.8	0.995

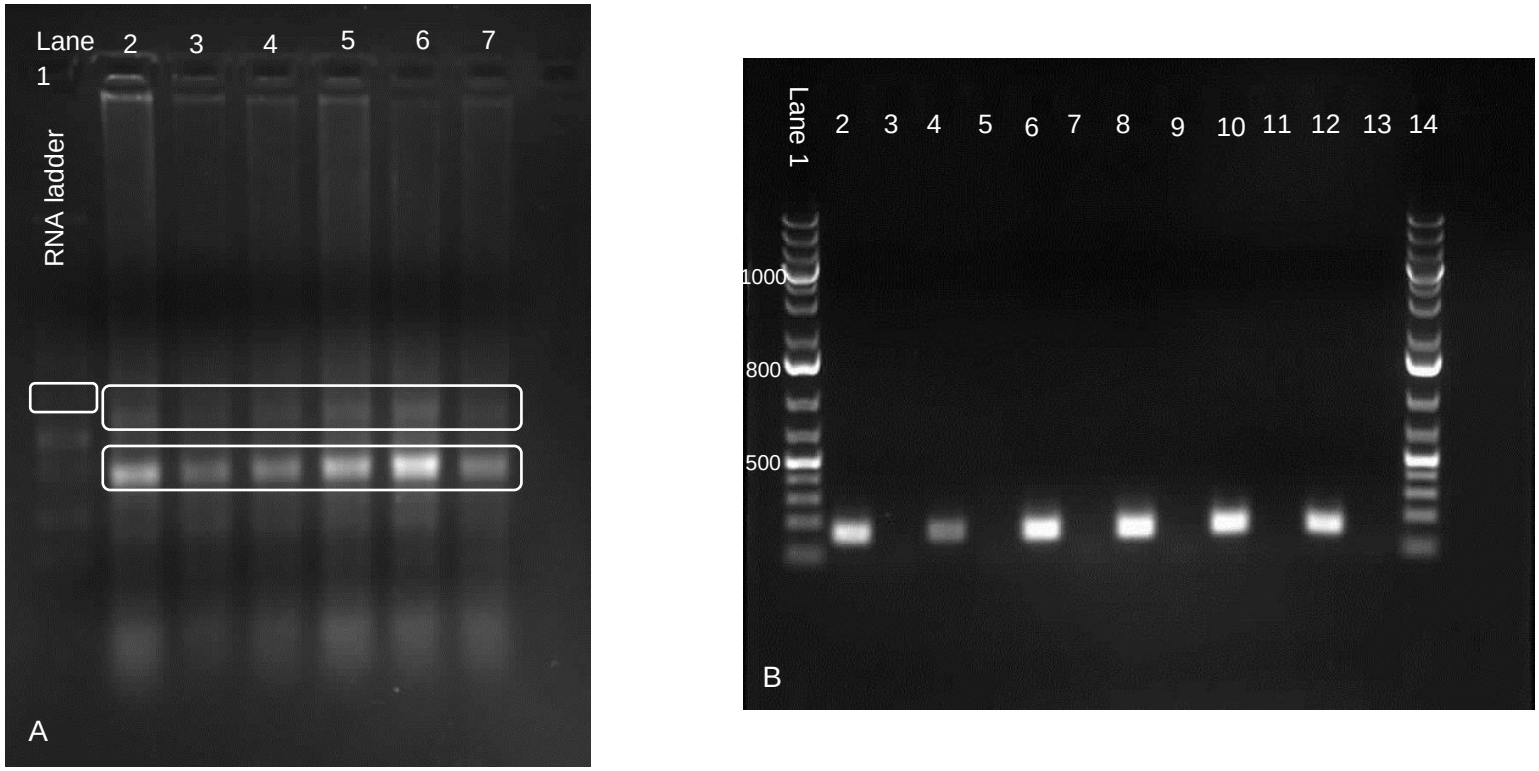

Figure 4: Assessment of RNA and cDNA quality for qPCR via gel electrophoresis a) RNA extraction on 1% agarose gel. Lane 1 = ssRNA ladder with major band marked at 3000 bp, 2-6 = RNA. White outlines highlight 28S (top) and 18S (bottom) RNA. *Drosophila* 28S rRNA is processed into 2 fragments that migrate in a similar manner to the 18S rRNA (Winnebeck, Millar & Warman 2010). Any degradation around these highlights is due to the use of non-autoclaved buffer when making and running the gel. b) Assessment of efficient synthesis of cDNA. Lane 1 and 14 show 100 bp DNA ladder with major bands size marked in base pairs. Lanes 2, 4, 6, 8, 10 and 12 show cDNA synthesis with reverse transcriptase (+RT) for the RNA extractions shown in a). Lanes 3, 5, 7, 9, 11 and 13 show cDNA synthesis without reverse transcriptase (-RT) for the same RNA extractions.

Quantification of transcript levels in flies relative to housekeeping genes (Ef1 and Rap21) were performed using the ΔΔCT method (Hellemans *et al.* 2007). For all genes, Ct values for samples were quantified against the lowest value across biological replicates, taking into account the efficiency of the primer used. For housekeeping genes this number was then used to calculate a geometric mean of both housekeepers in each sample. Each gene of interest was then divided by the geometric mean of the housekeepers for each sample before being averaged across biological replicates to get a relative expression for each gene when males were both single and with rivals (Hellemans *et al.* 2007).

6.3.7 Statistical analysis

Statistical analysis was performed using SPSSv14 and R 3.3.1 (Ihaka & Gentleman 1996).

6.3.7.1 Virgin finding

Behaviour was a binomial response (courting or not, courting the virgin or mated female), hence the effect of social treatment was analysed using a generalised linear mixed model with a binomial distribution with social treatment as a fixed factor and repeat as a random factor. A model with effect of treatment included was compared to a model relying solely on random factors to explain variation using Analysis of Deviance.

An activity index, or % courting, was generated for each individual male by dividing the number of times a male courted by the total minutes a male spent in the company of the females. A Learning index, or % correct courting (when a male courted the virgin, or “correct female”), was generated by dividing the number of

times a male correctly courted a virgin female from the amount of time that male spent courting. These indices were used to graph data.

6.3.7.2 Associative learning

To account for the differences in behaviour between test sessions, learning indices from 4 to 6 flies were analysed as the mean across individuals within one session. This gave one data point for each genotype for each training and testing session for between 8 and 10 sessions for each pairwise comparison. The Learning index from these 8 to 10 sessions was then compared in a pairwise manner between focal flies that had been kept singly or with rivals for all experiments. All experiments were normally distributed so were compared with independent T-tests.

6.3.7.3 Gene expression

For experiments with male focal flies exposed to *D. melanogaster* or *D. virilis* rivals, relative expression differences were established through a 2-way ANOVA with gene and social group as main factors. Pairwise comparisons were then performed to investigate the difference between males kept singularly and with a rival for each gene.

Table 2: Detailed function of important learning and memory genes examined in this chapter and predictions of how rival exposure for 10 days may affect expression of these genes.

Gene	Function	Prediction
Bruckpilot	Vesicle release at the synapse	Upregulated when focal males had previously been kept with rival *D. melanogaster* but not when kept with *D. virilis*
Dikar	LTM	Upregulated when focal males had previously been kept with rival *D. melanogaster* but not when kept with *D. virilis*
Dunce	Learning	Upregulated when focal males had previously been kept with rival *D. melanogaster* but not when kept with *D. virilis*
Futsch	Regulates synaptic growth and organisation	Upregulated when focal males had previously been kept with rival *D. melanogaster* but not when kept with *D. virilis*
Neurexin	Synapse development and function	Upregulated when focal males had previously been kept with rival *D. melanogaster* but not when kept with *D. virilis*

6.4 Results

6.4.1 Virgin finding

6.4.1.1 Males kept with D. melanogaster rivals

Males kept with *D. melanogaster* rivals for 10 days did not differ significantly in overall courting effort compared to single males (AOD: $X^2_1 < 0.0001$, $N = 165$, $p = 0.986$. Figure 5A). However, males previously kept with a rival male for 10 days were significantly more effective at targeting courtship towards virgin females over the length of the assay than males kept isolated (AOD: $X^2_1 = 29.212$, $N = 165$, $p < 0.001$. Figure 6A).

6.4.1.2 Males kept with D. virilis rivals

D. melanogaster males kept with *D. virilis* males for 10 days significantly increased their courting effort when compared to single males (AOD: $X^2_1 = 4.871$, $N = 176$, $p = 0.027$. Figure 5B) showing that males could sense the presence of a *D. virilis* rival. However, males kept singly were significantly more likely to effectively target their courtship towards virgin females compared to males kept with *D. virilis* rivals ($X^2_1 = 8.1616$, $N = 176$, $p = 0.004$. Figure 6B).

6.4.1.3 Males kept with D. melanogaster rivals for 50 days

When males were kept with rivals for 50 days they significantly decreased their courting rate over 20 minutes when compared to males kept single ($X^2_1 = 119.49$, $N = 157$ $p < 0.001$. Figure 5C). However, although there was a difference in how often males courted females generally, there was no difference in the ability of males to direct courting towards a “correct”, or virgin, female ($X^2_1 = 3.592$, $N = 157$, $p = 0.058$. Figure 6C).

6.4.1.4 Males kept in a variable environment

To establish whether the timing of sociality was important to the development of cognitive ability males were exposed to a *D. melanogaster* rival presence for 10 days before being socially isolated (deemed R,S for short). Males were also socially isolated for 10 days before being exposed to a *D. melanogaster* rival for a further 10 days (S,R). Males who had been exposed to rival males for the 10 days at the start of life before being socially isolated significantly increased courting effort when compared to males who had undergone social isolation first ($X^2_1 = 16.316$, $N = 172$, $p < 0.001$. Figure 5D). However, there was no effect of social environment on a males ability to court a virgin female in a complex environment ($X^2_1 = 1.027$, $N = 172$, $p = 0.311$. Figure 6D).

