INTERACTION BETWEEN THE IMMUNE AND NERVOUS SYSTEMS IN INSECTS

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By

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Declaration

I hereby declare that no part of this thesis has been previously submitted to this or other University as part of the requirements for a higher degree. The content of this thesis is the result of my own work unless otherwise acknowledged in the text or by reference.

The work was conducted in the Department of Biology, University of Leicester during the period January 2007 to July 2012.

Signed……..

Akram Saleh Alghamdi,

Interaction between immune and nervous systems in insects

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Abstract

In vertebrates, it has been established that interactions exist between the immune system, the nervous system, and behavior. A comparative examination of these interactions in other animals helps us understand the evolution of this interaction. It may also be possible to develop animal models of important human pathologies, which are due to the interactions between these two systems. In insects an immune-behavioral interactions similar to those seen in vertebrates has been shown to exist. This suggests that this interaction has a highly conserved function. For example, activation of immune response produces illness-induced anorexia, behavioral fever, changes in reproductive behavior, and decreased learning ability in different species. This thesis establishes further examples of this interaction between the immune and nervous system, examines the physiological basis between them and explores the evolutionary dynamics of the interaction. I establish this interaction between the immunity and memory in bumblebees in a free flying paradigm, where previously it had only been shown in artificial classical conditioning assays. Then, I checked the immunity of different bumblebee colonies of known learning ability to identify any evolutionary relationship between these two traits. I used *Drosophila melanogaster* to study the sleep phenomenon after activation of the immune system as a potential intermediary between immunity and memory. Finally, I checked the olfactory learning of *Drosophila melanogaster* after activating their immune system to see if the fruit fly would make a useful model for immune modulated memory reduction.

Additional Work Carried Out for This Version of The Thesis.

- Chapter 1: Revised
- Chapter 2:
    - Revised
    - Removal of undergraduate replicate
    - Four additional replicates carried out
    - Analysis now has a colony factor
    - New type of graphs used to represent data
    - Discussion rewritten
- Chapter 3
    - Revised
    - ANCOVA carried out to analyse data at individual level. Individual graph included for completeness.
- Chapter 4
    - Whole chapter extensively rewritten
    - Additional experiment carried out using an alternative method of stimulating the immune system (Geneswitch)
    - All data reanalyzed using a more powerful ANOVA model
    - Additional mean graphs included
    - Tables replace most of the writing in results section to make this section clearer.
- Chapter 5
    - Whole chapter extensively rewritten
    - Additional experiment carried out using an alternative method of stimulating the immune system (Geneswitch).

- Chapter 6
    - The scope of this chapter has been expanded from its original list of conclusions.

Contributions

- Chapter 2
    - Statistical analysis was designed by Dr. Mallon.
- Chapter 3
    - The learning ability of the bumblebees workers were measured by Dr. N. Raine in Queen Mary University in London, and frozen workers were collect for the immunity study in our lab.
    - Statistical analysis was carried out at the colony level by Dr. Mallon.
- Chapter 4
    - All flies used for sleep study were loaded using the monitors in Dr. Rosato's lab (Genetics Department). Dr. Rosato helped me load the flies in the heatshock experiment.
    - Statistical analysis was designed by Dr. Mallon. He also wrote the STATA script that carried out the analysis.

Acknowledgment

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Chapter One: Main Introduction

1. Introduction

In my PhD, I am interested in how the immune function affects memory in insects. My PhD is based on earlier work showing that an immune response elicited by lipopolysaccharide (LPS) affected memory in the proboscis extension reflex (PER) assay (Mallon, 2003). This is an example of an interaction between the immune system and the nervous system, the study of which has come to be called Psychoneuroimmunology (Adamo, 2002). This introduction is in four parts. First is an overview of the interactions of the immune system and the nervous system (Psychoneuroimmunology) in insects. Then I will outline the insect immune response to gram-negative bacteria. This is the response elicited by LPS. Thirdly I will detail the neurobiology behind memory formation in the PER assay. Finally I will outline the structure of the rest of this thesis.

1.1. Psychneuroimmunology

Bidirectional connections between the immune system and central nervous system are well studied and established in mammals, and there is an evidence for their existence in molluscs (Steinman, 2004; Sternberg, 2006; Ottaviani et al., 1997; Stefano et al., 2002). There is indirect evidence suggesting that an interaction between the two systems occurs in insects. These interactions allow the nervous system to combine and exchange information from both internal and external sources and produce the most adaptive behaviour and physiological set point. It has been noted that some molecules linking the systems are the same in the same phyla, and this suggests the possibility that the connections are ancient (Ottaviani and Franceschi, 1996). Below I separate the evidence into that which shows that the immune system can affect the nervous system and evidence that the nervous system can affect the immune system.

1.1.1. Evidence That the Immune System Can Affect Nervous System Function

There are few studies on immune-neural interactions in insects. These deal mainly with changes in host behaviour during an infection. The logic being that infection induced behavioural change must involve a change in neural system function and therefore indicates an immune-neural connection (Adamo, 2002, 2005). The difficulty with this evidence is that behaviour altering could occur due to some components correlated with infection as opposed to being caused by immune factors. For example, the host behaviour may be changed by some pathogen secretion or by pathological changes caused by the pathogen (e.g. damage to an insect host's sensory system) (Adamo, 2002, Moore, 2002).

1.1.1.1. Behavioural Fever

One of the earliest pieces of evidence that the immune system interacts with the nervous system was behavioural fever in cockroaches injected by LPS (Lipopolysacchrid) (a component of gram-negative bacterial cell wall) (Bronstein and Conner, 1984). In behavioural fever, insects move to warmer areas, which lead to increased resistance to different types of pathogens, providing the animal with an adaptive advantage (Moore, 2002). It increases some immunological functions and directly decreases pathogen lifespan (Ouedraogo, 2003).

Not all immune reactions elicit behavioural fever. Behavioural fever in crickets and locusts is not a general response to any infection, but is pathogen specific (Adamo, 1998, Springate and Thomas, 2005, Bundey et al., 2003). Different pathogens elicit various types of immune response (Brennan and Anderson, 2004, Gillespie et al., 1997). For example, crickets do not show behavioural fever when encapsulating sephadex beads, when infected with the tachnid parasitoid *Ormia ochracea*, when infected with *bacterium Serratia*

marcescens or infected with gregarines (gut protozoans) (Adamo, 1998). However, when crickets are infected with an intracellular parasite, *Rickettsiella grylli*, it moves towards warmer temperature (Adamo, 1998; Louis et al., 1986).

Eicosanoids may be involved in the induction of behavioural fever. Eicosanoids are involved in mediating cellular immune reactions (Stanley and Miller, 2006). Insect fat bodies and haemocytes are thought to be sources of eicosanoids (Stanley and Miller, 2006). In locusts, dexamethasone blocks the expression of behavioural fever (Bundey et al., 2003). Dexamethasone inhibits arachidonic acid which is a precursor molecule for eicosanoids such as prostaglandins (Stanley, 2000). Injection of either arachidonic acid or a commercially available prostaglandin (PGE1) rescues behavioural fever (Bundey et al., 2003, Cabanac & LeGuelte 1980).

Activation of the immune response by injection of bacterial cell wall does not produce behavioural fever immediately. It was noticed that there is no change in temperature 2.5-5 hours after injection, whereas a significant change can be noted, 24 hours later (Bundey, 2003). The cockroach *Gromphadorhina portenatosa* takes more than 10 hours after injection to develop a preference for warmer temperatures (Bronstein and Conner, 1984). LPS injection in the beetle *Onymarcis plana* is associated with an elevated temperature preference within one hour after injection and approaches the peak effect 5 hours post injection (McClain et al., 1988). However, in scorpions *Buthus occitanus* and *Androctonus australis*, the behavioural fever can be induced in 12 minutes after PGE1 injection (Cabanac and Le Guelte, 1980). The short period between the injection of PGE1 and the activation of the behavioural fever is constant with the hypothesis that prostaglandins exerts its effect on the nervous system after being secreted by the immune response

(McClain et al., 1988). Insects have peripheral thermal receptors, but how information is integrated within the central nervous system is still not clear (Chapman, 1988).

1.1.1.2. Illness-induced anorexia

Activation of the immune response (e.g. LPS injection) is adequate to elicit illness-induced anorexia in insects (e.g. *Manduca sexta*; Adamo, 2005; Adamo et al., 2007; Bedoyan et al., 1992; Dunn et al., 1994), although see Tyler et al. (2006) for a counter example. There is evidence that immune neural connection is responsible, at least, in part, for feeding reduction in immune-challenged *M. Sexta* (Adamo, 2005, 2006; Adamo et al., 2007).

The biogenic amine octopamine may play a role in this illness-induced anorexia. Octopamine has a major role in immune-neural connection. It is a neuroactive compound, that is neurons have octopamine receptors (Roeder, 2005). It has an important effect on neural activity (Orchard et al., 1993). Octopamine is also an immune modulator (Adamo, 2006; Roeder, 2005). Haemocytes have receptors for octopamine (Adamo, 2005), and it can affect phagocytosis (Baines and Downer, 1994; Baines et al., 1992). During a challenge to the immune response, octopamine concentration increases in the hemolymph (Adamo, 2005; Dunphy and Downer, 1994). The high level concentration of octopamine damages the neural circuits for swallowing located in the frontal ganglion, a part of insect central nervous system (Miles and Booker, 2000). Therefore, release of octopamine by the immune system could change neuronal firing in the frontal ganglion.

Another biogenic amine, Serotonin, may also play a part in producing illness-induced anorexia. It has been found in locusts that, an injection of laminarin, an immune activating agent will induce illness-induced anorexia (Beckage, 2008) Increased feeding can be

induced by releasing mianserin, an inhibitor of both octopamine and serotonin receptors in locusts (Hiripi et al., 1994; Molaei and Lange, 2003).

1.1.1.3. Changes in reproduction

Activation of immune response can lead to a rapid change in reproduction behaviour in insects; this rapidity suggests that the changes are caused by a direct immune-neural link. For example, LPS injection in male crickets, *Gryllus campestris* led to a reduction in their calling behaviour within a day (Jacot et al., 2004). Egg laying of the cricket *Acheta domesticus* increases after activation of the immune system by injection of LPS (Adamo, 1999). Bacterial infection of the cricket *Gryllus texensis* leads to an increase in egg laying (Shoemarker et al., 2006a).

1.1.1.4. Changing in learning behavior

Injections of LPS in honeybees led to a decrease in their ability to perform a classical conditioning task i.e. associate a novel odour with a food reward (Mallon et al., 2003). In bumblebees, Riddell and Mallon (2006) have found that LPS injection had no effect on learning, unless the bees were deprived of protein. Both studies indicate the possibility that an immune-neural connection is responsible for the effect. In vertebrates and molluscs, immune-neural connections do not need food deprivation for their activation. Both Mallon et al. (2003) and Riddell and Mallon (2006) suggest alternative explanations for the decline in learning, such as a competition for resources between the mechanisms underlying learning and those underlying an immune response. However the involvement of protein, does not necessarily mean these behaviours are not an example of psychoneuroimmunology. If the immune system affects the nervous system, but a good

supply of protein masks the effect, by perhaps repairing the damage, you would get the results Mallon et al saw.

1.1.2. Evidence for neural affects on immune function

In vertebrates, neurons of the sympathetic nervous system release norepinephrine that binds to receptors on immune cells leading to changes in their function (Webster et al., 2002). It has been noted that some vertebrate's neuropeptides have antimicrobial characteristics (Brogden et al., 2005). Insect's neurons have some of the same or related neuropeptides that have antimicrobial properties in vertebrates (e.g. Settembrini et al., 2003; Winther et al., 2006), suggesting that insects neurons could also play direct role in immune function.

1.1.2.1. Neuroendocrine influences on immunity

Beckage (2008) and Adamo (2006) show evidence that those hormones that are directly or indirectly regulated by the central nervous system (Nijhout, 1994) influence the immune system. Hormones that are important for the regulation of development also affect the expression of immune-related genes (Roxstorm-Lindquist et al., 2005).

1.1.2.2. Neural Innervation of immune organs

In insects, the fat body is the major organ of the immune system (Gillespie et al., 1997), and is innervated (Hazarika and Gupta, 1987) this innervation supports the possibility of direct neural affect on immune response function.

1.1.2.3. Changes in behaviour that a result in changes in immune function

Activating flight-or-fight behaviour in mammals results not in fleeing or fighting only, but also in the activation of the mammals stress response (Sapolsky, 1992). This neural neuroendocrine circuit can affect immune function using different pathways (Glaser and Kiecolt- Glaser, 2005; Sternberg, 2006; Webster et al., 2002).

Reproductive behavior

Both cellular and humoral immune changes can correlate with reproductive behaviour in insects (Adamo, 2006; Lawniczzac et al., 2007; Siva-Jothy et al., 2005). Several studies suggest that the steroid hormone 20-hydroxy-ecdysone (20E), a main regulator of insects development, metamorphosis, reproduction and aging (Nijhout, 1994; Kozlova and Thummel, 2000; Tu et al., 2006), adapts cellular and humoral innate immunity. In the mosquito *Anopheles gambiae*, 20E induces expression of prophenoloxidase 1 (PPO1), a gene containing ecdysteroid regulatory elements (Ahmed et al., 1999; Müller et al., 1999). In *Drosophila melanogaster*, 20E causes mbn-2 cells, a tumorous blood cell line, to differentiate into macrophages and to increase their phagocytic activity (Dimarcq et al., 1997), and injection of 20E in the mid third instar larvae increases the phagocytic activity of plasmatocytes (Lanot et al., 2001). In Drosophila, 20E signalling is also essential for lymph gland development and hematopoiesis, both necessary for pathogen encapsulation (Sorrentino et al., 2002).

Stress-induced immunosupression

Various stressors can affect immune function in insect (Brey, 1994). In crickets, flight-or-fight behaviour leads to a fleeting decrease in resistance to bacteria (Adamo and Parsons, 2006). In Orthoptera, during flight-or-fight there is a release of octopamine as a neurohormone (Orchard et al., 1993) this leads to increase energy supply, influencing muscles functions and sensory perceptions. Therefore, octopamine release could be analogous to the activation of the adrenergic system in mammals (Orchard et al., 1993, Roeder, 2005).

Octopamine appears to mediate a neural-immune connection in insects (Webster et al., 2002). In insects, the 'stress' hormone octopamine effects on immune function are not as simple as adrenaline in vertebrates (Adamo, 2006). Nevertheless, if crickets are injected by octopamine, they become immune-suppressed, mirroring the immune response after a flight response (Adamo and Parson, 2006). The ability of neurohormonal octopamine to change immune function supplies good evidence for the existence of a neural-immune connection in insect.

1.2. Insect response to gram-negative bacteria

An immune system is a complex network of cells and humoral factors whose function is to control and attack pathogens. Immune systems differs between the groups of animals in their level of complexity and even their basic constituent parts, but all function through the recognition of pathogens as non-self by specialized proteins known as pattern recognition proteins (PRPs). PRPs set off regulatory cascades that stimulate the activity of a series of effectors systems that destroy the pathogen. There are two systems that animals can use to defend themselves against infectious organisms, the innate and acquired immunity. Innate immunity relies on germline encoded factors for recognition and killing of foreign organisms, whereas acquired immunity can produce effector molecules that recognize specific antigens and development of an immunological memory (Fearon, 1997). Insects are thought to lack an acquired immune system, but they possess a well-developed innate response that is divided into humoral and cellular defense. Humoral defenses include soluble effector molecules such as antimicrobial peptides, and the enzymatic cascades that regulate melanin formation and clotting (Muta & Iwanaga, 1996; Cornelis & Soderhall, 2004; Blandin & Levashina, 2004; Theopold et al., 2004; Imler & Bulet, 2005). Cellular

immunity includes defense responses like phagocytosis and encapsulation that are mediated by blood cells (hemocytes) (Lackie, 1988; Strand & Pech, 1995; Gillespie et al., 1997; Irving et al., 2005). Here I will briefly discuss the immune response to gram negative bacteria, presumably the response initiated by Mallon et al, given that it was induced with LPS. I will look at Peptioglycan recognition proteins, the main gram-ve PRPs, then the signalling cascades involved and finally which effectors the insect immune system uses against gram -ve bacteria.

1.2.1. Peptidoglycan Recognition Proteins

Insects recognise bacteria by sensing bacterial peptioglycan with Peptidoglycan recognition proteins (PGRPs). Peptioglycan is an essential glucopeptidic polymer that is only found in bacterial cell walls. In fact it has been shown that induction of an immune response by LPS is actually caused by peptioglycan contamination (Kaneko et al 2004). Peptioglycan from gram-negative bacteria differs from most gram-positive peptioglycan by the replacement of lysine with meso-diaminopimelic acid (DAP) at the third position in the peptide chain (Lemaitre & Hoffmann, 2007). PGRPs are innate immunity molecules present in insects, mollusks, echinoderms, and vertebrates. In insects, there are 19 PGRPs, classified into short (S) and long (L) forms (Kaneko et al., 2004). The short forms exist in the hemolymph, cuticle, and fat-body cells, and sometimes in epidermal cells in the gut and hemocytes, whereas the long forms are mainly expressed in hemocytes. PGRP-LC is the particular PGRP that recognises DAP type peptioglycans. PGRP-LC has three alternative splice forms LCa, LCx, LCy. Signalling is induced by the close association of two PGRP-LC molecules caused by their binding to polymeric peptioglycan. PGRP-LCa is thought to act as an adaptor for monomeric peptioglycan, which could not bind two PGRP-LC molecules by itself

1.2.2. Signalling pathways

Innate immune responses are mediated by the activation of various signalling processes. The defense against gram-positive bacteria and natural fungi is mediated by the Toll signaling pathway. In contrast the defense against gram-negative bacteria is mediated by immune deficiency (Imd) pathway. Although there are interactions between them (Lemaitre & Hoffmann, 2007), for our purposes it is enough to present a brief outline of the Imd pathway.