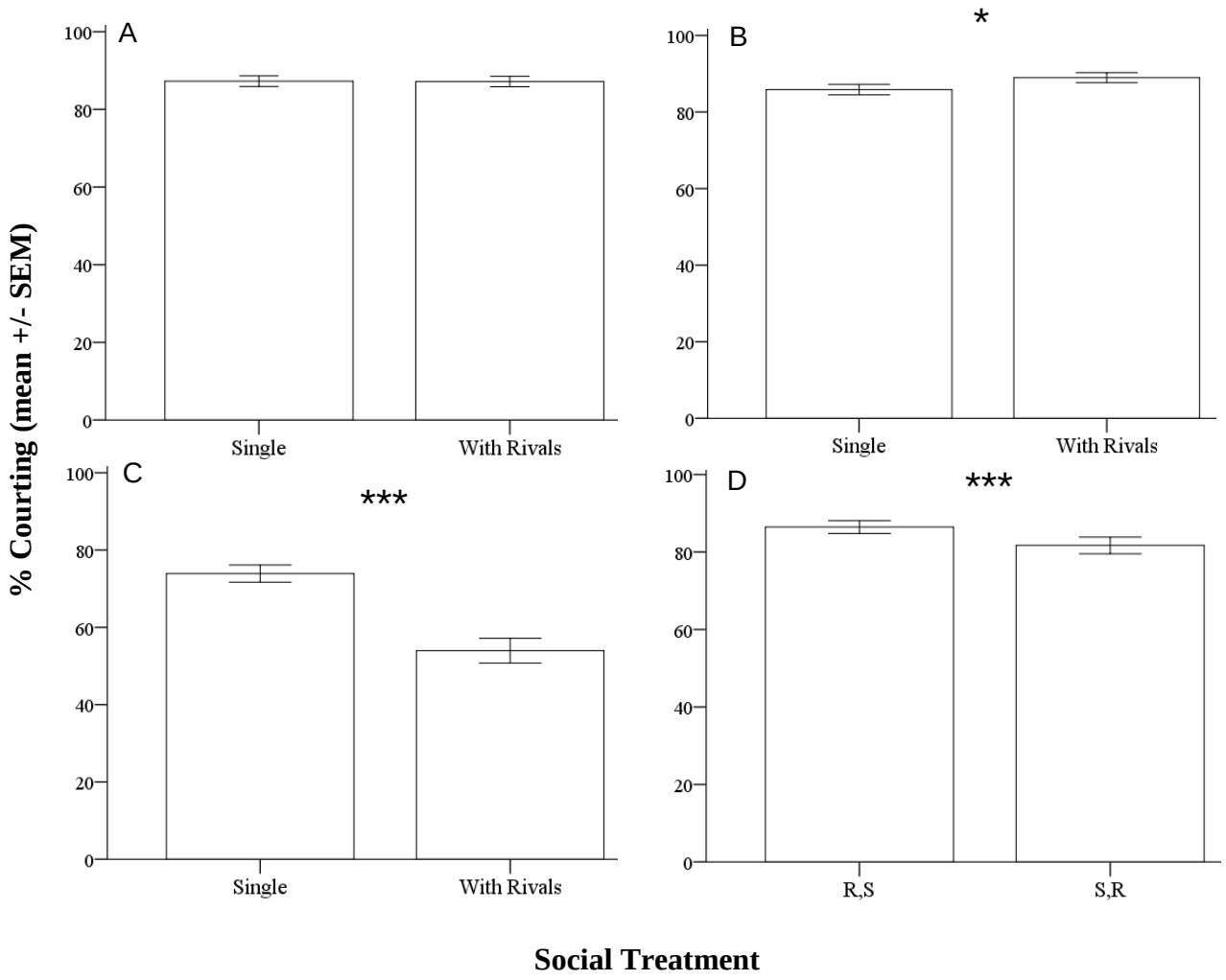

Figure 5: Effect of social environment on the amount of unspecific courting performed by *D. melanogaster* focal males in 20 minutes. All graphs show courting percentage towards any female (choice of one virgin female and four mated females) as a proxy for activity. A) Shows the difference between males kept singly and males previously kept with a rival *D. melanogaster* male for 10 days. B) Shows the difference between males kept singly and males previously kept with a rival *D. virilis* male for 10 days. C) Shows courting effort when males have been kept singly or with a *D. melanogaster* rival for 50 days and shows the effect of ageing on activity. D) Compares courting effort when males have been kept with rival *D. melanogaster* males for the first 10 days (R,S) or the second 10 days (S,R) of a 20 day cycle. All error bars represent SEM. * < 0.05, ** < 0.01, *** < 0.001.

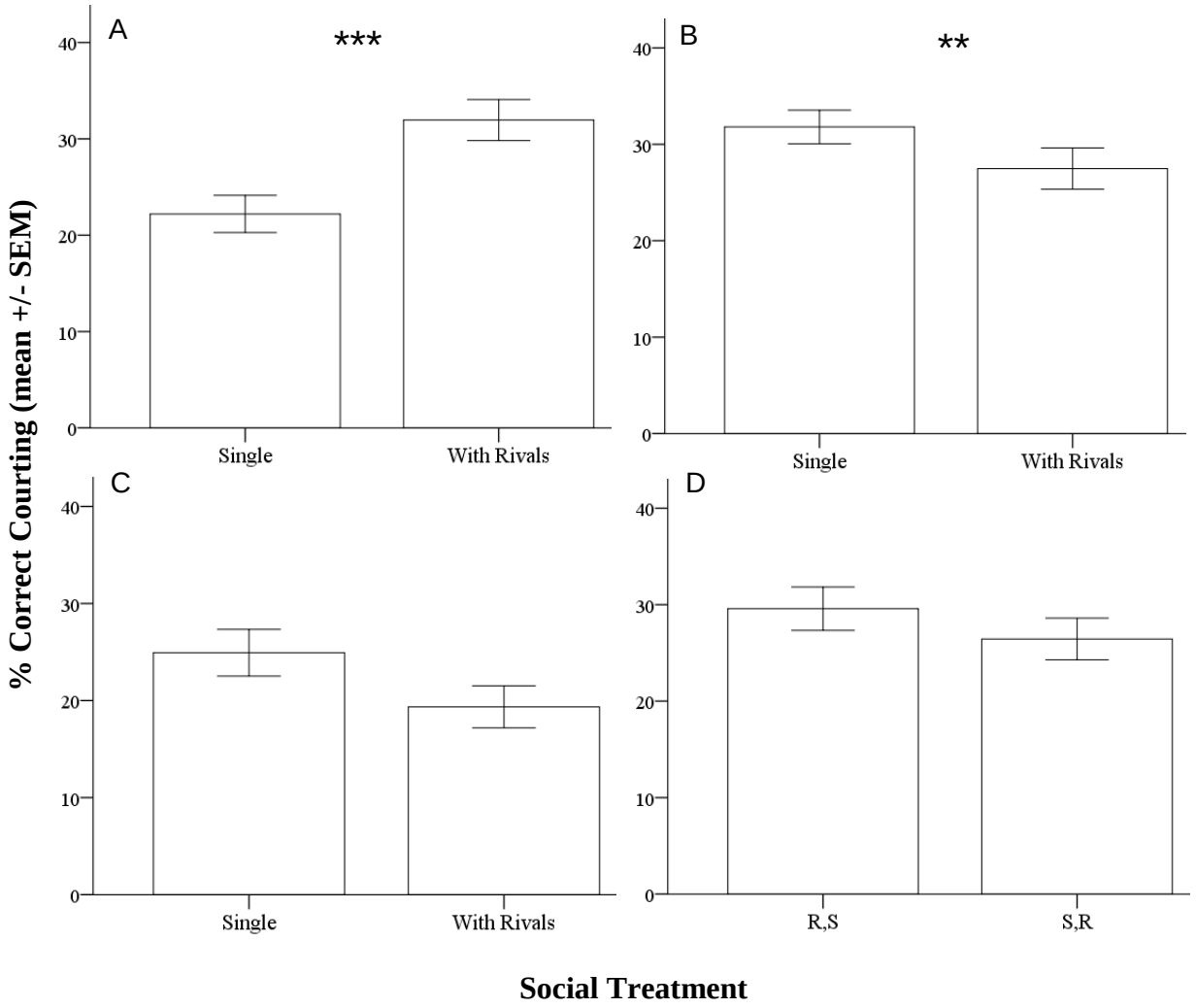

Figure 6: Effect of social environment on the ability of *D. melanogaster* focal males to identify and court a virgin female in a complex environment made up of four other previously mated females. All graphs show the percentage of courting which is directed towards a virgin female ("Correct" courting) as a proxy for cognitive ability. A) Shows the difference between males kept singly and males previously kept with a rival *D. melanogaster* male for 10 days. B) Shows the difference between males kept singly and males previously kept with a rival *D. virilis* male for 10 days. C) Shows ability to identify a virgin when males have been kept singly or with a *D. melanogaster* rival for 50 days and shows the effect of ageing on cognition. D) Compares virgin finding ability of focal males when kept with rival *D. melanogaster* males for the first 10 days (R,S) or the second 10 days (S,R) of a 20 day cycle. This compares social effects on the development of cognition. All error bars represent SEM. * < 0.05, ** < 0.01, *** < 0.001.

6.4.2 Associative learning

The environment an individual was kept in for 10 days significantly changed the way they were able to learn and memorise information taught to them in an olfactory T-maze.

When kept with a rival *D. melanogaster* male for 10 days, focal *D. melanogaster* males significantly increased their ability to learn and memorise an association compared to isolated males (t-test: t_{20} = -2.422, p = 0.025). In comparison, when *D. melanogaster* males were kept housed with a *D. virilis* male there was no difference in their ability to learn or memorise compared to single males (T-test: t_{12} = -0.063, p = 0.950 Figure 10).