1.2.2.1. Imd pathway

The Imd (immune deficiency) pathway controls defence reaction against gram-negative bacteria, and resembles the tumour necrosis-factor-receptor (TNFR) pathway in mammalian (Hoffmann et al., 1999, Khush et al., 2001). The Imd pathway leads to the expression of antimicrobial peptides such as Attacin, Cecropin and Diptericin that confer substantial immunity to gram-negative bacteria (Hoffmann, 2003; Brennan and Anderson, 2004). Imd pathway is totally intracellular and initiated by activation of the peptidoglycan recognition protein (PGRP) (Choe et al., 2002; Gottar et al., 2002). It's over expression activates antimicrobial peptides genes transcription in the absence of an infection (Georgel et al., 2001).

Genetic screening has identified eight canonical components of the Imd pathway: the PGRP-LC (Gottar et al., 2002, Ramet et al., 2002) Mitogen-Activated Protein 3 kinase (MAP3K) TAK1 (Vidal et al., 2001, Silverman et al., 2003); TAB2 (Gesellchen et al., 2005, Zhuang et al., 2006); DIAP2, a member of Inhibitor of Apoptosis protein (Gesellchen et al., 2005; Kleino et al., 2005, Leulier et al., 2006); IKKβ/ird5 and IKKβ/ird5 and IKKγ/Kenny (Silverman et al., 2000, Lu Y et al., 2001); the dFADD adaptor (Leulier

et al., 2002, Naitza et al., 2002) the Dredd caspase (Leulier et al., 2000) and Relish is the transcription factor (Hedengren et al., 1999).

After infection, the Imd pathway leads to the activation of the Relish factor, and it cleaves into two parts (Stoven et al., 2000). The amino (N)-terminal Rel- 68 fragment that contains the DNA binding Rel homology domain, translocates to the nucleus where it binds to NF-κB like trigger component of the antimicrobial genes, and the other fragment, Rel-49 is IκB-like and remains in the cytoplasm (Hultmark, 2003). See (Figure1.1). NF- κB is a transcription factor that regulates the expression of multiple target genes involved in various cellular processes including the immune system in vertebrates (Ghosh et al. 1998, Li and Verma 2002).

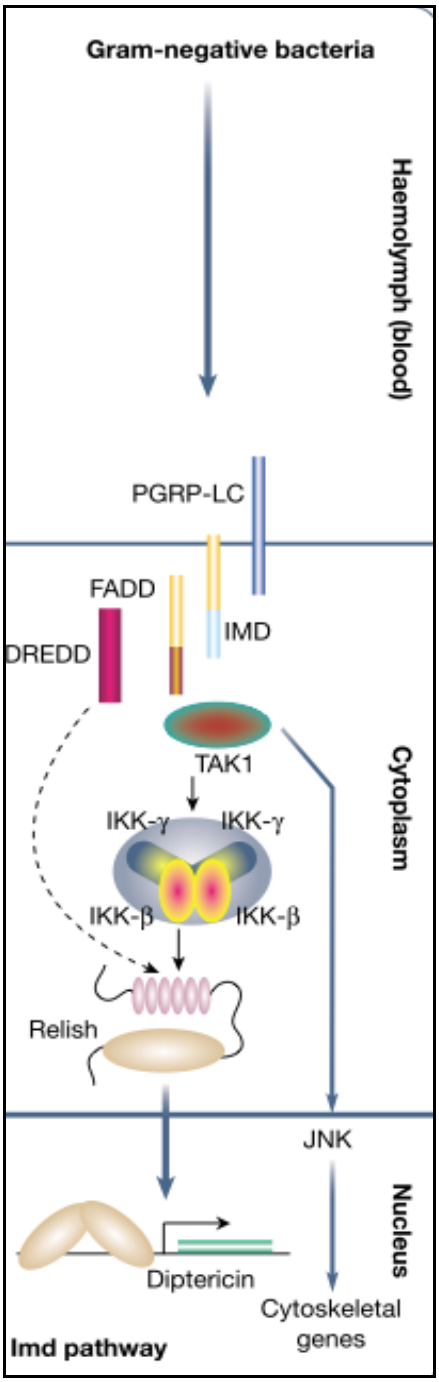


Figure 1.1

The Imd pathways in the control of expression of genes encoding antimicrobial peptides. For explanations, see the main text. Colour codes for common domains: yellow, death domains; red, TIR domains (Hoffmann, 2003).

1.2.3. Effectors of the insects' immune response to gram -ve bacteria

1.2.3.1. Cellular effector

Both cellular and humoral defense is activated when microbes and invading organisms successfully enter the body of insects. The cellular defense consists of three major kinds of blood cells, as characterized by morphology and function. Nearly 90% of all blood cells are plasmatocytes, which demonstrate a macrophage-like behavior by phagocytosing invading bacteria (Holz et al., 2003; Lanot et al., 2001; Rizki and Rizki, 1984).

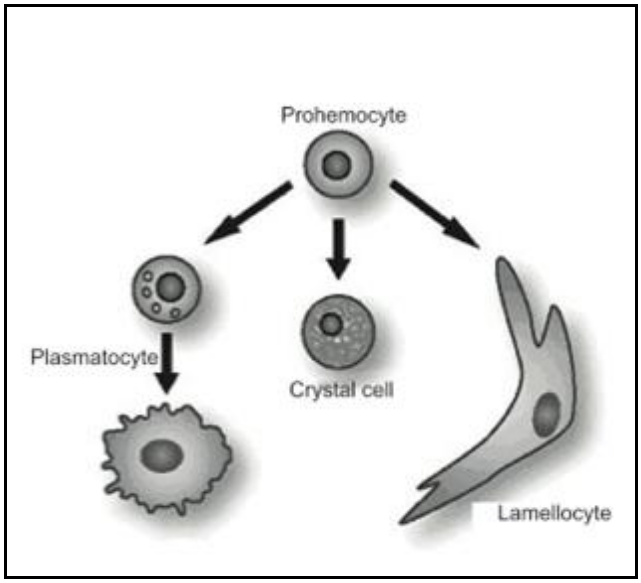


Figure 1.2

The three differentiated types of haemocytes in the larvae of Drosophila melanogaster (Strand, 2008).

Phagocytosis is an important process for different biological events including the removal of microorganisms and activation of innate and adaptive immune system. In mammals, macrophages are phagocytic cells that express a large numbers of surface receptors for recognizing engulfing microbes (Erturk and Silverman, 2005). Lamellocytes encapsulate larger invaders that cannot be phagocytosed, e.g. the eggs of endoparasitoid wasps. Finally,

crystal cells provide enzymes required for melanization reactions (Rizki and Rizki, 1984) (see Figure1.2).

In Drosophila, a number of genes have been shown to be involved with phagocytosis. The scavenger receptor dSR-CI was one of the first to be identified as a pattern recognition receptor that binds to bacteria (Rämet et al., 2001). This receptor is responsible for only a small amount of the total binding of bacteria by a phagocytically active Drosophila cell line. To look for additional genes involved in phagocytosis Rämet et al., (2002) conducted a RNA interference-based screen in the Drosophila immune-reactive S2 cell line and reported 34 genes possibly involved in the phagocytosis of bacteria. The peptidoglycan recognition protein LC (PGRP-LC) is one of these genes which are involved in the engulfment of Gram-negative, but not Gram positive bacteria. Similarly, the mosquito PGRP-LC was also found to be involved in the phagocytosis of bacteria (Moita et al., 2005). However, the loss of PGRP-LC only reduced the phagocytic capability of the S2 cells to 65% of normal, suggesting that other receptors must be involved in the recognition and engulfment of invading microbes.

Melanisation is the deposition of melanin and this can be induced when invading microorganisms break the cuticle barrier (Soderhull and Cerenius, 1998). There are three types of phenoloxidase existing in insects, granular, wound and haemolymph phenoloxidase. The importance of melanisation is healing of the wound and contributes to encapsulation. Phenoloxidase exists in inactive form and the required phenoloxidase for the production of melanin from dopamine can be activated in response to stress and infection by the serine protease cascade (Soderhull and Cerenius, 1998).

1.2.3.2. Humoral effectors

Besides the cellular responses, microbial challenge in Drosophila triggers the secretion of effectors molecules from the fat body into the hemolymph. Due to the open circulatory system hemolymph can freely surround the fat body, which in turn can recognize the microbial components in the hemolymph and initiate AMP production.

Antimicrobial peptides (AMPs)

Antimicrobial peptides are molecules that play a major part of the innate immunity in response to infections, and are produced from the fat bodies (equivalent to the liver in mammalian) which are a major immune responsive tissue that originate from the mesoderm during embryogenesis. Fat bodies are large and are located inside the open circulatory system of the insect body cavity so they represent an important site for synthesis and secretion of the AMPs in the haemolymph (Hoffmann and Reichhart, 2002). AMPs also can be synthesized and secreted from haemocytes (Dimarcq et al., 1997) and from the gut (Tzou et al., 2000, Boulanger et al., 2002b, Lehance et al., 1997). More than 170 insects AMPs have been discovered (Bulet et al., 1999). In Drosophila alone, 20 AMPs have been identified and utilized and are grouped into seven classes (Lemaitre and Hoffmann, 2007) (Figure1.3). AMPs are small in size, have a positive charges (cationic) and structurally varied molecules that work in combination to block the growth of attackers by disrupting their membranes (Lemaitre et al., 1996), so they are highly efficient and effective at managing any microbial invaders (Bulet et al., 1999).

AMPs structurally are diverse and can be classified into three main groups that described whether they attack gram-positive bacteria, gram-negative bacteria or fungi. Diptericin, Drosocin and Attacin are very effective against Gram-negative bacteria (Wicker et al.,

1990, Bulet et al., 1993). Defensin is active against gram-positive bacteria (Dimarcq et al., 1994) whereas Drosomycin and Metchnikowin are antifungal (Fehlbaum et al., 1994, Levashina et al., 1995).

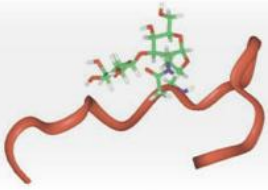
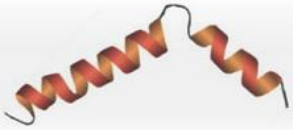
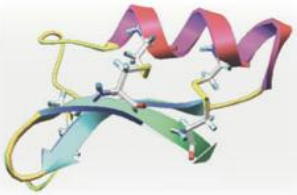
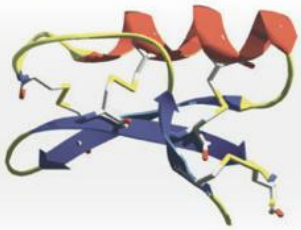
Diptericin	2	Gram-negative bacteria	0.5 µM	nd
Attacin	4	Gram-negative bacteria	nd	nd
Drosocin	1	Gram-negative bacteria	40 µM	
Cecropin	4	Gram-negative bacteria	20 µM	
Defensin	1	Gram-positive bacteria	1 µM	
Drosomycin	7	Fungi	100 µM	
Metchnikowin	1	Fungi	10 µM	nd

Figure 1.3

The Antimicrobial peptides in Drosophila name number of genes, concentration, and the three dimensional structure (Lamaitre & Hoffmann 2007).

Insects AMPs act on the cell membrane of the bacteria and fungi by creating small pores in the cell membrane or ion channels leading to an increase in the cell membrane permeability (Yang et al., 2000, Brogden, 2005, Lehrer et al., 1989), by depolarizing the bacterial membrane (Westerhoff et al. 1989), or by inducing hydrolases that degrade the bacterial

cell wall and reduce the biosynthesis cell activity (Bierbaum et al., 1985). Some AMPs inhibit intracellular targets by crossing the cell membrane (Kragol et al., 2001, Brodgen, 2005).

1.3.Neurobiology of the proboscis extension reflex

Learning is the acquisition of a neuronal representation of new information such as visual, auditory and olfactory features (Dukas, 2008). Insects rely on their learning and memory for a lot of activities in their life such as feeding, aggression, defending themselves from predators, social interaction and sexual behaviour (Dukas, 2008). Of course, learning and memory are not the same thing, with learning being the acquisition and memory being the retention of a neuronal representation.

Honeybees are the model species to study learning and memory in insects due to their extremely rich repertoire of behaviours (Frisch, 1967) and their small brains. The brain of a honeybee has a volume of approximately 1 mm^3 and contains around 960,000 neurons (Giurfa, 2007). A bee can travel several kilometres outside the hive between flowers looking for and collecting pollen / nectar, they have developed sensory and motor capacity and see the world in colour (Menzel and Backhaus 1991). In addition honeybees have a highly developed ability to differentiate between types of odours (Guerrieri et al., 2005). In nature, honeybees have the ability to recognize, learn and memorize the cues of the food source of the hive (Menzel 1985; Menzel et al., 1993). Honeybees can use a ‘‘waggle dance’’ to communicate and transmit the information about the important locations around the hive (Frisch, 1976). Another advantage to using bees as a model for learning is that appetitive learning is a strong and fast event in the honeybee in the laboratory (Menzel, 2001).

Appetitive learning is a form of associative learning (Hammer & Menzel 1995). The principle is that the subject becomes conditioned or learns to associate one stimulus, the conditioned stimulus (CS) with a reinforcing, unconditioned stimulus (US) which elicits a reflex response in the animal. The US is presented simultaneously with an initially neutral CS. The CS is neutral in that it carries no innate meaning, and thus the animal does not respond to it. After an appropriate time period, an association forms, so that the CS will signal the occurrence of US. The measure or indicator of learning is that where initially the unconditioned response (UR) occurred with US, it is now elicited by the CS alone and has become a conditioned response (CR). This is the classical conditioning paradigm first described by Pavlov. This paradigm can be applied in hungry bees, by conditioning the innate proboscis extension response (PER) to an olfactory stimulus. The PER is stimulated by contacting the antennae or the mouthparts with sucrose that represents the US. Simultaneous presentation between an initially neutral odour, the CS and the US provide a relationship between the stimuli and stimulate natural foraging behaviour (Menzel 1999). A successful association between the two stimuli can be tested at second trial, by looking for increased PER to the CS alone. After one trial, 80% of the trained honeybees will perform a conditioned response (Ray & Ferneyhough 1999).

In the associative learning paradigm, the PER is mediated by the US pathway, originating at the antennal chemoreceptors, and travelling directly to the suboesophageal ganglion (Hammer & Menzel 1995). The pathway terminates close to motor neurons that mediate movement of the proboscis. The CS pathway begins at the antennal olfactory receptors neurons terminating in the antennal lobe so, the antennae are the main chemosensory organs and olfactory recognition starts at the level of the antennae. The antennal lobes are

paired structures at the front of the brain in the honeybee, and the two lobes are connected with each other by nervous tissue called the supraeosophageal commissure and they are connected with the mushroom body via olfactorio globularis tract (Arnold et al., 1985; Flanagan and Mercer 1989; Galizia et al., 1999). Neurons projects to the lateral protocerebral lobe directly or indirectly via the mushroom body calyces. The lateral protocerebral lobe receives its input from all the sensory pathways, and projects into the supraeosophageal ganglion to generate movements of the proboscis. Normally, olfactory input is insufficient on its own to generate these motor movements. However, the US pathway has a second termination, onto the VUMmx1 (Hammer & Menzel 1995).

Figure 1.4

Representing the main structural parts of the brain in the Drosophila, yellow, suboesophageal ganglion; red, antennal lobes; blue, mushroom bodies; orange, central Complex; and the gray regions represent the neuropil regions surrounding mushroom bodies and central complex (Heisenberg, 2003).

In the case of the unconditioned stimulus pathway, VUMmx1 (ventral unpaired median neuron, of the maxillary neuromere 1) is a unique neuron that is necessary and sufficient to

substitute for the sucrose reward in honeybee (Hammer, 1993, 1997). This leads to a response to sucrose solution delivered at the antennae and the proboscis (Hammer, 1993).

VUMmx1 branches in the brain and combines with the olfactory pathway at three sites in the antennal lobe, the calyces (the olfactory input areas of the mushroom bodies, and the lateral horns. See (Figure 1.4). VUMmx1 forms the neural representation of the unconditioned stimulus in the proboscis extension reflex (PER), Hammer (1993) showed that the learning of an olfactory stimulus can be prevented by depolarizing the VUMmx1 after olfactory stimulating (forward paring of odor). Hammer explained that learning of an olfactory stimulus can be induced by the substitution the sucrose reward in olfactory conditioning of the proboscis extension reflex by an artificial depolarization of VUMmx1 immediately after olfactory stimulation (forward pairing) (Giurfa, 2003).These results explained the central role of VUMmx1 in the neural correlation of the US in associative olfactory learning (Giurfa, 2007). The VUMmx1 releases octopamine (OA) onto the CS convergence sites when the US pathway is activated. If activated within an optimal time interval of CS pathway activation, the VUMmx1 neurons will reinforce the CS signal to each convergence site. Octopamine also modulates the response to odour stimuli. After a conditioning trial, stimulation of the CS pathway alone is adequate to stimulate the motor neurons in the oesophageal ganglion, and elicit a PER. Octopamine inhibitors obliterate this and the sensitising effect of the US, but leave the response to sucrose stimulation via the US pathway intact. Injecting octopamine into the brain can restore conditioning but not sensitisation. Local octopamine injections into either the antennal lobe or mushroom body can substitute for US delivery during pairing with CS delivery and condition the PER (Giurfa, 2007).