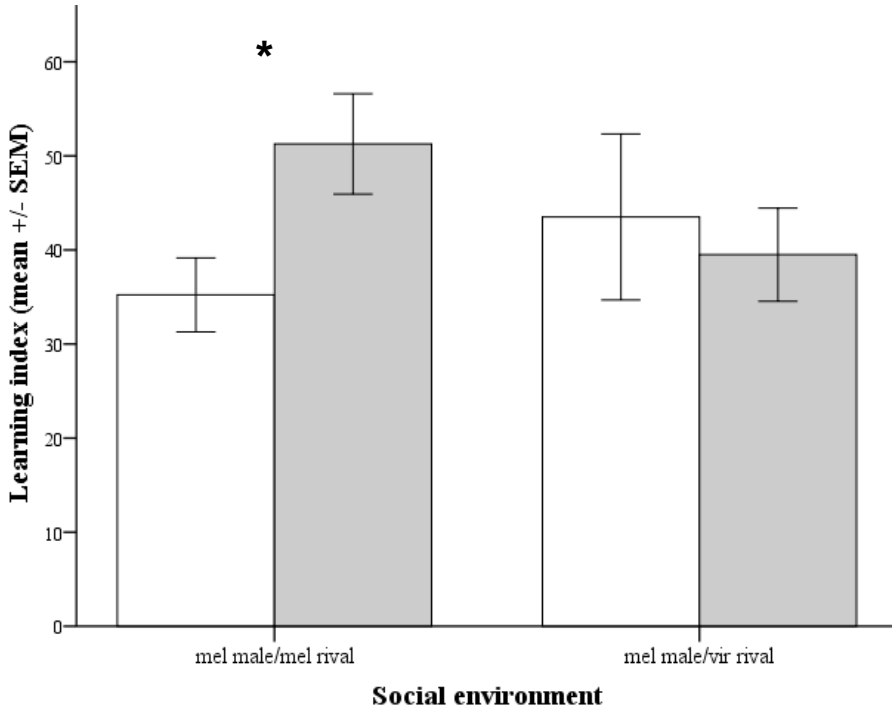

Figure 7: Learning index in an associative learning and memory task. A) *D. melanogaster* males were either kept singly (white bars) or exposed to either *D. melanogaster* or *D. virilis* (grey bars) as rivals. Error bars represent 1 SEM, * indicates a significant difference between paired treatments * < 0.05, ** < 0.01, *** < 0.001

6.4.3 Measuring gene expression via qPCR

6.4.3.1 Gene expression changes after exposure to a D. melanogaster rival

The social environment had a significant effect on gene expression tested across all genes (ANOVA: $F_1 = 11.178$, $p = 0.002$). When each gene was investigated individually using pairwise comparisons to test the effect of social housing with a rival, sociality significantly increased gene expression for two genes, *Futsch* (T-test: $t_9 = -3.299$, p = 0.012) and *Neurexin* (T-test: $F_{10} = -3.424$, p = 0.006; Figure 11), but not for any of the other genes tested (Table 2).

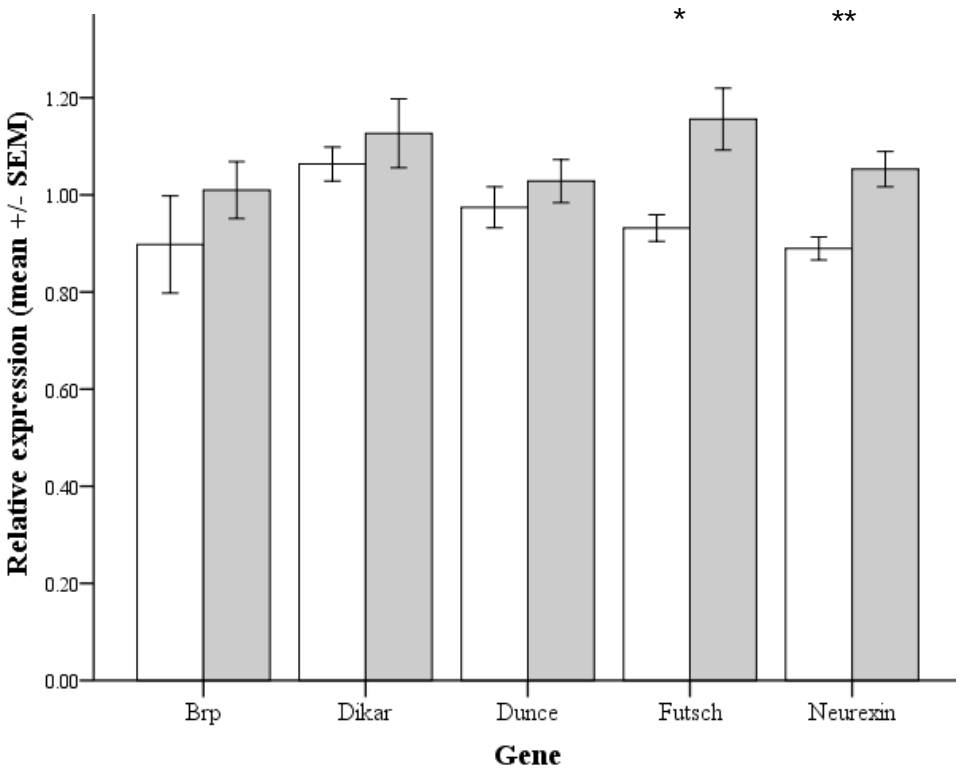

Figure 8: Relative expression of five learning and memory genes after exposure to conspecific rivals. Relative expression levels of five genes when males were kept with *D. melanogaster* rivals (grey bars) compared to when males were kept singly (white bars). Error bars represent SEM. * indicates a significant difference between paired treatments * < 0.05, ** < 0.01, *** < 0.001

6.4.3.2 Gene expression changes after exposure to a D. virilis rival

When *D.melangoaster* males were exposed to *D. virilis* rivals for 10 days there was no subsequent difference in gene expression for any of the genes tested (Figure 8; Table 2).

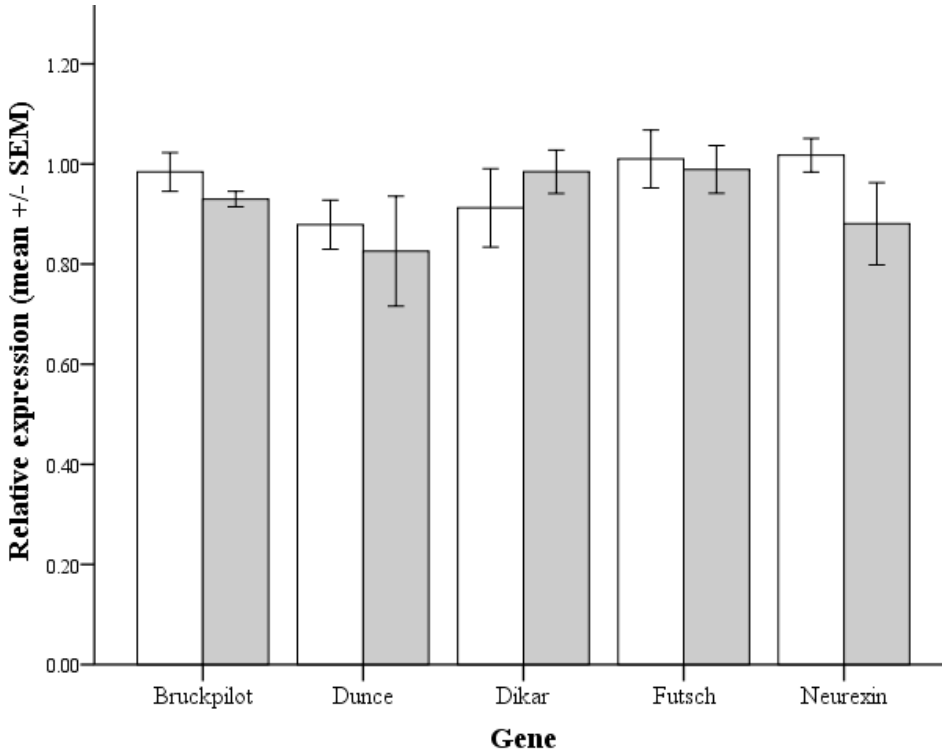

Figure 9: Relative expression of five learning and memory genes after exposure to heterospecific rivals. Relative expression values for five genes when males were kept with *D. virilis* rivals (grey bars) or singularly (white bars). Error bars represent SEM.

Table 3: Statistics for gene expression studies involving *D. melanogaster* (Mel) and *D. virilis* (Vir). Degrees of freedom are in parenthesis to the test statistic.

Experiment	Gene	Test	Test statistic	p
Mel x Mel		2 way ANOVA		
		Sociality	$F_{1,49}$ = 11.178	0.002 **
		Gene	$F_{4,49}$ = 1.957	0.116
		Gene x Sociality	$F_{4,49}$ = 0.718	0.584
	Brp	T-test	t_{10} = -1.030	0.327
	Dunce	T-test	t_{10} = -0.849	0.416
	Dikar	T-test	t_{10} = -0.703	0.498
	Futsch	T-test	t_{9} = -3.229	0.012 *
	Neurexin	T-test	t_{10} = -3.424	0.006 **
Mel x Vir		2 way ANOVA		
25°C		Sociality	$F_{1,39}$ = 2.233	0.083
		Gene	$F_{4,39}$ = 3.639	0.064
		Gene x Sociality	$F_{4,39}$ = 1.014	0.412
	Brp	T-test	t_{8} = -0.170	0.870
	Dunce	T-test	t_{7} = -0.480	0.646
	Dikar	T-test	t_{8} = 1.560	0.157
	Futsch	T-test	t_{7} = 0.899	0.398
	Neurexin	T-test	t_{7} = 1.236	0.256

Stars show level of significance * < 0.05, ** < 0.01

Table 4: Detailed function of important learning and memory genes used in this chapter, predictions of how expression may change with rival exposure for 10 days and actual results.

Gene	Function	Prediction	Result
Bruckpilot	Vesicle release at the synapse	Upregulated when focal males had previously been kept with rival *D. melanogaster* but not when kept with *D. virilis*	No significant difference in expression levels when males kept with rivals of any species compared to males kept isolated
Dikar	LTM	Upregulated when focal males had previously been kept with rival *D. melanogaster* but not when kept with *D. virilis*	No significant difference in expression levels when males kept with rivals of any species compared to males kept isolated
Dunce	Learning	Upregulated when focal males had previously been kept with rival *D. melanogaster* but not when kept with *D. virilis*	No significant difference in expression levels when males kept with rivals of any species compared to males kept isolated
Futsch	Regulates synaptic growth and organisation	Upregulated when focal males had previously been kept with rival *D. melanogaster* but not when kept with *D. virilis*	Males kept with *D. melanogaster* rivals have significantly upregulated expression compared to males kept isolated. Males kept with *D. virilis* rivals have no change in expression levels compared to isolated males
Neurexin	Synapse development and function	Upregulated when focal males had previously been kept with rival *D. melanogaster* but not when kept with *D. virilis*	Males kept with *D. melanogaster* rivals have significantly upregulated expression compared to males kept isolated. Males kept with *D. virilis* rivals have no change in expression levels compared to isolated males

6.5 Discussion

In this chapter I show exposing male *D. melanogaster* to competitive rivals of the same species for 10 days significantly increases their ability to perform two learning and memory tasks. One, an ethologically relevant complex association task requires males to learn about a female's mating status and apply this information over 20 minutes (Hollis & Kawecki 2014). The other examines a male's ability to create a simple association between a smell and shock and tests early-term memories (Claridge-Chang *et al.* 2009). Increased learning and memory ability in both these tasks couples with increased expression in genes associated with synapse morphology and cytoskeletal dynamics. In comparison, exposing males to rivals which are not seen as a competitive threat (*D. virilis* species) does not induce either increase in learning and memory ability or gene expression changes seen when males are kept with conspecific rivals.