1.4. Thesis Structure:

In the second chapter, I look at the immune memory interaction in a free flying paradigm. Using a floral choice paradigm, this chapter shows that learning performance is impaired in bumble-bees (*Bombus terrestris*) that are not infected but whose immune system is stimulated non-pathogenically. This was in response to a paper that suggested that infection with *Crithidia bombi* lead to a decrease in memory in this paradigm (Gegear et al., 2006). My result demonstrates that before it is assumed that a parasite has a direct effect on a host's behavior, the effect of the immune response stimulated by the parasite must first be quantified.

In chapter three, I looked at the evolutionary relationship between the immunity and memory in insects. I measured the immune response for bumblebees from different colonies of known learning ability (measured by researchers at QMUL). I took workers from these colonies and tested their immune response using the antibacterial zone of inhibition (ZOI) assay. This allowed me to identify any evolutionary relationship between these two traits.

Thus far in the thesis, I have treated the interaction between the immune and nervous system as a black box. Chapter four looks at a possible physiological mechanism to explain how stimulating an immune response leads to a reduction in learning. One promising avenue is that sleep plays a modulating role between the two systems. I looked at the effect an immune response had on sleep in *Drosophila melanogaster*. *Drosophila melanogaster* has been helpful to the analysis of molecular components and cellular pathways that mediate both associative learning, sleep and immunity because it provides a resource bank of genetic tools and transgenic mutants. The GAL4-UAS system is considered a powerful

technique for studying the expression of genes in fruit fly. Activation of the immune response in our transgenic *Drosophila melanogaster* requires only heat shock of or the addition of a chemical (Geneswitch) to the Gal4 gene that triggers the promoter region of the UAS region that causes the expression of peptioglycan receptor protein (PGRP) in our transgenic flies. The expression of PGRP sets off a cascade of interactions causing an immune response.

In chapter five, I attempted to develop *Drosophila melanogaster* as a model for studying the interaction between the nervous system and the immune system in insects. One of the most well studied paradigms in Drosophila learning is classical olfactory conditioning. I quantified the memory of *Drosophila melanogaster* after activating their immune system (using the systems detailed in chapter 4) to see if the fruit fly would make a useful model for immune modulated memory reduction.

Chapter six details my conclusions including some possibilities for future work. I have also attempted to explain the importance of my work to several fields.

Chapter Two: The interaction between immunity and memory in a bumblebee free flying learning paradigm

1. Introduction

Parasites can influence different host behaviors including foraging, mate choice, and predator avoidance (Moore 2002). Several recent papers have shown reduced memory abilities in infected insects (Gegear, Otterstatter et al. 2006; Iqbal and Mueller 2007). In nature this would have severe fitness costs (Raine and Chittka 2008). However, although parasites can act directly on hosts, a growing number of general pathologies have been shown to be the result of the immune response elicited by the parasite rather than the parasite itself (Moret and Schmid-Hempel 2000). Bumblebees (*Bombus impatiens*) infected by a protozoan parasite (*Crithidia bombi*), have an impaired ability to learn the color of rewarding flowers (Gegear, Otterstatter et al. 2006).

There is extensive communication between the central nervous system and the immune system (Dantzer 2004). Many behavioural responses to infectious agents, such as fever, increased slow-wave sleep, reduced activity, exploration and sexual behaviour in mammals are orchestrated by immune products called proinflammatory cytokines that are released in response to the detection of antigens (Maier and Watkins 1998). Links between nervous and immune systems are not unique to vertebrates. Work in the Mallon lab has shown that both the honeybee *Apis mellifera* (Mallon, Brockmann et al. 2003) and the bumblebee *Bombus terrestris* (Riddell and Mallon 2006) perform poorly in proboscis extension reflex (PER) memory tests (Bitterman, Menzel et al. 1983) when their immune systems have been challenged by lipopolysaccaride (LPS). LPS is a component of gram-negative bacterial cell walls which is a non-pathogenic elicitor of the immune response (Moret and Schmid-Hempel 2000). That is, they found that memory is impaired by the immune response directly with no parasite present.

In this study I will use a free flying floral choice assay to test the learning and memory abilities of bumblebees (*Bombus terrestris*). Instead of using a live parasite to affect memory (Gegear, Otterstatter et al. 2006) I will use LPS. Gegear et al. (2006) proposed that the *Crithidia* induced alteration in memory abilities that they found is most likely caused by the host's own immune system, and not the direct action of the parasite itself. If the Gegear result is replicated in my study, it will show that this is indeed the case. It will also show that the previously found immune induced memory reduction (Riddell and Mallon 2006) is reproducible in a free flying semi-natural paradigm, providing evidence that this connection between immune response and memory in insects is a general and vitally important part of their ecology.

2. Methods and Materials

Experiments were carried out on five bumblebee colonies from Koppert Biological Systems, UK. Colony 2 was the data presented in the first version of the thesis. The data from the undergraduate has been removed. Colony 1, 3, 4, and 5 are new to this thesis and not previously published. The experiments began when the colonies had a minimum of 30 workers, approximately four weeks old. Between observations, colonies were fed ad libitum with pollen (Percie du Sert, France) and 50% diluted glucose/fructose mixture (Meliose; Roquette, France). Before the experiments colonies were kept at 26°C and 60% humidity in constant red light.

2.1. Tagging and injection

Each bee was marked with an individual tag (Opalith tags; Christian Graze KG, Germany). I challenged the bee's immune system by injecting the bee with LPS. I injected into the haemocoel through the pleural membrane between the second and the third tergite, using a sterilized glass capillary that had been pulled out to a fine point using an Narishige PC-10 pipette puller, a dose of 5µl of Ringer solution, containing 4% lipopolysaccharide (0.5 mg/ml = 9 mg 4% LPS g-1 of bee) (LPS, Sigma L-2755) (Mallon, Brockmann et al. 2003, Schmid-Hempel, 2005, Moret and Schmid-Hempel, 2000, 2009, Sadd, B.M. et al. 2005) a highly immunogenic but non-pathogenic elicitor of the immune response (Moret and Schmid-Hempel 2000). Imler et al (2000) showed that LPS stimulated insects immune system. Half the workers in each colony were injected with LPS. To control for injection, the other half were injected with 5 µl of Ringer solution, a saline solution regularly used in insect physiology. It has been show previously that this control also leads to an immune

response, but at a much lower level than that elicted by LPS. Korner and Schmid-Hempel (2004) found that LPS bees had ZOIs ten times higher than Ringer injected bees. As new workers eclosed they were assigned to either the LPS or control group. Each bee was re-injected every 10 days to ensure their immune system remained stimulated (Korner and Schmid-Hempel 2004).

2.2.Learning assay

Bees were left for four days after injection before observations were begun to ensure that the immune system of LPS bees had been stimulated (Korner and Schmid-Hempel 2004). I connected the nest box to the flight arena, a 1200mm x 1000mm x 300mm plywood box with a removable Perspex lid (figure 2.1). All observations were carried out at 23°C, 60% humidity with a 12:12 light: dark cycle. The reduced temperature discouraged the colony from moving into the flight arena.

2.2.1. Training method

I used here the method outlined in Raine et al. (2006). During the pre-training, the bees foraged from 20 randomly placed blue and yellow flowers, both containing 15 µl of sugar water. The flowers consisted of an inverted Eppendorf tube on a small platform (20 mm in diameter), 50 mm from the floor of the arena. The bees completing at least five consecutive foraging bouts were selected for training. Ten blue flowers and 10 yellow flowers were placed at random locations within the arena. The yellow flowers were always rewarding, as the bees show an innate preference for the blue flowers (Raine et al. 2006). For each bee, we recorded 89 flower choices after the bee first chose a rewarding flower. That is, each bee visited 90 flowers. The data was analyzed using a repeated measures regression (see

below). Once a bee temporarily left the flight arena, the flowers were refilled. After each bee's 90 visits, the flight arena was cleaned using 70% industrial methylated spirit (IMS).

2.2.2. Statistical Analysis

The method of generalized estimating equations (an extension of a generalized linear model) was used to account for correlations among observations from the same subject in a repeated measures logistic regression (Hardin & Hilbe 2003). The dependent variable was 'visit to rewarding flower' (yes/no). The independent variables were colony (1 to 5), trial (1–90) and treatment (LPS or Ringer). All statistical analysis was carried out using STATA version 11. These figures were drawn by the XTGRAPH command in Stata 11.

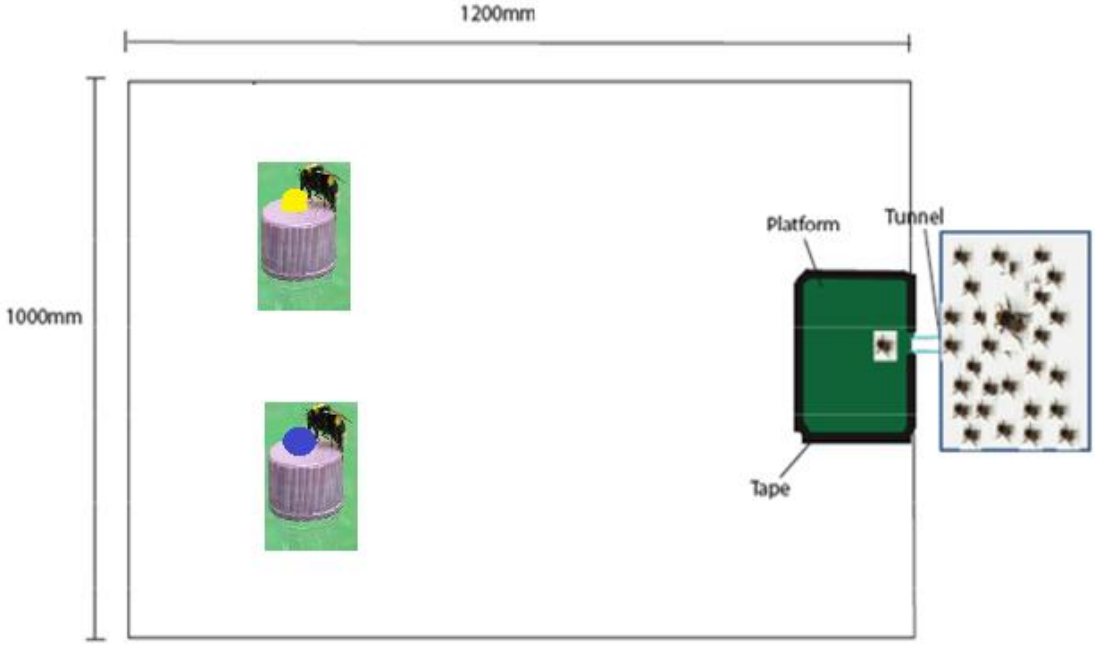


Figure 2.1

An aerial view of the fight arena box connected to the nest colony box, containing the two different kinds of flowers, i.e. the artificial flowers (rewarding) yellow and (not rewarding) blue.

3. Results

I tested 57 bees injected with LPS (Colony 1: 10, Colony 2: 15, colony 3: 11, colony 4: 12, colony 5: 9) and 56 injected with ringer (Colony 1: 7, Colony 2: 15, colony 3: 11, colony 4: 12, colony 5: 11). Colony has a significant effect on whether a bee visits a rewarding flower (colony $z = 5.01$, $n = 10170$ visits, $p < 0.0001$). Both trial ($z = 13.38$, $n = 10170$ visits, $p < 0.0001$) and treatment ($z = -6.69$, $n = 10170$ visits, $p < 0.0001$) have a significant effect on whether a bee goes to a rewarding or unrewarding flower, see figure 2.2 (A). I then analysed each colony separately. Trial had a significant effect on whether a bee went to a rewarding flower or not in all colonies (colony 1 $z = 2.65$, $n = 1530$ visits, $p = 0.008$, colony 2 $z = 14.48$, $n = 2700$ visits, $p < 0.0001$, colony 3 $z = 4.18$, $n = 1980$ visits, $p < 0.0001$, colony 4 $z = 4.21$, $n = 2160$ visits, $p < 0.0001$, colony 5 $z = 2.34$, $n = 1800$ visits, $p = 0.019$), see Figures 2.2 (B-F). Whether a bee was injected with LPS or Ringer had an effect on whether that bee went to a rewarding flower or not in colony 1, 2, 3 and 4 (Treatment: Colony 1 $z = -5.90$, $n = 1530$ visits, $p < 0.0001$, Colony 2 $z = -5.90$, $n = 2700$ visits, $p < 0.0001$, Colony 3 $z = -2.4$, $n = 1980$ visits, $p = 0.017$, Colony 4 $z = -4.75$, $n = 2160$ visits, $p < 0.0001$), see Figure 2.2, (B), (C), (D) and (E). For colony 5 there was no significant difference between ringer and LPS treated bees with regards to whether they chose a rewarding flower or not (Colony 5 $z = -1.57$, $n = 1800$ visits, $p = 0.117$), Figure 2.2 (F). Using the XTGEE algorithm there is no option to test interactions. This also appears to be the case for the GENMOD process in SPSS. For colony 2, treatment (LPS versus Ringer) had no effect on how quickly the bees first found a rewarding flower (Mann–Whitney: $U=-1.378$, $n=30$, $p=0.1681$, median value =7 visits). This data was not collected for the other colonies.

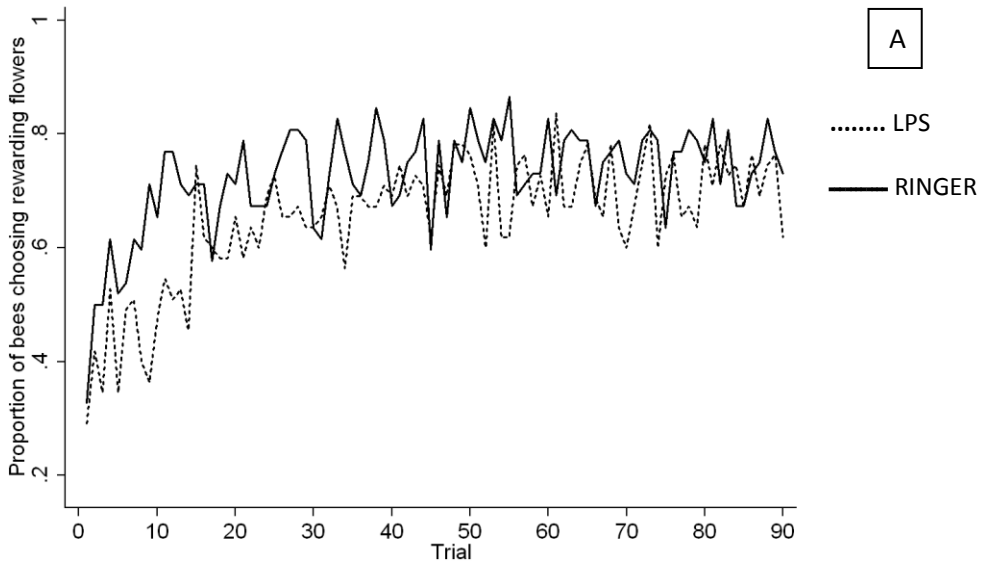


Figure 2.2A

The proportion of bees choosing the rewarding flower for each visit for the all 5 colonies. The dotted line represents bees injected with LPS. The solid line represents those injected with the Ringer control.

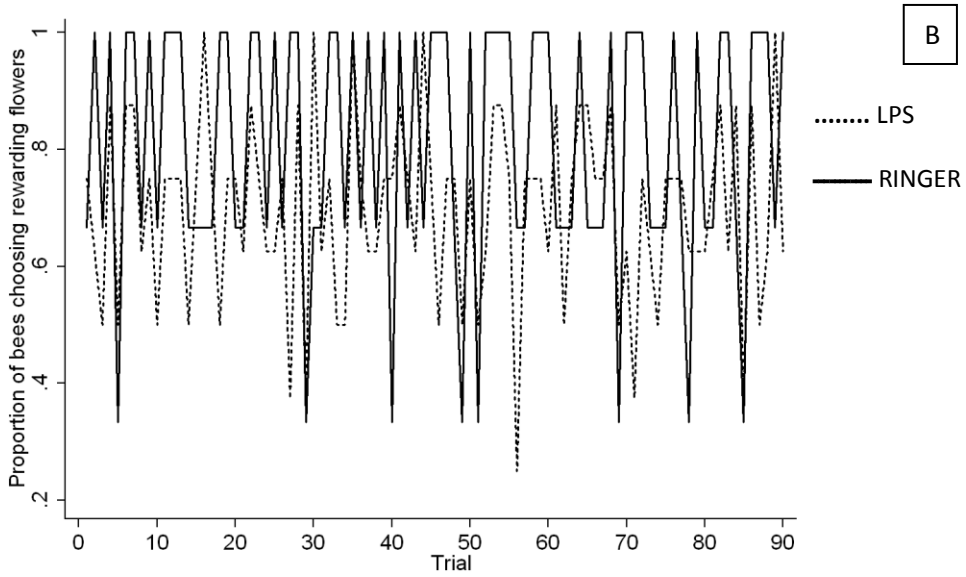


Figure 2.3B

The proportion of bees choosing the rewarding flower for each visit for colony1. The dotted line represents bees injected with LPS. The solid line represents those injected with the Ringer control.

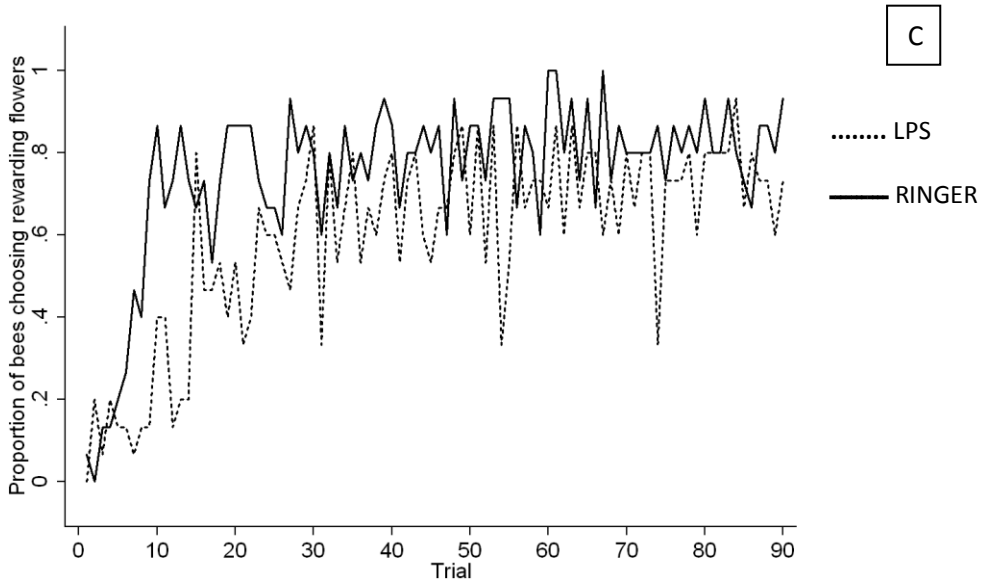


Figure 2.4C

The proportion of bees choosing the rewarding flower for each visit for colony2. The dotted line represents bees injected with LPS. The solid line represents those injected with the Ringer control.

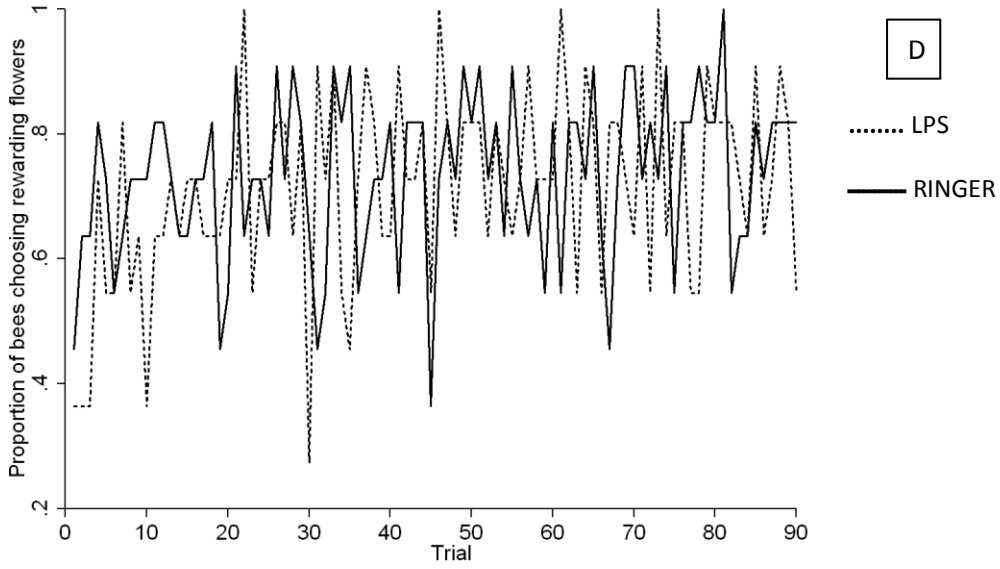


Figure 2.5D

The proportion of bees choosing the rewarding flower for each visit for colony3. The dotted line represents bees injected with LPS. The solid line represents those injected with the Ringer control.

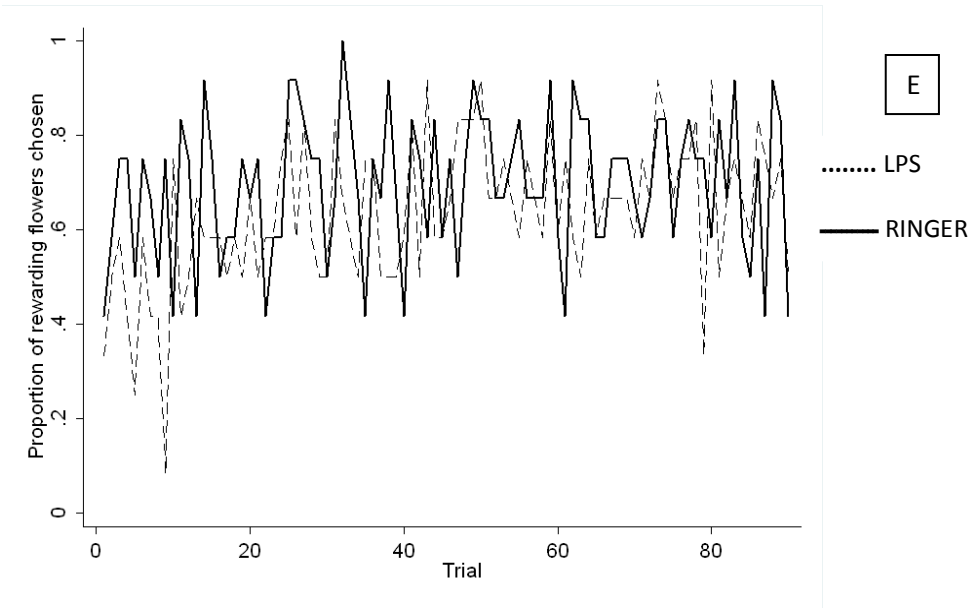


Figure 2.6E

The proportion of bees choosing the rewarding flower for each visit for colony4. The dotted line represents bees injected with LPS. The solid line represents those injected with the Ringer control.

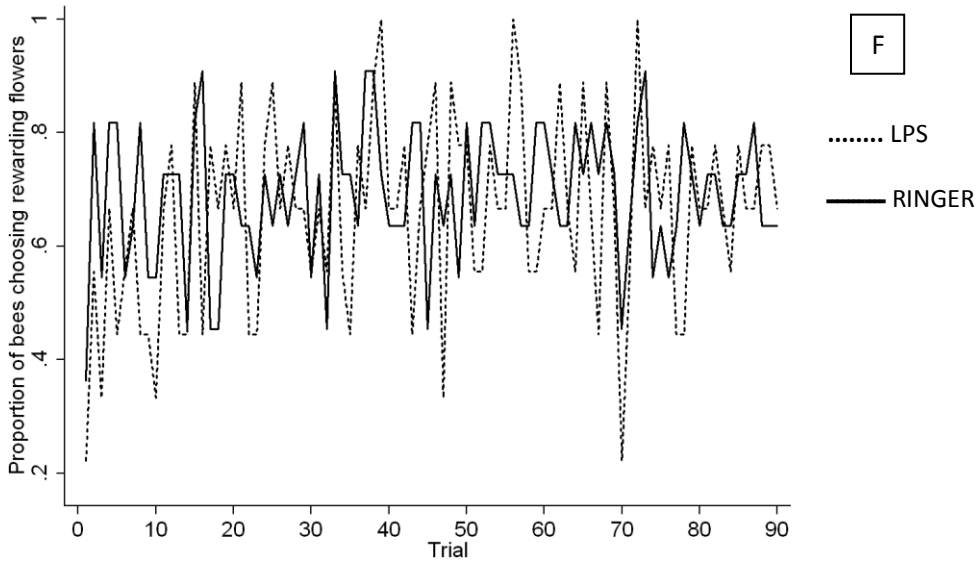


Figure 2.7F

The proportion of bees choosing the rewarding flower for each visit for colony5. The dotted line represents bees injected with LPS. The solid line represents those injected with the Ringer control.

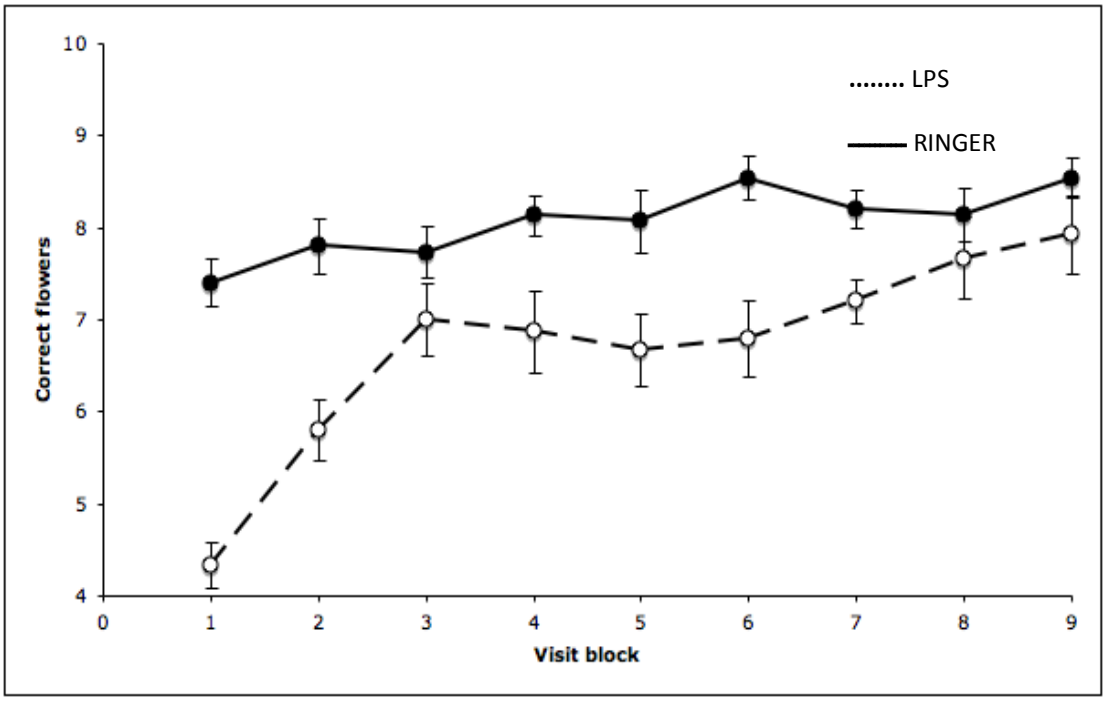


Figure 2.8

Data from colony two as represented originally in the thesis. The solid line represents the control bees, whereas the dashed line represents the LPS bees. The y-axis is the number of rewarding flowers chosen in that ten visit blocks.

4. Discussion

I found that bees whose immune systems were stimulated non-pathogenically by LPS, had an impaired ability to learn to discriminate between rewarding and non-rewarding flowers in four out of five colonies tested. This result mirrored the effects found both in PER assays using LPS (Riddell and Mallon 2006) and the performance of bumblebees in the flower choice assay when infected by *Crithidia* (Gegear, Otterstatter et al. 2006).

In the previous viva there was some confusion about the figures. Figure 2.3 shows one of the original figures. This is data from the same bees as is represented in Figure 2.2 (C). One comment during the viva was that Figure 2.3, rather than showing that LPS bees take longer to learn to discriminate rewarding flowers, actually shows that they in fact learn quicker. This was based on the steep slope of the dashed line (LPS) between visit block 1 and 3 compared to the corresponding solid line (Ringer bees). This misunderstanding is due to thinking that nothing happened between the 0 and 1 on the x-axis. Rather the control bees take about 10 visits to get to where it takes the LPS bees at least 30 visits. This can be seen clearly in Figure 2.2 (C). This is the main reason I have opted for the style seen in figure 2.2 rather than then Figure 2.3. Also note that the y-axis in figure 2.3 is not the same as in figure 2.2. In figure 2.3, it is the number of rewarding flowers visited in a given ten visit block for a given bee, this is then averaged over all the bees in that treatment to give us the data point and a standard error. In figure 2.2 it is the proportion of bees choosing rewarding flowers for a group of bees defined by treatment and trial. This is a single value and therefore there are no error bars. The statistical analysis, repeated measures logistic regression with ‘visit to rewarding flower (yes/no)’ as the dependent variable, remains the

same, with the addition of Colony as a factor, it is merely how it is graphically represented that is different.

Colony five did not show the expected immune related decrease in discrimination ability. This is surprising given that the immune effect was found in four of the five colonies presented here and in the other colony reported in Alghamdi et al. (2008) Moreover, qualitatively similar results in the three infected (rather than immune stimulated) colonies from Gegear et al (2006). One possibility is that it is just due to colony level variation in learning ability or immune response or both. Both of these have been found previously in the literature (Raine et al 2006, Riddell et al 2009)

Previous work has shown that there is good correlation between performance of honeybees in PER assays and in free flying experiments (Laloi, Bailez et al. 2000). One difference which I found between our PER work and our current study involves protein consumption. In both honeybees and bumblebees, immune-induced PER impairment was only recorded when protein consumption was restricted (Riddell and Mallon 2006). From this they suggested that increased protein consumption ameriolated the effects of the immune response on memory. However in this current free-flying experiment, protein was not controlled, yet a clear effect was present. Another study has shown that an immune response increases the food intake of bumblebees (Tyler, Adams et al. 2006). It is also known that if a bumblebee is allowed to forage, its immune response is decreased (König and Schmid-Hempel 1995). It seems likely to me that during the PER assays as the bees are harnessed their protein requirements are reduced, therefore to expose the immune induced reduction in memory protein must be controlled. In the free flying experiment, foraging as a physical act has a protein cost, therefore the protein requirements of the bee are

increased. The bee then exhibits the immune-induced memory impairment without artificial external protein reduction.

Despite the major importance of protein, studies of dietary effects on insect immunity are infrequent (Schmid-Hempel, 2005). Encapsulation, phenoloxidase (PO) activity, and lysozyme activity are all enzyme-based immune responses to foreign invasion or wounding (Siva-Jothy et al., 2005) that can affected by protein level. Spodoptera moth caterpillars fed protein-rich diets had enhanced immunity to a viral pathogen relative to those fed on carbohydrate-rich diets (Lee et al., 2006). Increased dietary protein increased PO activity and antibacterial activity, and bacterially infected *Spodoptera caterpillars* increased their protein intake in response to infection while ingesting similar levels of carbohydrates (Povey et al., 2009).

The findings in this chapter lead to two major conclusions. Firstly, my results demonstrate clearly that before it is assumed that a parasite has a direct effect on a host's behaviour, the effect of the immune response stimulated by the parasite must first be quantified. Parasite manipulation of host behaviour is a well-developed field (Lefevre et al 2009). However increasing it is been seen that many infected hosts' behaviours can be seen as being immune-mediated rather than caused directly parasites (Adamo 2006). This chapter's result also shows that the previously found decreased PER ability in immune stimulated bees can be generalized to more natural learning paradigm and a more realistic nutritional status. Recently it has been shown that learning ability has a direct effect on bumblebee colony fitness (Raine and Chitka 2008). This opens up the possibility that this cross talk between immune and nervous system could have vital fitness costs. In the next chapter I consider,

given these fitness costs, if an evolutionary relationship exists between the immune response and learning and memory.

Chapter Three: The evolutionary relationship between the immunity and memory in Bumblebees

1. Introduction

In chapter 2 I showed that there was a physiological interaction between memory and immunity in bumblebees during a free-flying assay. This strengthens evidence showing that both honeybees *Apis mellifera* (Mallon et al., 2003) and bumblebees (Riddell & Mallon, 2006) perform poorly in learning assays when their immune systems have been challenged by lipopolysaccaride (LPS). Given this physiological link between learning and immunity, and that learning and immunity have demonstrated fitness costs in insects (Raine & Chittka, 2008, Mery & Kawecki, 2003, Kraaijeveld & Godfray, 1997), it seems reasonable to hypothesise an evolutionary trade-off between learning and immunity. An evolutionary trade-off is where the evolution of an increase in a given trait leads to a reduction in a different trait. Whilst a recent paper showed no evidence of a trade-off between immunity and learning in different artificially selected Drosophilia lines in the laboratory (Kolss et al., 2006), it might be more ecologically relevant to examine natural levels of variation in these traits. Before I discuss the experimental design I would like to discuss some examples of evolutionary trade-offs and the costs and benefits of both an immune system and learning and memory.

1.1. Evolutionary trade-offs

Evolutionary theory is based on the assumption that trait evolution is restricted by fitness trade-offs. (Stephens & Krebs, 1986; Charnov,1989; Roff, 1992, 2002; Stearns, 1992;Futuyma, 1998; Houston & McNamara, 1999; Reznick et al., 2000). A trade-off arises when a fitness increase due to a change in one trait is opposed by a fitness reduction due to a change in another trait. The term trade-off can be used in the description between the functional relationship between traits or the statistical correlation between the traits.