6.5.1 Males, competition and cognition

D. melanogaster males previously housed with conspecific rivals for 10 days showed an increased ability to learn and memorise in two cognitive tasks. The ability to avoid an odour previously paired with a mechanical shock relies on associative learning (Mery & Kawecki 2003). Finding and successfully courting a virgin female in a complex social environment relies on a male's ability to apply previous experience, and requires learning and decision making under uncertain conditions (Ejima & Griffith 2011; Hollis & Kawecki 2014). We applied both assays in order to assess male cognition in a reproductive and non-reproductive context.

The ability of a focal male *D. melanogaster* to perform two cognitive tasks improved when exposed to *D. melanogaster* rivals, but did not show the same significant improvement when focal males were housed with *D. virilis* rivals. *D. virilis*

inhabit similar temperature parameters and cosmopolitan distribution as *D. melanogaster* suggesting the two species would interact in the wild (Spieth 1979; Throckmorton 1982; Mirol *et al.* 2008). In support of this, there is a significant decrease in courting effort of males housed with *D. virilis* compared to isolated males suggesting that *D. melanogaster* males can sense the presence of rival *D. virilis*. The two species are sexually isolated so cannot hybridise. In *Drosophila*, acoustic, tactile, gustatory, visual and chemosensory cues have all been implicated in sexual isolation (Cobb & Ferveur 1995; Greenspan & Ferveur 2000). However, recent work has highlighted auditory differences as the original driver of isolation (Yukilevich *et al.* 2016). Male *D. melanogaster* ability to sense a rival *D. virilis* may therefore be due to a multimodal sensory system that include differences in cuticular hydrocarbon make-up (Jackson & Bartelt 1986; Ferveur 2005) or incompatibility of courtship song (Saarikettu, Liimatainen & Hoikkala 2005). Despite the apparent ability for *D. melanogaster* males to identify *D. virilis* males, *D. melanogaster* do not extend mating duration when exposed to *D. virilis* males for three days (Bretman *et al. in prep*). This response has previously been shown to be induced by an increase in sperm competition (Bretman, Fricke & Chapman 2009). Considering the failure of *D. virilis* rivals to induce an increase in learning and memory ability on par with conspecific rivals, it is probable the increase in cognitive ability seen in this chapter is driven by male-male conspecific competition, potentially sperm competition. There have been previous examples of the need for the social environment to drive the full development of memory ability in bees. Honey bees (*Apis mellifera*) socially isolated for the first 3 days of adult development show decreased ability to perform an associative task compared to bees exposed to social cues (Ichikawa & Sasaki 2003). However, to my knowledge this is the first time conspecific competition has been shown to drive changes to within generational learning development.

A likely candidate for the underlying mechanism controlling this increase in cognitive ability might be structural responses within the MBs. Previously MB circuits have been shown to control extended mating duration, a plastic behaviour induced by an increase in sperm competition (Chapter 4). They are also responsible for decision making in uncertain conditions and olfactory memory (Akalal *et al.* 2006; Farris 2013). *D. melanogaster* MBs continue to develop during the first week after eclosion, gaining a net increase of 200-400 fibres, before remaining stable for another 2 weeks (Technau 1984). Adults deprived of olfactory cues within the first 3 weeks do not develop more MB fibres than the initial number present at eclosion (Technau 1984; Balling, Technau & Heisenberg 1987). This developmental pattern can be used to explain the increase in cognitive ability after males were housed for 10 days with a rival. It also suggests that behaviour induced by an increase in conspecific contact modulates the adult development of the MBs and so effects learning and memory ability. In a similar way, individual bees improve their foraging efficiency with experience (Dukas & Visscher 1994; Ismail, Robinson & Fahrbach 2006) by learning the spatial distribution of food sources (Dukas 2008b), with the change driven by an increase in the size of the mushroom body neuropil (Withers *et al.* 2008; Lutz *et al.* 2012). This pattern is repeated throughout many taxa. For example, the ant *Camponotus floridanus* denied social interaction for the first 30 days of life show decreased relative MB size compared to individuals allowed normal social interactions (Seid & Junge 2016). In mammals, macaque monkeys isolated for the first 6 months of life are behaviourally atypical (Harlow & Suomi 1971) while monkeys living in larger social networks increase relative brain size to a greater extent than monkeys limited to a normal social group (Sallet *et al.* 2011).

6.5.2 *D. melanogaster* males kept in variable environments

Males exposed to a variable environment for 20 days where exposure to rival males occurred in the first or the last 10 days did not differ in their ability to find and court a virgin female between these two environments. There is no loss of MB fibres in the first three weeks of *D. melanogaster* post-eclosion life (Technau 1984). Therefore, males exposed to rival males in the variable environments tested should both increase cognitive ability as already discussed above. For males that were exposed to rivals for 10 days prior to isolation this then shows that any socially induced cognitive changes that occur in the first 10 days are maintained beyond immediate removal from the environment that induced those changes. This supports ideas above, as well as in chapter 4, that structural plasticity is responsible for conspecific mediated behaviour. This is because if an increase in cognition was purely a transient behavioural change, 10 days away from the stimulus inducing that behaviour should see individuals return to previous iterations of behaviour (Cardoso, Teles & Oliveira 2015). Nethertheless, the lack of isolated male control within this experiment, I cannot say with certainty that males exposed to a rival after isolation for 10 days have increased cognitive ability. Therefore, more testing of cognitive reactions to variable environments in *D. melanogaster* is needed. In the honey bee (*Apis mellifera*) the later individuals are first exposed to a complex social environment the less able they are to learn a novel association (Ichikawa & Sasaki 2003). A similar time sensitive opportunity window for males exposed to conspecifics to increase cognitive ability may well be occurring here. This would then show that males exposed to rival presence for 10 days before isolation return to a cognitive level on par with isolated males. Overall, more work is required to separate these two possibilities.

6.5.3 Socially induced changes in gene expression

As well as an effect on cognitive ability, perceived sperm competition increased expression of two of the five genes tested within the central nervous system. *futsch* and *neurexin* significantly increased their expression levels when males had previously been kept with rivals compared to singly kept males or males kept with *D. virilis* rivals. *futsch* controls synaptic growth at the neuromuscular junction by regulating the synaptic microtubule cytoskeleton (Roos and Hummel 2000). *Nrx-1* controls synapse communication and assembly (Knight, Xie & Boulianne 2011). Both are specifically associated with the cytoarchitecture of synapses, either through the regulation of the microtubule cytoskeleton (Roos *et al.* 2000) or through synaptogenesis (Li *et al.* 2007; Larkin *et al.* 2015). Therefore, these genes could be seen as proxies to synapse functionality or number, especially *neurexin*, which shows evidence of increased adult synaptic development with overexpression (Larkin *et al.* 2015). The link between synapse number and cognition is well established, with decreases in synapse number linked to decreased cognition in multiple species (Spires-Jones & Knafo 2012), including humans (DeKosky & Scheff 1990) and chemically induced increases in synapse connectivity in mice linked to an increase in cognitive performance (Rogers *et al.* 2011). The increase in expression of genes linked to synapse functionality in this model can therefore be linked to the increase in cognition. Future work on the effect of sperm competition on the brain in *D. melanogaster* should therefore focus on revealing changes in synapse number or functionality with an increase in sperm competition.

dunce, *bruckpilot* and *dikar* did not show differential expression when compared between social environments. This is in line with previous results that show an increase in sperm competition does not induce expression changes of genes directly associated with learning and memory (Chapter 5). Despite this, there was a generalised tendency to increase gene expression in males that had

previously been kept with a rival. Although within this research a large scale study into neurogenomic differences brought on by competition was not performed, differences in gene expression linked to changes in the social environment are not unusual (Rampon *et al.* 2000; Greenwood & Peichel 2015). Small genomic changes spread across multiple genes may therefore have an additive effect causing small changes in gene expression. Indeed, 5 minutes of courting in *D. melanogaster* males is enough to significantly change transcriptional profiles, including genes involved in neurotransmission (Carney 2007), though these changes are reduced 24 hours after rival addition (Mohorianu *et al.* in prep). As qPCR is low throughput I have only assessed a small number of genes. Broad scale neurogenomic change could be assessed using transcriptomics. In addition to measuring differential gene expression transcription factors can be used to establish whether expression changes may be seen in downstream genes. In other models, conserved transcription factors regulate large portions of neural plasticity. For example the transcription factor *egr-1* is upregulated rapidly when fish compete for social dominance, going on to regulate genes involved in social dominance (Burmeister, Jarvis & Fernald 2005) but is also downregulated in socially isolated individuals (Matsumoto *et al.* 2012). Consequently, it may be beneficial in the future to assess the role of transcription factors or epigenomic modifiers as key regulators of brain plasticity in response to environmental enrichment.

6.5.4 Males 50 days

When males were exposed to a rival for 50 days they significantly decreased the amount of courting they engaged in, and no longer showed an increased ability to identify a virgin female compared to isolated males. This supports previous work that details substantial costs associated with males responding to the threat of sperm competition, including reduction in lifespan (Bretman *et al.* 2013). A likely

source of the costs associated with responding to rivals was thought to be in the upregulation of ejaculate proteins (Wigby *et al.* 2009). However, results in this Chapter and in Chapter 4 now also point to costs associated with an upregulation in cognitive abilities. The likely development of MBs with increased social interaction (Technau 1984) increase neural substrate within the MBs, a form of tissue likely to be very costly (Laughlin, van Steveninck & Anderson 1998; Rittschof, Grozinger & Robinson 2015). In addition, a male response to rivals is modulated by anaesthesia sensitive memory requiring protein synthesis (Tully *et al.* 1994)(Chapter 4), which itself induces a substantial cost leading to decreased lifespan and ability to cope with an environmental challenge (Mery & Kawecki 2005; Mery 2007). Therefore, in addition to increased ejaculate production, responding to a rival presence initiates both the cost of activating neural substrate and the development of new neural tissue.