Evolutionary trade-offs in performance are important in limiting the distribution and the profusion of organisms, these trade-offs are the products of genetic, physiological, and resource constraints that prevent an organism from simultaneously optimizing different traits (Stearns, 1992).

Trade-off has been demonstrated, between such traits as longevity and fecundity (Medawar 1952, Rose and Charlesworth 1981), reproduction and growth (Warner 1984, Reznick 1985, Bell and Koufopanou 1986), and competitive ability and resistance to parasites' exploitation of their host's resources (Lubchenco 1978, Lenski and Levin 1985). Trade-offs are also central to the hypotheses concerning the evolution of generalists vs. specialists (Huey and Hertz 1984, Jaenike 1990, Van Tienderen 1991).

Trade-offs are specified in quantitative genetics by a negative genetic covariance between traits, a covariance that could be caused by the pleiotropic effects of the genes involved, linkage disequilibrium with deleterious mutations or resource allocation during development (Roff & Fairbairn, 2007, Schmid-Hempel, 2005). The form and extent of pleiotropy is central to many theories in evolutionary biology, including the evolution of specialization (Futuyma and Moreno 1988; Jaenike 1990; Cooper and Lenski 2000), senescence (Rose, 1991), and limits to adaptation (Barton and Keightley 2002; Otto 2004). Fisher (1958) emphasized the importance of pleiotropy, and formalized its role in his geometric model of adaptation.

Mery and Kawecki (2003) demonstrated an evolutionary trade-off between learning ability and competitive ability in the fruit fly. They showed that the evolution of an improved learning ability in replicated experimental fly populations has been associated with a

decline of larval competitive ability comparing with control populations. The poor competitive performance of the selected populations seems to be due to pleiotropic effects of alleles that were favored by the learning selection regime (Mery and Kawecki, 2003). Experimental evolution studies with *Drosophila melanogaster* indicate that evolution of improved learning ability is associated with trade-offs with other ecological aspects of performance (Burger et al., 2008). These trade-offs seem to be symmetric, selection for better learning results in a reduction in larval performance and faster aging, whereas selection for improved larval performance or slower aging results in a reduction in learning.

For there to be an evolutionary trade-off between learning and immunity in bumblebees, there must be fitness effects due to both of these traits. In the following sections I will outline the cost and benefits of both immunity and learning.

1.2. Costs and benefits of Immunity

The immune system plays an important role in fighting parasites and different types of infections and is one of the main physiological mechanisms that regulate host survival (Zuk & Stoehr, 2002). One of the most important foci of the evolutionary ecology of immunity is the identification and understanding of the selective forces shaping and maintaining immune defences, focusing on both the factors that induce an immune response and the consequences of that response (Schmid-Hempel 2002). The cost of immune defence is one of the central limitations considered in these evolutionary ecology studies and results in trade-offs with other life-history traits, such as reproduction and development (Schmid-Hempel 2005).

The benefits of the immune system are clear. While parasites, by exploiting their host's resources affect its survival, growth or fecundity, an active immune system will help to decrease these negative effects. In Drosophila and other insects the immune system including humoral and cellular components that defend against foreign organisms, parasitoids, viruses, bacteria and fungi (Brey & Hultmark 1998, Khush & Lemaitre, 2000). In insects the cell-mediated immune responses are involved in encapsulation to defend against parasitoids (Carton & Nappi, 1997). On the other hand humoral immune response produces small antimicrobial peptides attacking any microbial pathogens (Brey & Hultmark 1998, Khush & Lemaitre, 2000). In *Drosophila melanogaster*, overexpressing the pathogen receptor molecule PGRP-LE leads to the activation of innate immunity, decreasing lifespan. This longevity decline occurred due to the continuous activation of the NF-κB factor *Relish*. Both enhanced resistance and decreased lifespan were dependent on *Relish* suggesting a link between NF-κB signaling and longevity, (Libert et al., 2006).

There are two main parts of immunity costs. The first part is the cost of having the immune system itself (including the cost of its evolution) and the second part is the physiological cost of its activation (Sheldon & Verhulst 1996). Variation in the expression of a component of the immune system may simultaneously affect another fitness relevant trait (e.g. growth, reproduction) of the organism. Over evolutionary time, this can become entrenched in negative genetic covariances, such that a trade-off between the immune trait and another fitness component is observed (Stearns, 1992).

This can appropriately be called the evolutionary cost of the immune system, and several studies have implied that the evolutionary cost of the immune response may be the causal link that underlies the crucial life-history trade-off between current reproduction and future

expected success (Richner et al., 1995; Deerenberg et al., 1997). Studies have shown that artificial selection for increased resistance (the end product of successful immune defenses) to parasites is associated with reductions in at least some other components of fitness. Yan et al., (1997) found that two laboratory strains of the mosquito *Aedes aegyptii*, which differed in their ability to defend themselves against the avian malaria parasite, *Plasmodium gallinacium* also differ in their longevity and fecundity or their time required to develop. More resistant Indian meal moths (*Plodia interpunctella*) have lower egg viability and longer development (Boots & Begon 1993). Parasitoid resistant Drosophila strains are weak larval competitors (Kraaijeveld & Godfray 1997). These results suggest the occurrence of genetic trade-offs between immune defences and life history traits that could potentially lead to evolutionary changes under selection (Bell & Koufopanou 1986). The evolutionary cost of immune defense can affect the dynamic of resistant and susceptible genotypes in a host population according to parasite predominance. Resistant host genotypes should only be maintained when parasites are abundant (Schmid-Hempel, 2005).

Physiological aspects of life history trade-offs have been reviewed previously (e.g., Townsend & Calow 1981, Bell & Koufopanou 1986, Sibley & Calow 1986, Ricklefs 1991, Sibley & Antonovics 1992). The cost of activating the immune response on some other fitness trait is easier to understand and to measure than are evolutionary costs. Examples are a lower tolerance for desiccation and starvation (Hoang, 2001) and reduced fecundity (Fellowes et al., 1999) after an immune response was activated. Moret and Schmid-Hempel (2000) found that when starved bumblebees activate their immune response, their subsequent survival is lowered compared with starved controls by 50 – 70%. Immune

response activation can affect other traits, but the reciprocal is also true. Intense reproductive efforts causes a reduction in the encapsulation rate in dragonflies (Siva-Jothy et al., 1998) and a reduced PO-activity and resistance to bacterial infections in male crickets (Adamo et al., 2001).

1.3. Costs and benefits of learning

Learning is defined as the ability to acquire a neural representation of a new association between a stimulus and an environmental state that may affect fitness (Dudai, 1989). Biologically, learning is usually defined as a change in an animal's behaviour resulting from a past sensory experience that the animal remembers (Dukas, 2008). Learning allows animal to adjust its behaviour in an adaptive way. Significant benefits of the learned information for fitness can only be realized through their behaviour (Johnston, 1982, Dukas 2008, Kawecki 2010).

It is thought that learning is an adaptation for managing with unpredictable environmental factors (Alcock, 2005). However learning is not fundamental, many organisms are unable to learn, but can respond to different types of factors that vary unpredictably with time and space. For instance, *Escherichia coli* contain chemoreceptors which can help to recognise the food substances and any harmful chemicals in their surrounding environment by a developed system of information processing and behaviour (Eisenbach & Lengeler 2004, Koshland 1980). This behavioural machinery can be also observed in fruit flies. Females fly towards the aggregation pheromones deposited in food by early reaching females (Wertheim et al., 2005). Male flies begin a rigid sequence of courtship activity in response to females producing sex pheromones (Hall 1994, Manoli et al; 2006, Spieth, 1974).

Many forms of behaviour, even in short-lived animals, are amenable to learning. The potential ways in which learning could contribute to fitness are diverse (Johnston, 1982; Papaj and Prokopy, 1989 and Dukas, 1998). A bee can identify a neural representation of the nest site, record the spatial position, odour and colour of flowers, and it can learn a new motor model for handling these flowers. Raine et al., (2007) showed that variation between bumblebee colonies in learning ability was correlated with their foraging ability, a proxy for fitness in bumblebees.

There are many costs associated with learning and memory. In animals dependent on learning, they pay costs for acquiring experience for examples in energy, time and mortality (Laverty & Plowright, 1988; Heinsohn, 1991; Dukas & Visscher, 1994). In a laboratory study, Mery & Kawecki (2003) found that artificial selection of improved learning ability of replicated experimental fly population has been associated with a decline of larval competitive ability under low food availability compared with replicated control populations. There is also a decline in egg-laying rate from lines selected for improved learning ability in fruit flies than flies from the unselected ones (Mery & Kawecki, 2004). In another study, it was found flies which are subjected to associative spaced condition producing long term memory died earlier than flies subjected to control treatment in the absence of food (Mery & Kawecki, 2005). This last set of results demonstrates that the high learning rate is costly owing to metabolic and energy expenditure.

1.4. Aims of chapter

Given the physiological link between learning and immunity, and that learning and immunity have demonstrated fitness costs in insects (Raine & Chittka, 2008, Mery & Kawecki, 2003, Kraaijeveld & Godfray, 1997), it seems reasonable to hypothesise an

evolutionary trade-off between learning and immunity. Bumblebees are an obvious candidate for this hypothesis as colonies show natural variation in learning performance (Raine et al., 2006a) and the physiological relationship between learning and immunity has been demonstrated in *B. terrestris* (Riddell & Mallon, 2006, Chapter 2).

The demands of foraging from many different flower species, which can vary dramatically in the quantity and quality of rewards they offer, and the need to find the nest after each foraging bout, mean that bees have highly developed cognitive abilities. Bumblebees also learn from conspecifics, so-called social learning (Leadbeater & Chittka, 2007). Furthermore, I would also expect that immunity would be a more important trait in social species that have high contact rates with genetically close individuals leading to a greater chance of infection (Cremer et al., 2007).

In this chapter my Queen Mary collaborators used a free-flying floral choice assay to test the learning abilities of bumblebee colonies. I took workers from these colonies and tested their immune response using the antibacterial zone of inhibition assay. This allowed me to identify any evolutionary relationship between these two traits.

2. Methods and Materials

Twelve bumblebee colonies (*Bombus terrestris dalmatinus*) were obtained from Koppert Biological Systems (Berkel en Rodenrijs, The Netherlands). The colonies were fed *ad libitum* with pollen (Percie du Sert, France) and 50% diluted glucose/fructose mixture (Meliose; Roquette, France). Before the experiments, the colonies were kept at 26 ^{o}C and 60% humidity in constant red light. All workers were uniquely marked with Opalith tags (Christian Graze KG, Germany).

2.1. Learning assay

All work involving learning was carried out by Dr. Nigel Raine, while at Queen Mary's, London. Results from this associative learning assay are reported in a previous paper (Raine & Chittka, 2008). Bees were pre-trained to forage from 20 bicoloured, blue and yellow, artificial flowers in a flight arena. During pre-training all flowers were rewarded with 50% (w/w) sucrose solution providing previously colour-naïve bees with an equal chance to associate both colours with reward (Raine et al., 2006). Bees completing at least 5 consecutive foraging bouts were selected for training. These foragers were trained individually, in a flight arena containing 10 blue (Perspex® Blue 727) and 10 yellow (Perspex® Yellow 260) artificial flowers (each 24 x 24mm). Yellow flowers were rewarding (each contained 15µl of 50% (w/w) sucrose solution), whilst blue flowers were empty (unrewarding). Bees were regarded as choosing a flower when they either approached (inspected), or landed on it. Choosing a yellow flower was regarded as 'correct', whilst choosing a blue flower was deemed to be an 'error'. They recorded the choice sequence made by each bee from the time it first entered the flight arena. Recording flower choices ceased once a bee made 99 flower choices after the first time it probed a

rewarding (yellow) flower (Raine et al., 2006b). Flowers were changed and their positions re-randomized between foraging bouts to prevent bees using scent marks or previous flower positions as predictors of reward. Flower colours were selected so that bees had to overcome their strong, unlearned preference for blue, before associating one of their innately least favoured colours (yellow) with reward (Raine et al., 2006a, Chittka et al., 2004).

2.2.Learning curves

The starting point for each bee's learning curve was the proportion of errors made (blue flowers chosen) before the bee first probed a rewarding (yellow) flower. Flower choices made by each bee after (and including) the first time it probed a rewarding (yellow) flower were evaluated as the number of errors (blue flowers chosen) in each group of 10 choices. Learning curves (exponential decay functions: $y = y0 + Ae^{-x/t}$) were fitted to these eleven data points (i.e. the start pointing and subsequent 10 groups of ten flower choices) for each individual bee, using Microcal Origin (Chittka et al., 2004; Raine et al., 2006b). Here, x is the number of flower choices the bee made, starting with the first time it probed a yellow flower, and y is the number of errors. The saturation performance level (y0) is the number of errors made by a bee after finishing the learning process, i.e. when reaching a performance plateau. The decay constant (t) is a measure of learning speed: with lower t values corresponding to faster learning speeds. A is the curve amplitude: the maximum displacement (height) of the curve above y0. Both amplitude (A) and saturation performance (y0) were constrained between 0-10 for curve fitting.

2.3. Zone of inhibition assay

This assay measures antibacterial activity: it is based on the ability of immune proteins to inhibit bacterial growth when placed onto an agar plate seeded with bacteria (*Arthrobacteur globiformis* 10^5 bacteria per ml of agar). Workers from all twelve colonies were sacrificed after the learning assay and stored at –20^{o}C for later analysis. Each thorax was homogenised in 300 μL of sodium cacodilate/CaCl2 buffer. 2 μL of the supernatant from the centrifuged solution (1300g for 10 minutes at 4°C) was pippetted into a hole on the agar plate. This was incubated over night (28°C). The resultant zones of inhibition (mm) were measured as the mean of its longest and shortest axis (ZOI value).

Antibacterial test plates (diameter 9 cm, Sterlin) were prepared by adding 0.05 ml of live *Arthrobacter globiformis* bacteria suspension (10^7 cells/ml) to 5 ml of sterile broth medium (10g Bactotryptone, 5g Yeast extract, 10g NaCl, 1000 ml of distilled water, pH 7.5), with 1% of bacto-agar at 45°C. Plates were swirled to disperse the bacteria and left to settle at room temperature. Eight holes (diameter: 2 mm) per plate were made in the agar.

The heads width of the bumble bee's samples was measured. Maximum head width is the distance between outer margins of left and right eye measured using calipers under a binocularmicroscope.

3. Results

As reported in Raine & Chittka (2008) there was significant variation among colonies in learning speed (t value: one way ANOVA: $F_{11, 160} = 1.900$, $p = 0.043$).

I tested the immune response of 55 bees from 12 colonies (mean number per colony (± S.D.) = 4.58 ± 0.67) using the zone of inhibition assay. I tested the effect of colony and headwidth on ZOI using an analysis of covariance model (ANCOVA). The dependent variable was ZOI with colony as a factor and headwidth as a covariate. There was a significant difference between colonies in their immune response ($F_{11,43} = 2.30$, $p = 0.026$), which could not be attributed to the effect of body size (headwidth $F_{1,43} = 0.73$, $p = 0.397$). The nonsignificant result for headwidth shows it has no effect on a bee's immune response and it will not be considered in further analyses. For completeness please see Figure 3.3 for a plot of headwidth vs ZOI.

There was a significant negative correlation between the median t value of a colony and its mean ZOI value (Spearman's rank: $r = -0.608$, $n = 12$, $p = 0.036$: Figure 3.1). As high t values correspond to slower learning speeds, this is a positive relationship between the ability of a colony to learn and the strength of its immune response.

I also tested this correlation on an individual level. I carried out an analysis of covariance (ANCOVA) with square root of t (Sharpiro-Wilks normality test: n=55, $z = 0.439$, $p = 0.33$) as the dependent value and colony as a factor and zone of inhibition measurement as a covariate. There was no relationship between sqrt t and zoi ($F_{1,54} = 1$, $p = 0.3226$). Figure 3.2 shows this data arranged by colony.