The trade-off between the energy needed to create a response to sperm competition and energy needed for other traits is shown here in a reduction of effort when courting females, but may also show in cognitive ability. Males kept with conspecific rivals for 10 days increased their level of cognition compared to single males but this difference was abolished after 50 days of exposure to a rival. A single male's cognition did not change between the two time periods, making it unlikely the reduction in cognition is solely a response to ageing. Instead, it seems males kept with rivals are unable to continue to increase cognition past a certain age. Considering the cognitive pressures enforced by responding to rivals, cognitive ability may therefore act with age in a similar vein to other measurable factors as shown in Bretman et al (2012). That is, increased cognition brought about by neurogenomic and synaptic changes early in life is favoured due to the benefits associated with the ability to respond to an environment, however, with the caveat of decreased energy levels later in life.

D. melanogaster show reduced learning and memory ability with increased age (Tonoki & Davis 2015), even showing cognitive decline within 10 days (Tamura *et al.* 2003). However, an increase in social complexity can help to protect against cognitive ageing and disease in multiple taxa (Seeman & Crimmins 2001; Ryff & Singer 2005; Amdam 2011). In *D. melanogaster* an increase in sociality can lead to the restructuring of areas of the brain affected by disease (Xu *et al.* 2016), and subsequent rescue of cognitive function (van Praag, Kempermann & Gage 2000; Carulli, Foscarin & Rossi 2011). However, very little work has been performed in insects to study how sociality affects cognitive ageing without prior induction of disease in a model. A *D. melanogaster* model to investigate cognitive declines would therefore be valuable, especially considering the malleable genetic and behavioural system. For males kept with rivals for 50 days before learning and memory was measured it is hard to elucidate whether males show increased cognitive ageing with rivals. This is due to control flies never exposed to rivals unable to show normal cognitive ageing due to depressed cognition early in life (discussed above). It would therefore be interesting to probe the temporal dynamics of cognitive stimulation and decline in more detail.

Evolution of insect cognition

The results in this chapter support the social brain hypothesis that cognitive ability is shaped by social interactions, but with caveats. Instead of a general increase in social interactions promoting brain evolution, I show conspecific interactions (perceived risk of sperm competition) are an important mechanism promoting the evolution of cognition in *D. melanogaster*, as first suggested by Hollis and Kaewecki (2014). Within this previous study males were kept in a strict monogamous mating system or allowed to mate freely for 100 generations. Learning and memory ability were then measured and enforced monogamy was found to depress cognitive

ability in males. This study directly tests how the evolution of cognition is influenced by sperm competition, and through my data a mechanism can now be suggested. That the sperm competition environment affects the developmental process of male *D. melanogaster* to elicit phenotypes with different cognitive abilities, with these phenotypes then under selection after an initial 3 week development window. Overall, this is in conflict with previous work on mammals suggesting that male-male competition decreases the cognitive ability of males over evolutionary time (Schillaci 2008), due to increased investment in testes decreasing investment opportunities in the brain (Pitnick, Jones & Wilkinson 2006). This highlights that different cognitive pressures imposed by different mating systems may depend on species specific detail such as access to mates and potential competition within the environment. In addition, complexity in primate social groups, thought to have an effect on cognitive ability (Schillaci 2008) varies with levels of parental care, a behaviour not often seen in insects (including *D. melanogaster*).

Considering MBs seem to modulate reactions to the sperm competition environment (Chapters 4 and 5), and previously have been the focus of studies investigating the evolution of the insect brain, the experimental evolutionary work discussed above would be an excellent system to investigate sperm competition as an evolutionary pressure on brain structure. I would predict that males kept in a monogamous environment would show a significant decrease in MB complexity compared to a polygamous environment. One way to test this theory would be to measure complexity or size of the MB lobes in flies undergoing experimental evolution similar to that seen in Hollis and Kawecki (2014). Another way to examine how quantity of sperm competition may affect a male's cognitive ability is through the use of hyper-promiscuity. This would involve measuring male cognition under the pressures of a hyper-promiscuous environment. Previously, males that have undergone experimental evolution within this environment decreased their courtship

frequency, though the quality of courtship as a proxy of cognition was not investigated (Perry *et al.* 2016). If conspecific competition drives the evolution of cognition in *D. melanogaster* as suggested it may be that further cognitive developments would be seen under hyper-promiscuous environmental pressures. Conversely, the removal of female rejection allowing the development of hyper-promiscuous females within this study (Perry *et al.* 2016) may have also removed the advantage males gain from mating experience (Dukas 2005). This would potentially lead to a decrease in cognitive ability over evolutionary time.

Previous work on the evolution of cognition in insects has suggested sociality (Kamhi *et al.* 2016; Seid & Junge 2016) or novel visual inputs such as those gained from spatial learning as drivers of cognition (Riveros, Seid & Wcislo 2012; Muscedere *et al.* 2014; Amador-Vargas *et al.* 2015; Farris 2016). Both suggestions gain evidence from eusocial societies, creating confusion over whether a main driver of insect cognitive/brain evolution can be defined. Within individualised societies, the picture is clearer, with the evolution of the MBs primarily thought to be reliant on complex foraging behaviour (Lihoreau, Latty & Chittka 2012). In addition to this, results in this chapter and previous work (Hollis & Kawecki 2014) now suggest that sperm competition may also be one of the evolutionary pressures behind an increase in cognition in insects.

General discussion

Despite plastic behaviour being ubiquitous and important we know very little about the mechanistic underpinnings that control how behaviour reacts to the environment. It is theorised that different types of behavioural plasticity will require different proximate mechanisms depending on the time in development plasticity occurs, and the amount of plasticity shown by the behaviour i.e. how reversible the behaviour is. However, it is not known whether these discrete types of plasticity require different controlling mechanisms and hence this field is gaining more attention (Cardoso, Teles & Oliveira 2015).

To investigate mechanisms underlying plastic behaviour I have used a *D. melanogaster* model that is consistent, predictable and has a direct link to fitness. Male *D. melanogaster* have been shown to plastically respond to an increase in sperm competition by extending their mating duration, enabling males to gain fitness benefits due to an increased transfer of sperm and seminal fluid protein quantity (Wigby *et al.* 2009; Moatt 2014). The cues required for males to react to increased sperm competition are well known (Bretman *et al.* 2011), as are the costs of the plastic behaviour (Bretman *et al.* 2013).

This thesis has contributed to our understanding of the proximate mechanisms that can control a plastic behaviour. It has also highlighted one of the potential evolutionary drivers behind insect cognition.

7.1 Main findings

7.1.1 Males use exposure time to rivals to modulate dynamics of plastic behaviour

I investigated the temporal dynamics of extended mating duration seen in *D. melanogaster* as a response to increased sperm competition (Rouse & Bretman

2016). The study showed significant effects of changing the exposure time of males to the sperm competition environment. After 24 hours in a high sperm competition environment males significantly increased mating duration for 1 hour, after which males reduced their mating duration to pre-exposure levels. The time extended mating duration was maintained was modulated by exposure time to rivals. At its full extent, extended mating duration lasted for 12 hours, before slowly returning to pre-exposure levels. As males were able to reduce mating duration quickly after only 24 hours with rivals this shows males are able to react quickly to changes in competition pressure, but do not if they have undergone increased exposure time to a rival. Considering the removal of an immediate rival does not inform the focal males about the population level sperm competition threat, it seems likely that males use time with a rival to inform them whether further risk of sperm competition is high. This would suggest a memory mechanism is required to remember the length of time males spend in a rival presence. A cognitive mechanism is also supported by the temporal dynamics of the plastic behaviour which parallels known time periods of learning and memory (Margulies, Tully & Dubnau 2005). This suggests cognition could be a productive avenue of research to investigate the mechanism behind extended mating duration.

7.1.2 Accuracy of behavioural plasticity is dependent on a multimodal sensory system

I investigated how sensory removal impacted the temporal dynamics of extended mating duration, as discussed in the previous chapter. Some of this work was also published by Rouse and Bretman (2016). Sense removal showed significant effects on the time it took to build-up extended mating duration, revealing the importance of a multimodal sensory system which was not addressed in a previous study (Bretman *et al.* 2011). Previously, the need for multiple senses was seen as a form

of adaptive redundancy, whereas here I highlight the need for an accurate and reliable multimodal sensory system to effectively respond to increased sperm competition risk on the same time scale as unmanipulated males. In comparison to the build-up of the plastic response, the maintenance of the plastic response was not affected by all the sensory manipulations performed in the study. Reduction of single senses did not affect the maintenance of the response, but removal of the 3rd segment of the arista meant males responded to rivals for longer than unmanipulated males. This manipulation potentially removed some olfactory and auditory sensory ability which may have had the effect of essentially removing two sensory pathways. In addition, the maintenance of extended mating duration was not investigated at the same temporal level as the build-up of the plastic behaviour, potentially masking differences that would be revealed with a more detailed study. Overall, the result focused on the need for accurate and reliable sensory stimuli leading to time sensitive plastic behaviour, and supported previous studies suggesting that the build-up of mating duration was costly compared to reducing mating duration too early.

In addition, I examined a previous report that highlighted vision as the only sense *D .melanogaster* males required to respond to rivals. I found that vision was not involved in extended mating duration and in light of other research (Bretman *et al.* 2011; Maguire, Lize & Price 2015) this called into question genetic studies also performed into elucidating how extended mating duration was controlled (Kim, Jan & Jan 2012). It is important to know the cues involved in a plastic behaviour as this strongly relates to the possible memory mechanisms that may be used to control behaviour. For example, in *D. melanogaster* the ellipsoid bodies are required for visual place learning (Ofstad, Zuker & Reiser 2011), whereas olfactory memory requires action in MB neurons (Akalal *et al.* 2006). Both types of learning require the action of the cAMP second messenger system though mutants for genes within this

system differ in their effect on memory between visual and olfactory memory (Folkers 1982).

7.1.3 Anaesthesia sensitive memory controls extended mating duration

Within this chapter I investigated the cognitive mechanisms controlling extended mating duration and demonstrated a need for anaesthesia sensitive memory, but not anaesthesia resistant memory to control this plastic behaviour. In addition, I demonstrated that the MBs, the centre for olfactory associative learning (Akalal *et al.* 2006), were needed for extended mating duration to take place. The finding that extended mating duration relies on ASM is in part in line with previous reports (Kim, Jan & Jan 2012) and the predicted training males would receive from the environment (McInnis, Schaffer & Mettler 1982). In contrast, the importance of the MBs in controlling the behaviour is in direct opposition to previous reports (Kim, Jan & Jan 2012). Interestingly, the neural mechanisms predicted by my results point to the use of the most malleable ASM type available. This suggests that although memory needs to be stable (Tully *et al.* 1994), it must be able to follow a time-scale on par with the speed of change within the environment. Associative learning and memory use nearly identical genetic underpinning as extended mating duration (McGuire, Deshazer & Davis 2005), though they work on different timescales. This opens up the possibility that learning and memory mechanisms have been co-opted by other behaviours such as extended mating duration, potentially reducing the costs needed to develop separate nervous control for individual plastic behaviours. However, this raises the question of how behaviours are controlled in a context specific manner so plastic behaviours modulated by similar memory mechanisms stay defined. One way may be the physical separation between neurons used for one memory over another. In *D. melanogaster*, STM and LTM can be induced in parallel through a single session of appetitive conditioning, after which memory

types can are separated by the different neurons they depend on to control a reaction to the trained cues. STM is γ neuron dependent and LTM is α/β neuron dependent (Trannoy *et al.* 2011; Yamagata *et al.* 2015). This mirrors vertebrates that separate memories through the use of distinct brain areas (Izquierdo *et al.* 2006). Therefore, a behaviour such as extended mating duration, which uses γ neurons to produce a long term memory, could be separated from other ecologically relevant behaviours induced by separate cues that induce memory in different neuronal subsets. In vertebrates induction of different memory types is also separated by biochemical pathway (Izquierdo *et al.* 2002), something which has not been shown in insects.

7.1.4 Transient changes in gene expression do not control behavioural plasticity

I tested whether extended mating duration relied upon changes in expression levels of genes that in chapter 4 I had previously shown to control plastic behaviour. I also tested genes involved in LTM and synapse development. I found no significant evidence for transient changes in gene expression controlling extended mating duration. However, I only explored a subset of cognitive genes based on a previous transcriptomics study highlighting some of these genes as being responsive to a rival presence (Mohorianu *et al.* in prep). These genes covered very little of the genetic architecture underlying learning and memory and were focussed on to allow me to split the behaviour into time-periods I thought would parallel the temporal dynamics of learning and memory phases.

My experiment would benefit from the use of a high-throughput method to compare gene expression, even up to the point of testing for different transcript levels within genes. However, this would also reduce the temporal detail gained from the experiment due to funding restrictions. Overall, the study focussed on

possible neurogenomic mechanisms that theory held as important for behavioural plasticity (Cardoso, Teles & Oliveira 2015). My results provide some evidence that extended mating duration may be controlled more by physiological and neural mechanisms other than changes in gene expression.

7.1.5 Cognitive ability is modulated by conspecific interactions

In my final data chapter I investigated whether keeping males in a high sperm competition environment (kept with a *D. melanogaster* rival) for 10 days would affect the cognitive ability of a male. This was investigated through the use of two learning and memory assays designed to test both associative and operant conditioning. Furthermore, in order to distinguish between the effects of heterospecifc competition and conspecific competition on cognitive ability I also measured responses to *D. virilis* rivals acting as a general resource competitor. In addition to behavioural testing, I measured whether there was an effect of this long-lasting increase in sperm competition on the expression levels of a subset of genes involved in learning and memory. Experiments revealed an increase in cognitive ability and expression levels of two learning and memory genes associated with synaptic plasticity when males were kept in a high sperm competition environment. These changes were not seen when kept with heterospecific rivals that did not provide a sperm competition threat.

In addition to the main part of the study, male reactions to a variable environment were also tested to establish whether cognitive ability fluctuated with the presence or absence of a rival. Males were exposed to a high sperm competition environment for 10 days before being isolated, and vice versa, before undergoing a test of learning and memory. Male cognitive response to variable environments did not differ from each other, suggesting that once males were exposed to a high sperm competition environment any cognitive increases were maintained beyond

immediate environmental variability. Whether increased gene expression is also maintained without the constant presence of a rival remains to be established.

This provides an insight into the possible evolutionary drivers for an increase in insect cognition and is supported by previous experimental evolutionary work (Hollis & Kawecki 2014). Future work could focus on whether females show similar responses to competition and if not, whether the evolution of insect cognition could be constrained by sex differences in the evolutionary drivers of cognition.

7.2 Implications and conclusions

7.2.1 Genetic mechanisms of extended mating duration

The main objective of this thesis was to elucidate a possible cognitive mechanism modulating extended mating duration in *D. melanogaster*. Results from Chapter 4 would suggest that extended mating duration is controlled by ASM via the cAMP 2nd messenger system. In addition, memory is modulated via the γ neurons in the MBs, thereby using the most malleable ASM type available. To my knowledge this is the first time an ecologically relevant behaviour has been shown to be controlled through induction of plasticity via specific structural physiology i.e. specific neurons are responsible for the length of time of a specific behaviour.

From chapters 2-4, an overall mechanism for extended mating duration can be proposed. In *D. melanogaster*, males learn and acquire 'competitive' memory from a specific set of stimuli over 24 hours, consolidate this memory in the γ neurons of the MBs before expressing this memory for between 1 and 12 hours depending on the time they were exposed to rivals. From the geographical and temporal dynamics of the ecological system, this suggests that a single neuronal subset can control a complex behaviour independent of other distractions.

7.2.2 Mechanisms of social plasticity

The genomic and neural mechanisms controlling plastic behaviour are theorised to be specific to the type of plastic behaviour induced (Cardaso et al 2015, table 1). Within this thesis I tested the hypothesis that behavioural flexibility shows transient changes in gene expression and found no clear effect of changes in behaviour on the neurogenomic state over 2 days (Chapter 5). However, when males were exposed to rivals for 10 days (Chapter 6) there was an increase in gene expression of two genes involved with synapse development. This suggests that neurogenomic change does not control extended mating duration, which instead may be controlled by neural mechanisms such as switching between neural circuits. However, that neurogenomic change may be a consequence of a continued signal from the environment and may affect the mechanisms of plastic behaviour at a later developmental point (Figure 1). Neurogenomic change is also required for sequential MB development that potentially occurs when males have been exposed to a competitive environment for an extended amount of time (Technau 1984). This potential change in brain structure and increase in genes associated with synapse development parallels the pattern that occurs in bee MBs. In these brain structures accumulated foraging experience increases neutrophil growth associated with behavioural change detectable between the first and second week of foraging (Farris, Robinson & Fahrbach 2001; Ismail, Robinson & Fahrbach 2006). This correlates to neurogenomic changes on a similar timescale, with greater expression of genes associated with neutrophil growth the older and more experienced an individual becomes (Lutz *et al.* 2012).

The behavioural and development change occurring in bees has previously been treated as sequential behavioural plasticity, where structural and behavioural change is not reversible (Zayed & Robinson 2012; Cardoso, Teles & Oliveira 2015). Considering this, any behavioural plasticity induced by rival presence in *D.*

melanogaster, such as an increase in learning seen in chapter 6, may well be a type of sequential plasticity controlled by epigenetic change (Cardoso, Teles & Oliveira 2015). Research into differences in the epigenome between males exposed to rivals and single males would therefore be fruitful as a way to gain knowledge about how extended mating duration, and general plastic behaviour, is ultimately controlled. Previous studies into epigenetic changes have uncovered interplay between experience and the changing genome to control plastic brain development (Fagiolini, Jensen & Champagne 2009) and learning and memory (Day & Sweatt 2011). Considering both these areas seem to have a role to play in how males react to both short term and long term changes in the sperm competition environment the model used within this thesis would be ideal to investigate how epigenetics may control plastic behaviour further.

In addition to expanding this model of extended mating duration to include epigenetic dissection of behavioural control, investigating the response to rivals through the proteome should be considered. The *Drosophila* proteome is highly plastic (Zhou *et al.* 2012) and has already been shown to change after the induction of learning and memory (Zhang *et al.* 2014). Differential expression of proteins are also known to affect phenotypic plasticity in other species such as caste differentiation in the honey bee *Apis mellifera* (Fang *et al.* 2012). Behavioural plasticity might therefore benefit from investigation into how the proteome can help control behaviours in response to environmental change.

One potential way to test for neuro-plasticity and whether it may affect extended mating duration without complex imaging or genomic techniques would be use the temporal dynamics already known about the behaviour. Extended mating duration could be induced via 5 days in the social environment followed by isolation for 3 days. Males would then be tested for the speed at which they increase mating duration. This would provide insight into whether neurological and cognitive

changes seen after association with a rival are transient, or whether the structural plasticity of the brain has been irreversibly changed by rival exposure and can be used to inform the speed of future behavioural plasticity.