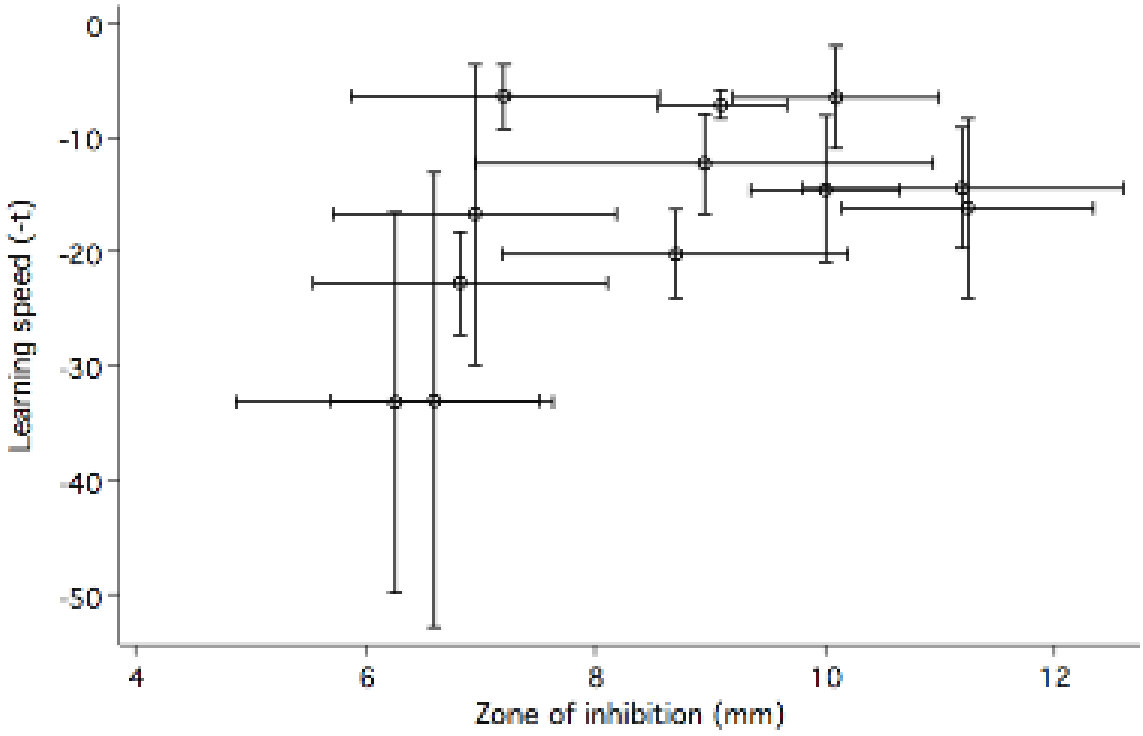


Figure 3.1

The relationship between median learning speed (negative t-values) and mean ZOI response of the 12 colonies. As high t-values correspond to slower learning speeds, I have plotted negative t-values to make clear the positive relationship between the colony learning ability and the strength of its immune response. Each point represents a colony. Vertical error bars represent median absolute deviation (MAD). Horizontal error bars represent standard error. Medians and MADs were used for t values as this data was not normally distributed.

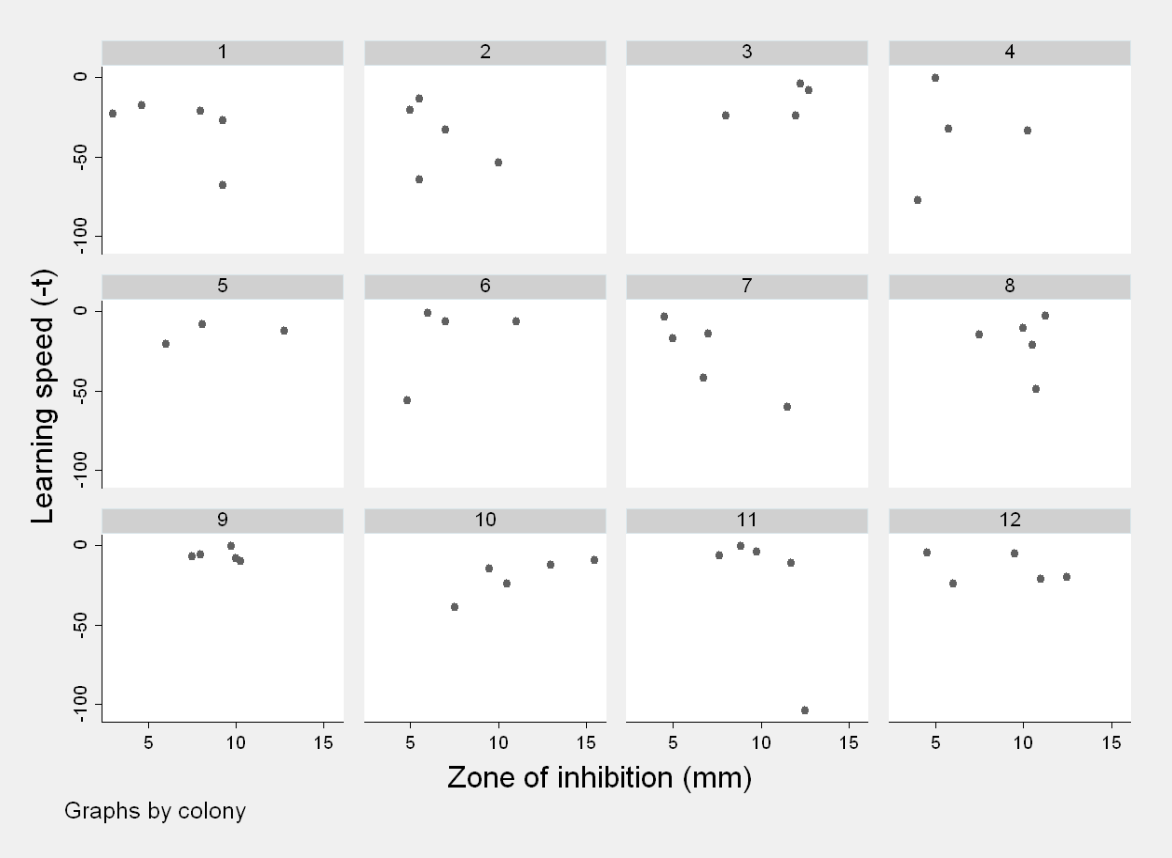


Figure 3.2

Learning speed versus zone of inhibition per colony. None of these correlations was significant except colony 10. As the ANCOVA covariate term was not statistically significant, this is considered a false positive. This figure is presented for completeness.

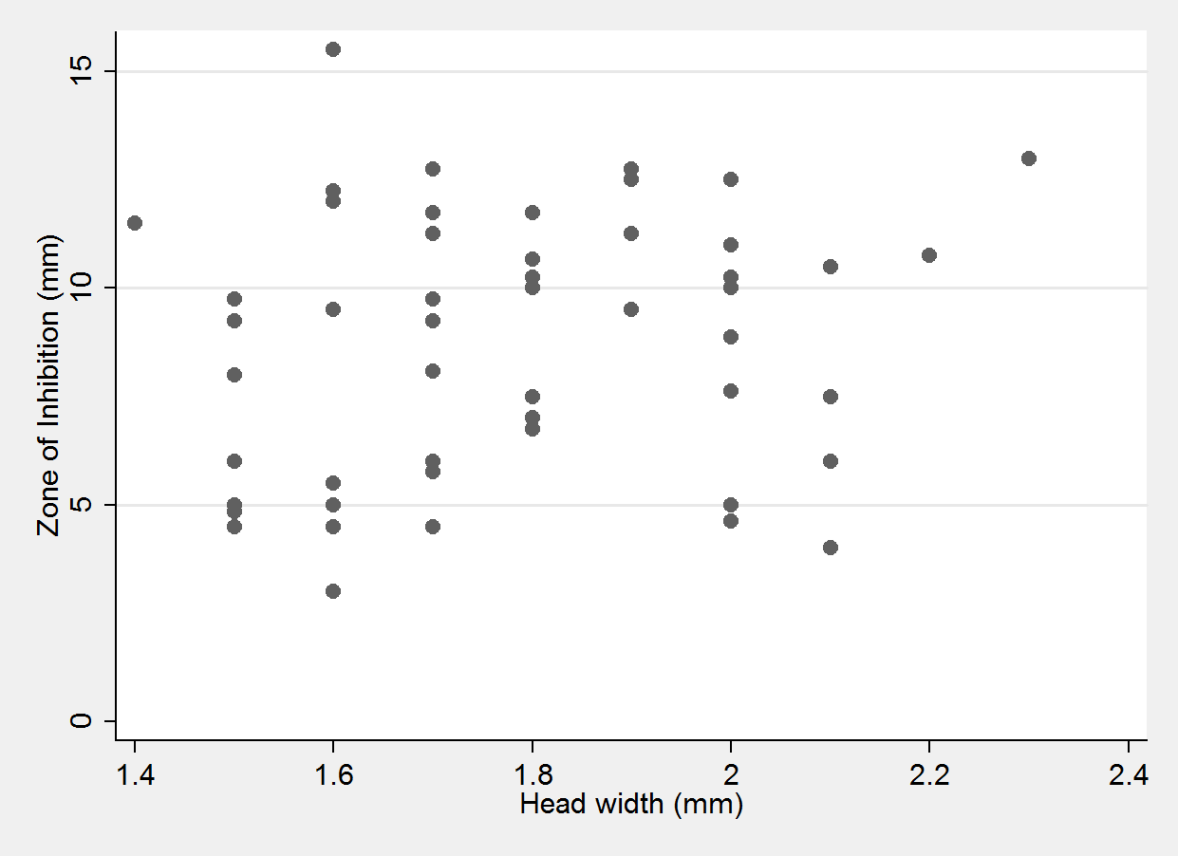


Figure 3.3

Showing the relationship between the head width versus the zone of inhibition. As shown there was no significant difference between the head width of the bumblebee's samples and their zone of inhibition.

4. Discussion

I found a positive correlation between the ability of a colony's workers to learn and the strength of that colony's workers' immune response. However, if the data is analysed at the individual bee's level, no relationship is found between learning ability and immune response. My initial hypothesis that learning ability and immune response would be in an evolutionary trade-off, demonstrated by a negative correlation was not supported.

Which result, the colony level or individual result is the true measure? This chapter was concerned with looking for an evolutionary trade-off between learning and memory in the bumblebee. There is a long history of treating the whole social insect colony, rather than the individual member of the colony, as the selectional unit (Queller and Strassmann 2002). Given that, the colony level result would seem the more appropriate. However, there is actually very little to choose between these two results. The individual level result finds no relationship between learning and immunity. The colony level result finds a positive relationship based on, as pointed out in the viva, two extreme values. A positive relationship in and of itself is quite uninteresting. A colony has high learning and immunity scores. These would then just be measures of it being a 'good' colony. The only interest in getting a positive correlation in this chapter is that it is not a negative correlation, as would have been expected if an evolutionary trade-off existed. My result is in broad agreement with the conclusions of Kolss et al (2006), that there is no evolutionary trade-off between immunity and learning.

Variation could be due to non trade-off factors. Foraging activity has been shown to decrease the immune response of bumblebee workers (König & Schmid-Hempel, 1995).

All workers tested in the learning assay and subsequently used for ZOI assays had similar levels of foraging experience in the laboratory flight arena. Hence, as all our bees were foragers this could not explain variation in immune response. Potential exposure to pathogens that could induce stimulation of the immune system was identical for all twelve colonies which came directly from the bee breeder and were not exposed to field foraging conditions before this experiment.

Phenotypic correlations are generally seen as weak evidence for evolutionary trade-offs (Reznick et al., 2000). Lack of significant bivariate correlation is insufficient to demonstrate the absence of a trade-off. Some interacting factors can affect the relationship between the bivariate correlation and the underlying functional trade off leading to the loss of the trade-off. (Roff & Fairbairn, 2007). However along with Kolss et al.'s selection experiment, I can ask why is there no evidence for an evolutionary trade-off when a physiological connection has been found repeatedly? Below I discuss three mutually nonexclusive possibilities.

Firstly, it could be argued that I have incorrectly generalised antimicrobial response to some measure of overall immunocompetence. The various parts of the insect immune system (antimicrobial peptides, encapsulation, NO production, etc.) are known not to necessarily correlate (Mallon et al., 2003). It is not simple to estimate the disease resistance, and recently many researchers have shown that connection between immunity and disease resistance is complex (Norris and Evans, 2000; Zuk and Stoehr, 2002). Assessment of an animal's relative disease resistance is critical for many questions in ecological immunology. (E.g. Lochmiller 1995; Siva-Jothy 1995; Sheldon & Verhulst

1996; Zuk 1996; Apanius 1998; Westneat & Birkhead 1998; Norris & Evans 2000; Zuk & Stoehr 2002). The main problems in interpreting the immunity assay are:

(1) Correlation between immunity and disease resistance depends on the pathogen itself. (E.g. Gross et al., 1980; Pinard-van der Laan et al., 1998; Boa-Amponsem et al., 1999; Yunis et al., 2000; Parmentier et al., 2001; Casadevall & Pirofski 2003). For example, in chicken *Gallus domesticus*, high antibody producers were significantly more resistant to *Mycoplasma gallisepticum*, *Ornithonyssus sylvarium* (mite), Newcastle virus, and Splenomegalia virus. However they were more susceptible to *Escherichia coli* and *Streptococcus aureus*, and equally susceptible to *Eimeria necatrix* (Adamo, 2004)

(2) Immune assay results may not reflect disease resistance. Statistically significant decrease in an immune measure frequently do not always result in a decline in disease resistance (e.g. Luster et al., 1993; De Waal et al., 1998; Oliver & Fisher 1999; Sinclair & Lochmiller 1999, 2000; Keil et al., 2001; Wilson et al., 2001). This could be due to the decrease in immune response although statistically significant not having reached the threshold at which it would affect disease resistance. Changes in immune component levels may have no effect on resistance or immune performance until this threshold is reached (Luster et al., 1993; Keil et al., 2001). Alternatively, the immune response to a given parasite might involve numerous components, switching off one may have no discernible effect on disease resistance (Adamo, 2004).

(3) The immune response can change itself to optimize to different types of conditions (Berczi & Nagy 1998; Kusnecov & Rossi-George 2002; Smith 2003). If some parameters

increase, others decrease and others stay the same, then the more appropriate interpretation may be that there has been a shift in the distribution of immune resources (Adamo, 2004).

It has been shown that using host resistance tests to confirm the relationship between different immune parameters and resistance to different pathogens is especially important when working with insects. Ecological immunologists use insect's species in which there have been few immunological studies, in addition, resistance tests have the advantage of assessing the immune system as a whole, an important feature given the complex interactions within the immune system (Descotes, 1999; Köllner et al., 2002). Hence, other parts of the immune response may show an evolutionary trade-off with memory if tested. However as the physiological trade-off has been found repeatedly with ZOI measures, I felt this was the most likely place to find an evolutionary trade-off.

Secondly, there is the possibility that genetic variation exists not only in resource allocation but also in resource acquisition (Reznick et al., 2000). Variation in acquisition could arise due to a genetic disparity, previous studies showed that larger bumblebees are both more successful nectar foragers (Spaethe & Weidenmüller 2002; Ings et al., 2005) and have more sensitive eyes with better visual acuity (Spaethe & Chittka 2003). If there were more variation in allocation and less in acquisition, I would expect to see a negative correlation between any two life history traits. Vice versa, I would expect to see a positive correlation (Van Noordwijk & Dejong, 1986). See figure 3.2.

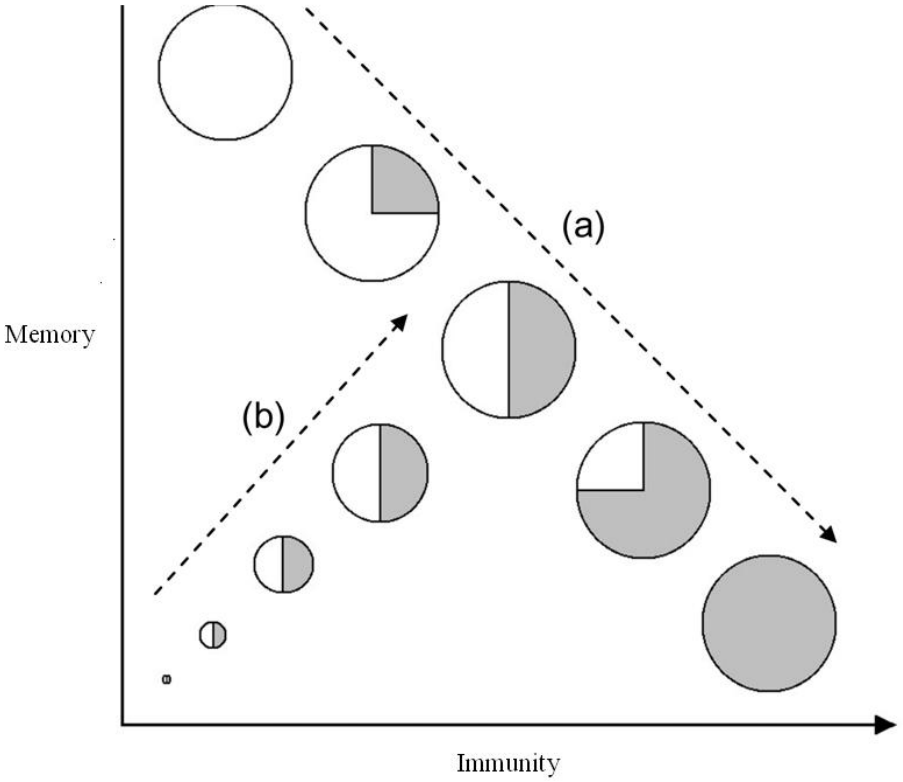


Figure 3.4

Schematic representation of possible relationship between memory and immunity, where there is no loss of resources and the absolute amount of available resources is always maximal, as the proportion diverted by the immunity increases, the portion left to the memory decreases (Relationship a). However, if there is a variation in acquisition i.e., the amount of maximal resource varies. An increase in resources will increase both memory and immunity and lead to a positive relationship between immunity and memory (relationship b). (Salvaudon et al., 2007).

Thirdly, Schmid-Hempel outlined the differences between the evolutionary and the activation cost of the immune system (Schmid-Hempel, 2005). Evolutionary costs are the fitness effects of possessing an immune system of a given strength. Activation costs are simply the effect on other physiological systems of generating the immune response from an organism's immune system. Although a physiological connection may lead us to look for an evolutionary trade-off there is no a priori reason to demand one.

To conclude, in this chapter I found no evidence for an evolutionary trade-off between learning and immunity. This does not negate the previous findings of a physiological trade-off. The remaining chapters begin the search to understand the mechanism of this physiological trade-off.