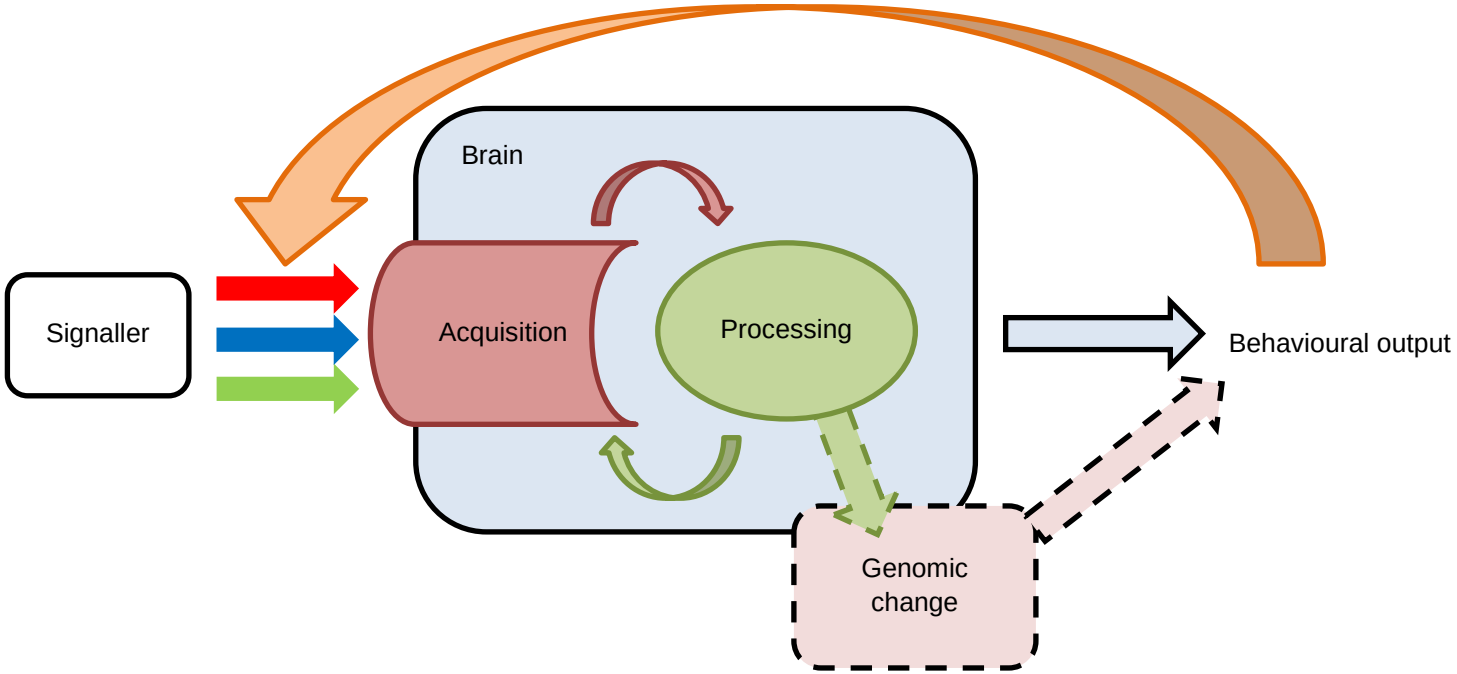

Figure 1: Potential mechanism controlling socially induced plastic behaviour. The receiver, here represented by a brain (blue) acquires information from the environment (red, blue and green arrows). This is processed through physiological mechanisms specific to that plastic behaviour such as specific neural networks. Once an individual is sure of an environment a behavioural output will be expressed, this will also feed back into the acquisition and processing of environmental cues. If the signal of environmental change is transient there will be no significant genomic change linked to controlling the plastic behaviour. However, if environmental change is sustained the processing of the environmental signal will feed into genomic change (green dotted arrow), which may have lasting implications on how behavioural output is controlled (red dotted arrow). For example, genomic change leading to the development of a more complex neural network controlling the plastic behaviour. Whether there are any repercussions to this development such as a faster behavioural change when an individual is exposed to a changing environmental signal is get to be investigated.

7.2.3 Competition and the evolution of the insect brain

The evolution of the brain to enhance cognitive ability is naturally a source of great interest. In mammals, increases in the number of individuals in the social group paired with increased complexity of social interactions seem to have driven the evolution of a larger relative brain and therefore and increase in cognitive ability. However, drivers of insect brain evolution have been harder to pin down due in part to disagreements about what constitutes sociality in an insect society (Sokolowski 2010). In chapter 6 I show that sperm competition is important for males to increase cognitive ability within a life-time, and suggest that this is one of the pressures by which insect cognition may have evolved. This is supported by previous work that used experimental evolution to show that enforced monogamy reduced cognitive ability in males (Hollis & Kawecki 2014) and lab work showing males require cognitive abilities to mate effectively within a complex environment (Dukas 2004b; Dukas 2005; Griffith & Ejima 2009). This is in direct opposition to how sperm competition is thought to act as an evolutionary pressure in mammals. Comparative analyses in mammals have shown that an increase in sperm competition correlates to a decrease in cognition, thought to be a factor of increased investment in testes size (Pitnick, Jones & Wilkinson 2006). Why effects of sperm competition seem to act so differently on cognition in insects is unknown, but highlights different cognitive pressures imposed by differing mating systems.

As well as more general social interactions such as the change in sperm competition felt in an environment that work on a population level and which I have focussed on in this thesis, some social interactions also work on a more individualistic level. This occurs when individuals can identify and react to genetically more similar individuals (like siblings) in a different manner to less genetically similar individuals, or strangers (Waldman 1988). In *Drosophila melanogaster* olfaction allows male and females to identify novel partners and

therefore increase the genetic diversity of their offspring (Tan *et al.* 2013). In a similar manner males are able to identify brothers (Lize, McKay & Lewis 2014) and social groups containing brothers' fight less and cause less harm to females after mating (Carazo *et al.* 2014). Considering this, there may be an effect of relatedness on the interaction of males, potentially effecting extended mating duration and/or some of the cognitive work investigated in this thesis. Taylor (Taylor 2014) has shown relatedness does not influence the extent to which males extend mating duration, though it may affect the time taken for males to mate with females. However, in Chapter 6 relatedness may well effect the extent males increase cognitive ability due to the potential of higher relatedness meaning a more complex social relationship (similar to a pair bond). To avoid this possibility all flies were randomised when assigned to a social environment and therefore were extremely unlikely to come into contact with a sibling, however, this cannot be totally ruled out.

What would be interesting is to evaluate whether similar competitive elements drive the evolution of the female brain. As sperm competition is not relevant when discussing females possible competitive behaviours that could be tested include egg laying or feeding. As an extra challenge these behaviours are controlled by both olfactory (Zrelec *et al.* 2013) and spatial memory (Navawongse *et al.* 2016), a known driver of brain development in honey bees (Menzel 2012). Separating competition and spatial memory would therefore be a challenge, but could be achieved with the use of different *Drosophila* species in a similar manner to this thesis.

It may also be informative to undertake a comparative analysis of the mechanisms behind cognitive evolution. Many insect species produce a similar response to sperm competition as *D. melanogaster*, changing their behaviour and ejaculate investment in response to competitive change. For example, *Gryllus bimaculatus* males seem to use a form of learning and memory to respond to a rival

presence (Lyons & Barnard 2006). In addition, multiple taxa seem to employ quantity to assess environmental change such as is seen when assessing sperm competition threat (Carazo, Fernandez-Perea & Font 2012; Shifferman 2012; Pahl, Si & Zhang 2013; Larsdotter-Mellstrom *et al.* 2016). It would therefore be interesting to evaluate if any mechanisms are conserved between species that use learning and memory or quantity assessment in their natural environments.

Given the research I have undertaken in Chapters 5 and 6 future work could focus on how synapse complexity is affected by sperm competition in a similar manner to work on sleep (Donlea, Ramanan & Shaw 2009; Donlea & Shaw 2009). Within this research, brain complexity is measured by counting the number of synapses within a certain neuronal tissue. I would recommend starting in the MBs, especially the γ neurons, which have been shown to remodel having undergone STM via appetitive associative learning (Marin *et al.* 2005) and underpin extended mating duration. In addition to this work, it is becoming increasing evident that social environment can drive ageing patterns in multiple taxa (Blakemore 2008; Cacioppo & Hawkey 2009; Baarendse *et al.* 2013). Therefore, as *D. melanogaster* respond to changes in sociality there is scope to investigate how this occurs through mechanistic studies. This simple insect model system for sociality and age would then be important for predicting and potentially preventing human age-related cognitive decline.

7.2.4 Tailor made memory

Previous work has suggested the theory of tailor made memory where learning and memory is species or population specific. Within this work, the learning and memory type for a population is described by the properties and temporal dynamics of memory acquisition, consolidation, waning, forgetting and retrieval after learning

(Smid & Vet 2016). This is supported by work highlighting where species differ in their memory type depending on a reward (Kruidhof *et al.* 2012).

From my work, I suggest that this theory is taken one step further, in that a learning and memory type can be specific within a population to the plastic behaviour memory supports. I feel this thesis lends support to my argument. Previously, *D. melanogaster* memory types have been described as STM, MTM, LLTM or ARM (Margulies, Tully & Dubnau 2005), with a very specific set of acquisition variables defined. Within this thesis, I have shown that a plastic behaviour modulated by sperm competition depends on similar genetic and neurological structures as associative learning. However, I also show that the acquisition, consolidation and retrieval of that behaviour do not fit into common memory dynamics. These assays are not ecologically relevant due to a researcher's control of the associative cues delivered to the flies.

Therefore, as extended mating duration is a natural behaviour, it is expected the brain's response to competition should occur on a time scale consistent with the life history of the fly and consistent with memory suitable to this particular ecological niche. Extended mating duration may be therefore tailor made specifically to address geographical and temporal dynamics of *D. melanogaster* using neural circuits that match the speed of the behaviour.

It is important to know how learning and memory may be tailor made to insects depending on their environment as this information could potentially be applied to areas of insect control. Vector insects have been shown to learn associations between host defence strength and a volatile odour (Vinauger, Buratti & Lazzari 2011a; Vinauger, Buratti & Lazzari 2011b), allowing them to avoid hosts with strong defences and develop host preferences (Bouyer *et al.* 2007; Vinauger, Pereira & Lazzari 2012). However, this learning ability can be modified by cue type and strength similar to other learning paradigms (Vinauger *et al.* 2016). For

example, the mosquito *Aedes aegypti* has been shown to employ LTM after being trained twice to associate a blood meal with an unconditioned odour (Vinauger, Lutz & Riffell 2014). However, in other studies using colour as the unconditioned stimulus individuals only remembered an association for 60 minutes after one training cycle (Menda *et al.* 2013). Knowledge about how vector insects control learning and memory and how it is advantageous may allow learning and memory in vector insects to be targeted as a potential control strategy.

Appendix I – Time of day effects on extended mating duration and determining smell in associative learning assays

I.I Extended mating duration and time of day

Due to the time periods some of my work spanned it was necessary to carry out experiments in the afternoon instead of in the morning. Usually mating duration experiments were performed at 9am, however, when males had to be removed from rivals at night to allow for longer periods of isolation or rival exposure (from 9 to 6 hours) prior to calculating mating duration (as when investigating the build-up of the mating duration response when individual senses are removed in chapter 3) mating duration was calculated at 2pm.

I.I.I Materials and Methods

Males were kept singularly or with rivals for 3 days before being exposed to a virgin female and mating duration calculated. Experiments were carried out at 9 am or 2 pm, requiring that social environments be set up at 9 am and 2 pm 3 days previous.