Chapter Four: The effect of the immune system on sleep in *Drosophila melanogaster*

1. Introduction

1.1. The immune-sleep-memory axis

In previous chapters I have shown that when an insect's immune system is stimulated this impairs its memory abilities. This effect has been shown with both Honeybees and Bumblebees and with free-flying and PER memory assays (Mallon et al., 2003; Riddell & Mallon 2006 and Chapter 2) Thus far this phenomenon has been studied as a black box. It is still unclear how an immune response affects memory. In this chapter, I will look at the possibility that a third system, namely sleep is involved. Why have I chosen sleep as an intermediary? I will present evidence from the literature showing that 1) reduced sleep leads to an increased immune response and that 2) a reduction in sleep leads to a reduction in learning ability and memory. My hypothesis is that an increased immune response leads to reduced sleeping (the reciprocal interaction compared to 1) that in turn leads to a loss in learning and memory. As a first step in this study, this chapter aims to test if an immune response leads to reduced sleeping in *Drosophila melanogaster*.

1.2. What is sleep?

Sleep is a physical and mental resting state where the sleeper exhibits relative inattention to the environment and is usually immobile (Siegel, 2003). In mammals, normal sleep is characterized by a general decrease in body temperature, blood pressure, breathing rate, and most other physiological functions (Cirelli et al., 2005). Generally sleep can be defined as rapidly reversible state of immobility and reduced sensory responsiveness (Siegel, 2008).

The duration and position of sleep vary greatly between different species. For example, humans sleep lying down, elephants standing up, and the hippopotamus sleeps under water.

Cows stand up even during REM sleep, and can keep their eyes open while sleeping. Dolphins continue swimming since only half of their brain sleeps at a time. Elephants sleep only 3.3 hours, humans 7 hours, rats 13.2 hours (Campbell and Tobler, 1984).

Sleep even persists in animals whose circadian rhythm has been eliminated (Siegel, 2008). In mammals, two types of sleep have been defined; non-REM sleep (slow-wave sleep) that is characterized by reduced activity in brainstem and REM sleep is characterized by a pattern of discharge that is similar to waking in most regions of the brain (Siegel, 1990; 2005). Although waking is usually characterized by low voltage cortical activity, it can be accompanied by high voltage similar to that seen in non- REM sleep (Vanderwolf, 2000).

1.3. What is sleep for?

Although sleep is a biological process that is necessary for survival in vertebrates and invertebrates, the underlying biological functions of sleep are currently unknown. Why do we sleep? Many theories try to explain the importance of sleep. Disrupting sleep in any organism is harmful to their performance, adaptive learning, working memory, and health (Rechtschaffen, 1998).

In mammals, sleep is a form of restoration or replenishment. Although this theory seems to be true, what is being restored and replenished is unclear. For example there is more oxygen consumed during REM sleep than while awake. Also not as much energy is conserved during sleep as was once thought, in fact it is only a 15% savings, something that could be easily made up with just a small increase in energy intake (Siegel, 2005).

Sleep deprivation in rats correlates with an increased oxidative stress and membrane disruption in some parts of the hypothalamus, a part of the brain that has the highest rate of protein synthesis and presumably the highest generation rate of reactive oxygen species (Siegel, 2005). It was noticed that sleep deprived rats dramatically increased their food intake, but lost weight and experienced increased heat loss, heartbeat, and energy costs. Additionally, the rats became messy and sick, developed digestive ulcers, skin lesions, and hair loss, despite maintaining the same amount of grooming activity; and furthermore host defense systems appeared to break down (Rechtschaffen and Bergmann, 1995). It has also been shown that *Drosophila melanogaster* die after chronic sleep deprivation totaling 60-70 hours (Shaw and Franken, 2003).

Another proposed role for sleep is that it is essential for memory consolidation, and thus learning. Sleep deprivation in human and other animals leads to a significant defect in hippocampal plasticity (Yoo et al., 2007). In addition, sleep loss causes learning impairment in *Drosophila melanogaster,* and short sleep mutants showed memory defects (Seugnet et al., 2008 & Bushey et al., 2007). All these studies demonstrate that sufficient sleep is important in learning and memory.

1.4. Sleep in Drosophila

Drosophila melanogaster like vertebrates has been shown to have a defined behavioural sleep state. The description of this sleep condition revealed striking similarities to sleep in humans. *Drosophila* on a daily basis showed extended durations of immobility lasting up to 10 hours. They are awake during a day time (Hendricks et al., 2000; Shaw et al., 2000), whereas at night when singly housed they prefer resting location close to their source of

food with a relaxed posture conveyed by lowering their abdomens to the floor, and away from food when housed in groups. Food would be an area of social interaction and activity (Hendricks et al., 2000). Sleep in flies is defined as a period of immobility longer than five minutes (Shaw et al., 2000; Hendricks et al., 2000). Huber et al. (2004) further examined this behavior in *Drosophila*, they independently confirmed that after 5 minutes of immobility, arousal threshold was increased, and reached a plateau, defining 5 minutes of immobility as a time unit of sleep. Importantly, they showed that arousal threshold was further increased following sleep deprivation.

Fly sleep behavior has been monitored using 3 methods: visual observation, an ultrasound activity monitoring system, and an automatic infrared system (Hendricks et al. 2000; Shaw et al. 2000). All provided similar results and confirmed that during the night flies show sustained periods of complete immobility that can last several hours. The most critical feature of sleep is the presence of a reduced ability to respond to the external world. This decreased responsiveness is reversible, a feature that allows sleep to be distinguished from coma (Cirelli and Bushey, 2008).

Arousal thresholds in flies have been measured using vibratory, visual, or auditory stimuli (Shaw et al. 2000; Nitz et al. 2002; Huber et al. 2004). In all cases it was found that flies that had been moving around immediately before the stimulus readily responded to low and medium stimulus intensities. By contrast, flies that had been behaviorally quiescent for 5 min or more rarely showed a motor response, although they quickly responded when the stimulus intensity was increased. Thus, sleep can be operatively defined in flies as any period of behavioral quiescence longer than 5 minutes.

There are two groups of processes that have been proposed to explain how sleep amount and timing are related, circadian and homeostatic processes (Borbely and Achermann, 1999). A circadian process is responsible for the sleep tendency change that is tied to the time of the day, for example, that flies tend to sleep more at night. Homeostatis is the characteristic of a system that attempts to maintain properties of the system (e.g. temperature) at a constant level. In the sleep case, any disturbance to sleep will be compensated for by later sleep periods, e.g. keeping fly awake causes it to eventually rebound in the amount of sleep it requires (Shaw et al., 2000). Another aspect of sleep homeostatis was discovered by Huber et al. (2004), they showed that arousal threshold was further increased following sleep deprivation.

Fly sleep decreases with age, is associated with changes in brain electrical activity (Nitz et al., 2002), is reduced by several drugs like caffeine and modafinil and increased by antihistamines (Hendricks et al., 2000, 2003a; Shaw et al., 2000), indicating the existence of conserved neurochemical mechanisms between fly sleep and its mammal equivalent. This conservation between fly and mammal sleep suggest something might be learned by studying the interactions between sleep and immunity in mammals.

1.5. Sleep and immunity in mammals

Does an immune response affect sleep and does sleep or the deficient thereof, affect the course of an immune response? Several studies have shown that infections as well as low-dose lipopolysaccharide administration increase sleep in humans and other mammals, most likely through induction of proinflammatory cytokines (Bryant et al., 2004). Cytokines are peptide-signaling molecules that are synthesized by immune cells activated by Pathogen-

associated molecular pattern (PAMP) recognition (Majde and Krueger, 2005). It has been shown that several cytokines and their receptors are present in the CNS (Eriksson et al., 2000 and Garden & Muller, 2006). Cytokines may also be synthesized and released in the CNS by both neurons (Breder et al., 1998 and Ignatowski et al., 1997) and glia (Garden & Muller, 2005). Some neurons are immunoreactive for interleukin 1 beta (IL-1B) and tumour necrotic factor alpha (TNFa) both cytokines implicated in the regulation of sleep–wake behavior and located in brain regions (Silver et al., 1996).

Cytokines affect sleep through a neuromodulatory route (release of growth hormone releasing hormone, prostaglandin D2, adenosine, and nitric oxide) and a direct effect on neurotransmitters. Particularly well studied is the action of two immune generated cytokines interleukin 1 beta (IL-1B) and tumour necrotic factor alpha (TNFa) on the seratonin system (Imeri and Opp, 2009).

In mammal's exogenous application of IL1 or TNFα increases sleep, depending on the dose, time of day, and the site of injection (Obal and Krueger, 2003). In addition, loss of either the IL-1 Type I (Fang et al., 1998) or the 55kDa TNF (Fang et al., 1997) receptor in mice reduces baseline sleep during the light hours or during the dark hours, respectively. On an applied level, it was established that neutralizing TNF causes significantly reduced sleepiness in obstructive sleep apnea patients (Vgontzas et al., 2004). In humans, infections with rhinoviruses and, with *Trypanosoma brucei,* decrease sleeping times (Drake et al., 2000 and Buguet et al., 1993). However, rhinoviruses often cause respiratory problems which themselves can affect sleep. These interactions are thought to be mediated by cytokines.

1.6.Sleep and immunity in flies

Given the presence of sleep in flies and their well-studied immune systems (Hoffman, 2007), is there an interaction between them mirroring that in mammals? Flies manually deprived of sleep or transgenic flies with reduced sleep phenotypes both show increases in immune gene transcription and increased resistance to disease (Cirelli et al., 2005, Williams et al., 2007).

The *Drosophila* innate immune response is mediated by two highly conserved pathways, *Imd* and *Toll* signaling pathways. *Relish,* a member of the NFκB family of transcription factors which is most similar to NF κ B or p100 in humans is a central component of the *Imd* pathway, and another NFκB, *Dif* is a central component of the *Toll* pathway (Hoffmann and Reichhart 2002). Both by mutational screens and RNAi, it has been shown that flies deficit in *Relish* expression exhibit reduced sleep (Williams et al., 2007). A preliminary model could be that knocking out *Relish* and the subsequent decrease in sleep shows that Relish is required for sleep. It follows that the sleep deprivation data means *Relish* expression isn't used up in sleep (as the flies aren't sleeping) so more is available for its immune role. But I am interested in the reciprocal interaction, what effect does inducing an immune response has on sleep. From the preliminary model I would suggest that the immune response would use up Relish leading to a reduction in sleep.

Two studies have examined the reciprocal effects that of immunity on sleep in flies. Kuo et al. (2010) injured or infected female flies with gram-negative bacteria *E.coli* and *P. aeruginosa,* and they found an increase in sleep occurred during the morning hours after treatment and the magnitude of the effect was dependent on the time-of-day of inoculation

or injury such that night-time treatment had a stronger effect than that during the daytime. On the contray Shirasu-Hiza et al. (2007) infected male flies with gram-positive bacteria *Streptococcus pneumoniae* or *Listeria monocytogenes* and found that they slept less.

This discrepancy between the two results could be due to them using either male or female flies. Sleep in of *D. melanogaster* is studied in both males and females, but there is a large sex difference between the amounts of sleep experienced during daylight hours, with females sleeping for only 40 per cent of the time that male flies sleep (Huber et al., 2004; Andretic &Shaw, 2005). This sexual dimorphic sleep has been observed in several strains of *D.melanogaster* and probably reflects the greater need of females in a natural environment to stay awake to forage for food and to select sites for egg laying, while males can spend more time conserving energy and avoiding physical and predatory dangers by remaining still.

Another obvious difference between the Kuo and Shirasu-Hiza studies is their choice of immune stimulators. This dependency on the type of pathogen is mirrored in mammalian studies, where the precise effects on sleep depend on the pathogen (bacteria, viruses, fungi or parasites), the host and the route of infection (Imeri and Opp, 2009).

1.7. Sleep affects memory

I have a clear hypothesis that inducing an immune response in flies will lead to them sleeping less. What effect would this have on learning and memory? One of the main proposed hypotheses for sleep function is that sleep periods are favourable for brain plasticity and in the adult brain for learning and memory (Maquet, 2001). In humans and other animals, sleep deprivation causes a significant deficit in hippocampal plasticity

(Ishikawa et al., 2006 & Yoo et al., 2007). Like humans, flies with a fragmented sleep show impaired learning compared with flies with consolidated sleep (Seugnet et al., 2008). Flies also exhibit decreases in learning with 6 and 12 hours of sleep deprivation (Seugnet et al., 2006). Li et al., (2010) demonstrated that 1-day sleep deprivation, but not stress impairs 1-h memory in *Drosophila*, and this effect can persist for at least 2 h in the Pavlovian olfactory conditioning paradigm.

In *Drosophila melanogaster*, several genes involved in sleep regulation has been effectively identified (Hendricks et al., 2000; Shaw et al., 2000). cAMP/ PKA signaling has been classically linked with learning and memory in Drosophila (Dudaí et al., 1983). Several genetic mutants that induce a down regulation in cAMP signaling result in decreased sleep time and, on the contrary, manipulations that increase cAMP signaling increase sleep compared to genetic controls (Hendricks et al., 2001). It has been shown that in the mushroom body a structure that is important for associative processing, the disruption of the cAMP/PKA signaling cascade can strongly modulate sleep time (Joiner et al., 2006).

Cirelli et al., (2005) found that fly mutants for the voltage-dependent potassium channel (*Shaker (Sh)*) sleep only 2–4 hours every day comparing to wild-type controls. In addition genetic mutation for the beta modulatory subunit *Hyperkinetic (Hk)*, which influences *Sh* conductance in flies, also sleep less than controls (Bushey et al., 2007). Using the heat-box assay for testing memory of these flies (Putz and Heisenberg, 2002), flies with mutant alleles for *Sh* or *Hk* that resulted in decreased sleep time also display memory impairments while alleles that did not change sleep had no significant effect on memory performance (Bushey et al., 2007). These results provide evidence that genetic mechanisms that

influence sleep in the fly strongly associated with pathways that are important for learning and memory.

1.8. Sleep, Memory and Immunity

I propose sleep as an intermediate between immunity and memory. There is a link between immunity and sleep and between sleep and memory in *Drosophila*. I predict that an increased immune response will lead to a decrease in sleep, which in turn could explain the decrease in memory abilities of immune stimulated insects. My prediction would agree with Shirasu-Hiza et al. but go against the findings of Kuo. How could this dicotomy arise? Male and female flies are known to sleep differently (Huber et al., 2004). In this chapter I will begin by looking initially at male and female flies. Another obvious difference between the previous studies is the investigator's choice of immune stimulators. In this chapter I overcome this ambiguity by directly stimulating the immune system without parasites. Peptioglycan receptor protein LC (PGRP-LC) is known to lead to expression of the constituent parts of the IMD pathway (Gottar et al., 2002). The IMD pathway leads to the production of antimicrobial peptides that defend against gram negative bacteria (Gottar et al., 2002). Here, I use transgenic fly lines to stimulate an immune response using two different methods, the heatshock pathway and the Geneswitch pathway, thereby removing the confusion caused by using pathogens.

For the heatshocked flies, I also looked at the effect of immune response on sleep during constant darkness (DD) and constant light (LL) lighting regimes. Unfortunately, I did not also have time to do this for Geneswitch flies. DD will allow the flies to free-run, meaning any effect I see should be due to the effect of the immune response on the circadian control

of sleep. The LL experiment will tell us if the effect is on sleep itself or the ability of the flies to be aroused by light. Continuous light and continuous dark regimes become more irregular the longer they run, so as well as testing the whole period, I also tested just the first 24 hours of the continuous period.

Methods and Materials

2.1. Fly stock

hsGAL4 and Geneswitch P{Switch1} lines were obtained from the Bloomington Drosophila Stock Centre at Indiana University (http://flystocks.bio.indiana .edu). The w^{1118} line were obtained from Dr. Howard Lipshitz (Department of Molecular Genetics University of Toronto) and the *UAS–PGRP* line were obtained from Dr. Jean-Marc Reichhart (University of Strasbourg). All 4 lines were maintained in vials containing agar, sugar, and Brewer's yeast media in a 12 h: 12 h light: dark cycle at 25°C.

2.2. Age of Flies

Sleep experiments are quite long, our longest experiment (LL) lasts 360 hours. As the probability of death increases with time (Mair et al., 2003), older flies are less likely to reach the end of the experiments. To reduce variation between experiments and to increase the number of flies alive for the full duration of the recording, I used flies that were 1–3 d old at the beginning of the experiment. As the normal lab life span of fly is 30 days, I would expect most of the flies to still to be alive at the end of the experiment.

2.3. Gender of Flies

In *Drosophila melanogaster*, males and females differ in the profile of their sleep. For instance, under LD 12:12, males show less activity around the times of light transition and a more pronounced ‘siesta’ in the middle of the day (Helfrich-Förster, 2000). Mated females have the added complication that the development of progeny will compromise the detection of activity.

So I sexed the flies by collecting the emerged flies in the morning and collecting the new emerged flies in the afternoon 8 hours later. The new emerged females flies at this time are still virgin. There are several other visible criteria are used to identify the sex of adult *D. melanogaster*. In general, females are larger than male flies; the abdomen of females is often noticeably swollen with maturing eggs and males are more darkly pigmented on the posterior portion of the dorsal side of the abdomen

2.4. Activation of the immune system using Gal4

Several techniques exist in Drosophila that gives both temporal and spatial control over gene expression (McGuire et al. 2004). Many of these approaches are derived from the widely used GAL4-upstream activating sequence (UAS) system adapted from yeast (Brand & Perrimon, 1993; Duffy, 2002). In this system, the transcriptional activator GAL4 is expressed in a spatially restricted pattern using a tissue specific promoter, and the advantage of this system is that the transcriptional activator ("driver") and the UAS-based transgene ("target") are carried in different parental lines, thus ensuring their viability and enabling a combinatorial approach with different driver and target lines to the biological question of interest (Nicholson et al., 2008).