I.I.II Results

There was no difference between a males ability to significantly extend mating duration dependent on the time of day tested. When exposed to rivals, males significantly increased mating duration at both 9am (Student T-test: $t_{62} = -2.668$, $p = 0.010$) and 2pm (Mann Whitney U test: $Z = -3.348$, $N = 62$, $p = 0.001$) in line with previous reports (Figure 1).

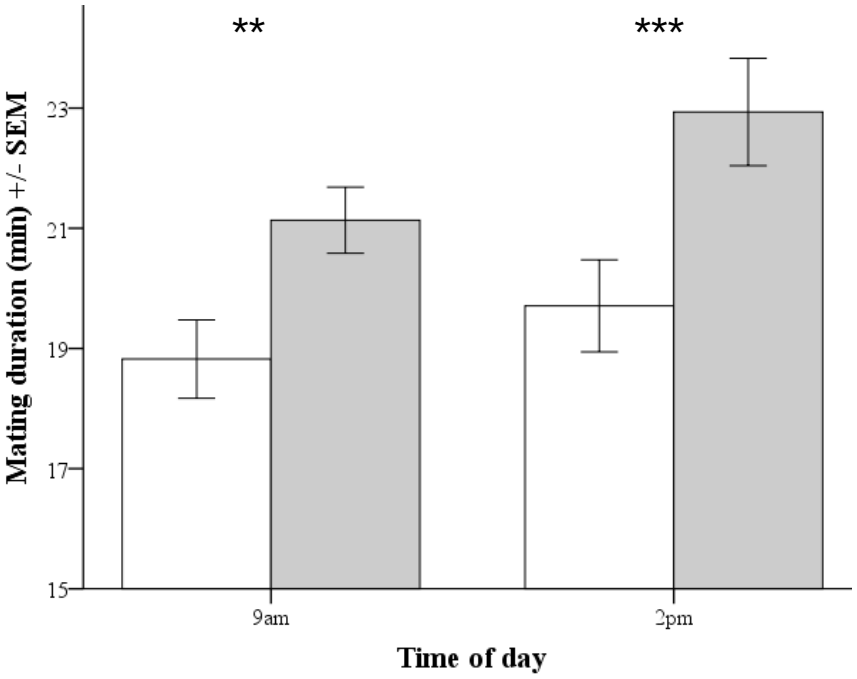

Figure 1: The ability to significantly extend mating duration does not change depending on the time of day. Single males (white bars) are directly compared to males kept with rivals (grey bars) with data collected at 9am and 2pm. Error bars represent 1 standard error mean (S.E.M.). * ≤ 0.05, ** ≤ 0.01, ***≤ 0.001

I.II Associative T-maze smell sensing with group males

Associative learning and memory allows the training of flies, both as a group and singularly, to associate a shock or reward with a smell (Mery & Kawecki 2003; Krashes & Waddell 2011). Traditionally, odours used are 3-Octanol (Procter, Moore & Miller) and 4-Methylcyclohexanol (Tomchik & Davis). To carry out T-maze associative learning and memory testing requires MCH and OCT to be balanced so that there will be no effect of odours on the behavioural response of the fly. This was a trial and error process using smell concentrations from previous experiments (Mery & Kawecki 2003; Krashes & Waddell 2011) as a starting point. In addition, once smells had been balanced, they were tested independently against air to confirm that each odour was equally weighted in their effect on the behaviour of the flies.

I.II.I Balancing 3-Octanol and 4-Methylcyclohexanol

All odours were delivered to flies with the use of a vacuum pump. 3-Octanol and 4-Methylcyclohexanol were diluted in 10 ml of light mineral oil before being vortexed and placed to bubble for 10 minutes. These odours were then used in experiments.

Groups of 50-60 flies were placed into a T-maze and exposed to air for 30 seconds; they were then transferred to a choice point between the 2 smells. Flies were given 2 minutes to choose between the smells, at the end of which they were removed and counted.

To calculate the percentage of flies moving towards 3-Octanol (an arbitrary choice of smell to compare against) the number of flies that moved towards OCT were counted and divided from the total number of flies in the test before being multiplied by 100 (figure 2a)

I.II.II Ability to sense smells

Groups of 50-60 males were placed into a T-maze and exposed to air for 30 seconds. They were then transferred to a choice point where they were allowed to choose between either OCT or MCH and air for 2 minutes.

To calculate the percentage of flies moving towards to smell the number of flies moving towards a smell was counted and divided from the total number of flies. This number was then multiplied by 100 (figure 2b).

I.II.III Results

Male flies separated equally between 3-Octanol and 4-methocyclohexanol when 18.5 µl of Oct and 20 µl of Mch was each diluted in 10 ml of light mineral oil (One-samples T-test: $t_{17} = -0.052$, $p = 0.959$, Table 1). There was no difference in the ability of males to sense either smell when compared to air (Students T-test: $t_{19} = 0.229$, $p= 0.821$, Figure 2c). Therefore, any group experiments undertaken used an 18.5/20 (µl/µl) Oct/Mch split.

A $\left[\frac{\text{Total number of flies}}{\text{Number of flies in 3-Octanol}} \right] \times 100$

B $\left[\frac{\text{Total number of flies}}{\text{Number of flies in smell}} \right] \times 100$

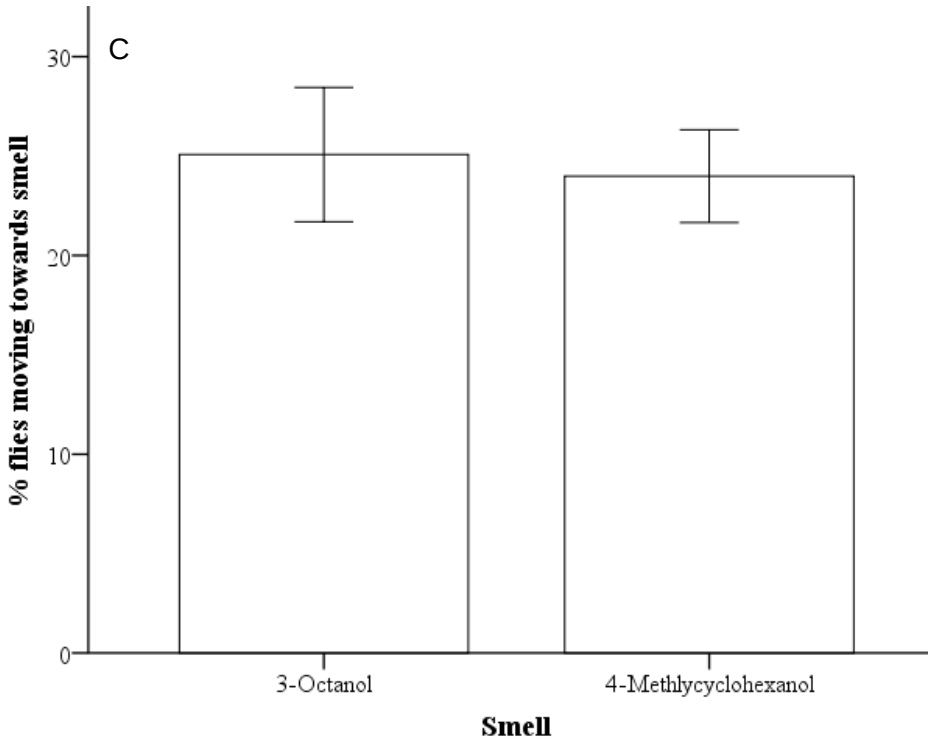

Figure 2: Smell sensing in group males. (a) is the equation used to calculate the Oct preference of male flies when exposed to both Oct and Mch. (b) is the equation used to calculate either Oct or Mch preference when exposed to a smell and air. (c) shows the % males flies moving towards smell when given a choice between a smell and air. Error bars show 1 standard error mean.

Table 1: T-maze smell sensing. Oct/Mch shows the amount used (in µl) when diluting in 10 ml of light mineral oil for the two smells. % Oct shows the percentage of flies choosing 3-Octanol out of the total number of flies who made a choice.

Oct/Mch (µl/µl)	% Oct	Sig. value
16/10	68.6 +/- 2.36	≤ 0.000
17/10	61.17 +/- 4.15	0.004
20/20	40.67 +/- 1.77	0.001
18.5/20	49.84 +/- 2.92	0.959

Sig. value shows the significance value of a one-sample T-test when % Oct was compared to 50 %.

I.III Associative T-maze smell sensing with single males

In addition to balancing the smells when males are tested in groups, males undergoing learning and memory testing in the T-maze individually should also have an equal likelihood to choose both smells (Claridge-Chang *et al.* 2009). Although this should theoretically be the same balance of smells as when males were tested in groups, group living may change the ability of males to sense one or both of the 2 smells used differently. Therefore, similar experimental methods were used to balance smells in the T-maze when only 1 male was present at a time. Again, a trial and error process was used beginning with the 18.5/20 OCT/MCH split used to balance males tested in groups.

I.III.I Smell sensing with single males

Single males were placed in a T-maze and exposed to air for 30 seconds before being moved to a choice point. Here, males were exposed to 3-Octanol and 4-Methlycyclohexanol for 2 minutes. The time males spent in either smell was timed and the overall 3-Octanol score calculated by dividing the total time spent making a choice by the time spent in 3-Octanol then multiplying this number by 100.

I.III.II Results

Single male flies spent equal amount of times in 3-Octanol and 4-Methlycyclohexanol when 27 µl of Oct and 10 µl of Mch were each diluted in 10 ml of light mineral oil (One-sample T-test: $t_{129} = -0.977$, $p = 0.330$, Table 2).

Table 2: Smell sensing for a single fly. Oct/Mch shows the amount in µl of each smell diluted in 10 ml of light mineral oil. Oct index shows the percentage of time out of 120 seconds single flies spent in Oct.

Oct/Mch (µl/µl)	Oct index (%)	Sig. value
10/30	98.8 +/- 1.19	≤0.000
10/25	67.2 +/- 20.73	0.444
10/20	87.8 +/- 12.18	N/A
20/20	74.9 +/- 18.81	0.257
25/20	97.4 +/- 3.52	0.003
25/15	100 +/- 0	N/A
40/20	97.6 +/- 2.35	≤0.000
20/10	77.5 +/- 16.22	0.151
25/10	76.5 +/- 11.31	0.079
27/10	46.1 +/- 4.0	0.33
30/10	8.6 +/- 5.0	0.004

Sig. value shows the significance value of a one-sample T-test when % Oct was compared to 50 %

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