2.4.1. Heatshock

One approach to regulate temporal expression of a UAS-responder is to make use of the yeast protein GAL80, which binds the transactivation domain of GAL4 and prevents GAL4 from activating transcription in yeast (Elliott and Brand, 2008**)**. GAL80 can also repress GAL4 in *Drosophila***,** and when expressed ubiquitously under the control of the *tubulin 1*α promoter, represses GAL4 activity in all tissues. GAL80 repression of GAL4 is alleviated

by a simple temperature shift, giving the researcher exact control of the onset of expression. See Figure 4.1

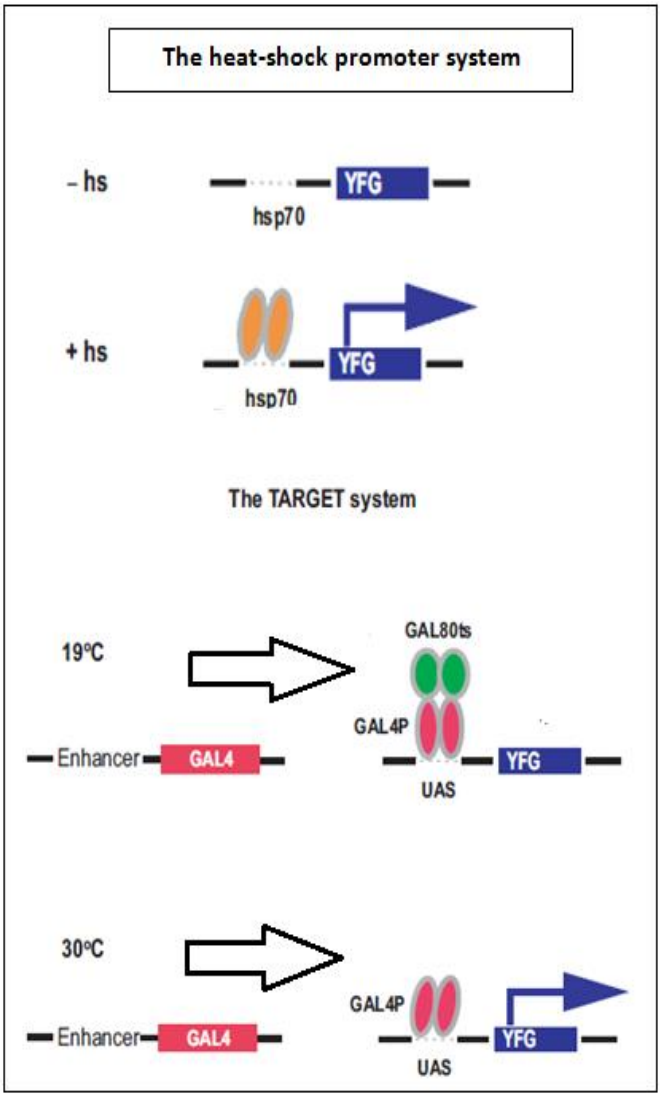


Figure 4.1

The heatshock promoter, GAL4-UAS systems. In this system, a copy of your favorite gene (YFG) is cloned downstream of the *hsp70* heatshock promoter. The expression of YFG is induced ubiquitously by exposure of the fly to a heatshock regimen. In the TARGET system, conventional GAL4-UAS system is conditionally regulated by a temperature-sensitive allele of GAL80. At 19°C, transcription of YFG is repressed, whereas this repression is relieved by a temperature shift to 30°C, leading to high levels of expression of YFG in a specific tissue. Adapted from (McGuire et al., 2004).

2.4.2. Geneswitch

GAL4 activates the expression of transgenes under UAS control in tissues that express GAL4. Several newer gene expression systems were developed, adding temporal control to the GAL4-UAS expression system to control the timing of transgene expression (Han et al. 2000; Osterwalder et al. 2001; Roman et al. 2001; Mcguire et al. 2003). One of these methods is the use modified steroid activated versions of GAL4 to achieve inducible expression. Steroid-activated chimeric GAL4 proteins have been independently developed by three different groups (Han et al. 2000; Osterwalder et al. 2001; Roman et al. 2001). Each protein combines the GAL4 DNA binding domain with a steroid hormone receptor transcriptional activation domain, which requires ligand binding to become transcriptionally active. In the Geneswitch system, the binding domain of the GAL4 protein is fused to the p65 activation domain and a mutant progesterone receptor ligand binding domain to generate ligand-stimulated chimeric activators. In the absence of hormone, the Geneswitch is in the "off" state. In the presence of hormone, the Geneswitch molecule changes to an active form in which it can bind to a UAS sequence and activate transcription of a transgene. The antiprogestin, RU486, the chimeric molecule binds to a UAS and provides for ligand-inducible transactivation of downstream target genes, see Figure 4.2. The steroid ligand can be administered by either feeding or immersing the animals in a steroid solution. Transgene expression can be detectable after 3–5 hr using Geneswitch and maximal expression is reached in 21–48 hr (Han et al. 2000; Osterwalder et al. 2001; Roman et al. 2001).

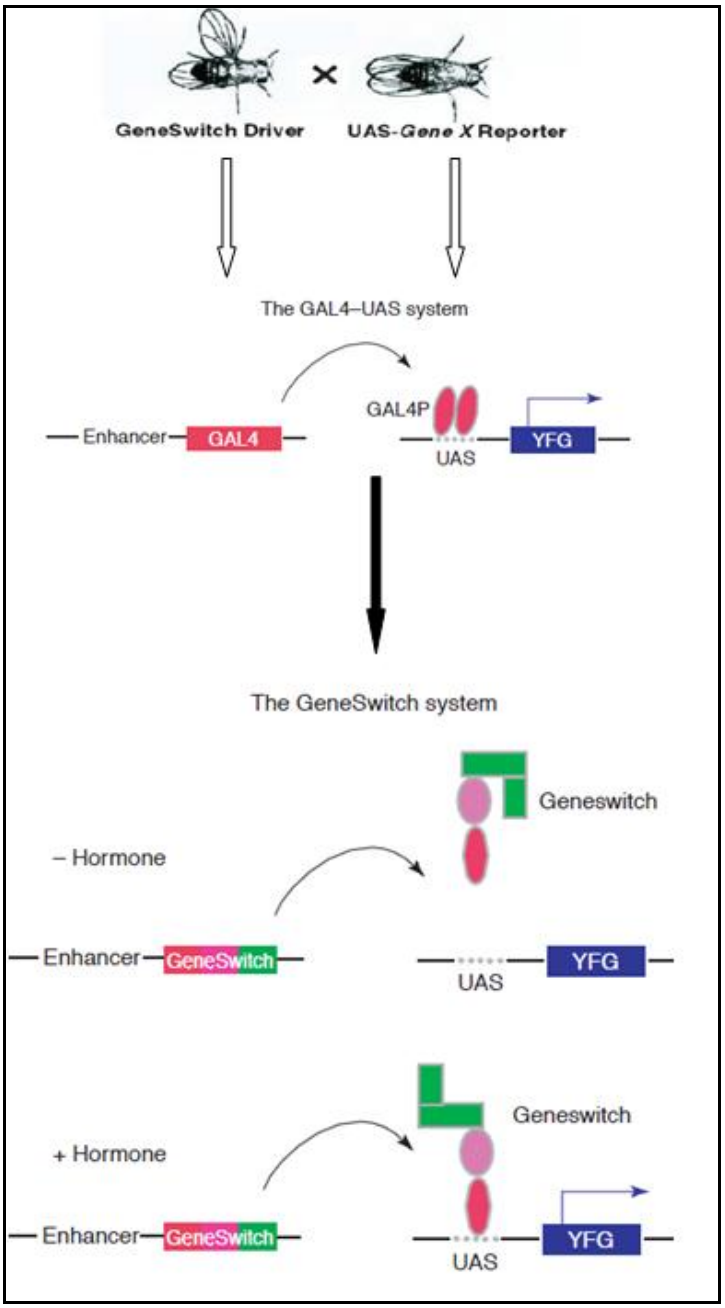


Figure 4.2

The Geneswitch UAS expression system in *Drosophila* Driver lines expressing the transcriptional activator Geneswitch in a tissue-specific fashion are crossed to UAS-reporter lines with genomic inserts of a target gene fused to GAL4-binding sites. In the absence of an activator, the Geneswitch protein is expressed in target tissues but remains transcriptionally silent. No expression of downstream UAS-linked genes therefore occurs. However, after systemic application of RU486 (induced) the Geneswitch protein becomes transcriptionally active, resulting in expression of UAS-linked genes. Adapted from (Osterwalder et al. 2001).

2.4.3. Experimental Genotypes

Both the Geneswitch and heatshock experiments use 3 genotypes each. The first genotype is the GAL4-UAS-PGRP cross. Heatshocking or feeding RU486 (Geneswitch) to this genotype will lead to the expression of peptioglycan receptor protein LC (PGRP-LC), which is a pattern recognition protein for the ImD pathway. That is, its expression will lead to the upregulation of several antimicrobial peptides (Gottar et al., 2002). The other two genotypes are both controls for genetic background. Heatshock/RU486 on these lines should not lead to the expression of immune genes.

For heatshock the three genotypes were the immune activated genotype (1) hsGal4 UAS-PGRP, and two control genotypes, (2) w^{1118} UAS-PGRP and (3) hsGAL4 w^{1118}.

(1) ♀ **(vg)** UAS-PGRP **x** ♂ hsGal4 (immune activated)

(2) ♀ **(vg)** UAS-PGRP **x** ♂ w^{1118} (control)

(3) ♀ **(vg)** W^{1118} **x** ♂ hsGal4 (control)

To heatshock the flies, they were put in the incubator at 37°C for an hour followed by two hours rest at room temperature. This was repeated three times.

2.4.4. Geneswitch

The Geneswitch system relies on a GAL4-progesterone receptor chimera activated by the ligands of progesterone receptor and expresses in adult fat body. See figure 4.2

For Geneswitch experiments the three genotypes were the immune activated genotype (1) Geneswitch gal4 UAS-PGRP, and two control genotypes, (2) Geneswitch gal4 w^{1118} and (3) w^{1118} UAS-PGRP.

(1) ♀ **(vg)** UAS-PGRP **x** ♂ Geneswitch gal4 (immune activated)

(2) ♀ **(vg)** W^{1118} **x** ♂ Geneswitch gal4 (control)

(3) ♀ **(vg)** UAS-PGRP **x** ♂ W^{1118} (control).

I made a 10mM stock solution of Mifepristone (RU486, Sigma Aldrich) (0.13 g of RU486 in 32 ml of 80% ethanol). This solution can be stored at 4°C for a few months as stock solution. I mixed 200 ml of RU486 stock solution with 980 ml molten *Drosophila* food. This ratio (1:49) makes a 200µM RU486 solution. This was poured immediately into food vials. Flies were put in these vials for two days for feeding. After two days flies were immediately load into DAMS tubes containing the RU486 food. Non-activated flies were obtained using genetically identical animals cultured on standard fly medium that lacked RU486. Food containing RU486 can be stored at 4°C for several weeks (McGuire et al., 2004).

2.5.Behavioural assay

For sleep studies, individual flies (males and females) on the day of eclosion were transferred to monitor tubes containing the media, described above. Their locomotors activity was monitored by the *Drosophila* Activity Monitoring System (DAMS) (Trikinetics, Waltham, MA), see Figure 4.3, at 25°C, continuously for different amounts of time depending on the availability of the monitoring system. By introducing an infrared beam into the path of the fly, a computer can count every time that the beam is broken, giving a measure of the pattern of activity and rest of the fly. The experimental setup consists of a number of activity monitors connected to a computer and housed in a light and temperature-controlled incubator in a quiet room. A single activity monitor consists of

several channels, each one having one or two pairs of infrared emitters and receivers positioned alongside a small (5 mm diameter, 80 mm length) glass tube, with the associated electronic components necessary for converting analog information (a reduction in the infrared light reaching the receiver) into binary data. Activity counts are stored in a computer, usually grouped in 30 min bins, although shorter or longer time bins are possible. Sharper time resolution is particularly important for the emerging field of sleep research in Drosophila. Physiological, pharmacological and behavioural assays have shown that uninterrupted inactivity lasting 5 min or more corresponds, in flies, to a sleep-like state (Rosato and Kyriacou, 2006). Measuring activity in 1 min bins offers a convenient way for estimating the amount of daily sleep in wild-type and mutant strains.

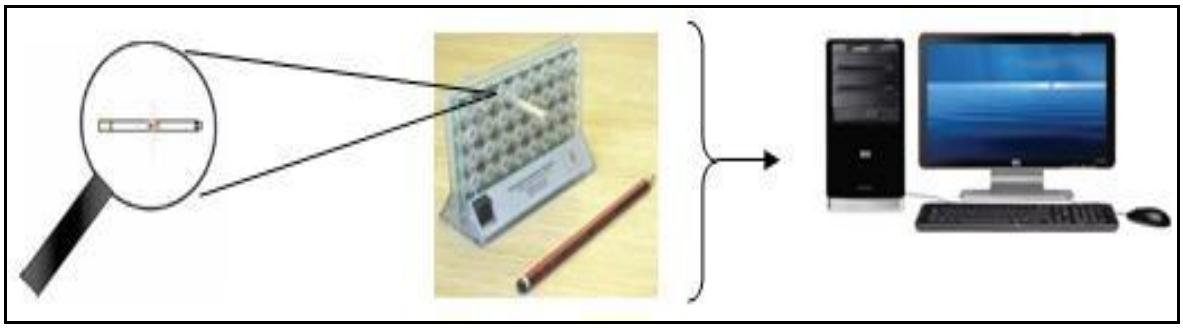


Figure 4.3

Setup for sleep analysis, Individual flies is inserted in small glass tubes. These are loaded in activity monitors (green boxes) housed in a light- and temperature-controlled incubator and connected to a computer. The activity monitors contain the infrared emitters and receivers and the associated electronic components. When a fly breaks the infrared beam across its path, the event is recorded by the computer. (Modified from (Rosato and Kyriacou, 2006).

2.6.Statistical analysis

The DAMS measures only one thing, when a fly crosses an infrared beam in the middle of the tube. This is outputted as the number of crossings per minute. Using an excel plugin, Befly designed by Ed Green (Department of Genetics, Leicester), I converted this output to

three measures. 1) Sleepbins per hour: five minutes of continuous inactivity is considered sleep, so six minutes of inactivity is six minutes of sleep whereas four minutes of inactivity followed by activity is considered no sleep. The maximum value of this measure is, of course, 60 minutes per hour. 2) Mean waking activity: the mean of the number of times a fly crosses the beam in 1 minute bins that are classified as waking, i.e. not part of at least a five minute period where there is no activity. If my treatments have an effect here, it suggests that any effect found on sleep was actually just due to a general decrease in activity. 3) Bouts of sleep: This is the mean of the number of sleep bouts the flies have in a given hour. If all the flies of a given group slept for the whole hour the value of this measure would be 1. This measure gives us an idea of how interrupted the sleep was. The more bouts, the more interrupted the sleep.

The above are my dependent variables. Independent variables are time, sex, genotype (immune stimulated, 2 controls), treatment (heatshock: yes/no **OR** Geneswitch:yes/no). Whether it was day or night could also be included as an independent variable, as it is known flies sleep differently during the day and night. Day/night and time could not be included simply in the same analysis due to problems of colinearity. Time could have been nested in Day/Night. Time is also a repeated measure. Time and Day/Night are potential sources of variation and are included in the ANOVAs so that this variation can be accounted for. Treatment and Genotype are main effect variables but what is really of interest is their interaction. Heatshocking or feeding RU486 (Geneswitch) to this genotype will lead to the expression of peptioglycan receptor protein LC (PGRP-LC), which is a pattern recognition protein for the ImD pathway. That is, its expression will lead to the upregulation of several antimicrobial peptides (Gottar et al., 2002). The other two

genotypes are both controls for genetic background. Heatshock/RU486 on these lines should not lead to the expression of immune genes.

So I have 3 dependent variables and 4 (plus 1 nested repeated measure) independent variables. The ideal analysis would begin with a 4-way (plus 1 nested repeated measure) MANOVA with 3 dependent variables and then if this was significant carry out the respective ANOVAs. In the original version of the thesis I instead carried out an ANOVA for each dependent variable with time (repeated) and treatment as the independent variables. This was replicated for both day and night, each sex and each genotype. A bonferroni correction was applied to compensate for the multiple comparisons. This inelegant analysis was carried out due to an inability to calculate the between subjects error (B.S.E.) term for the more complicated ANOVAs.

A new analysis has been carried out on all data for this current version of the thesis. A MANOVA was not carried out due to the B.S.E. problem. Also it could be argued that my three dependent variables are just different constructions of the same basic data, as outputted by DAMS. This would lead to an incorrect MANOVA. So instead ANOVAs were carried out for each of the three dependent variables. Again due to the B.S.E. problem I could not run ANOVAs with time as a repeated measure nested inside day/night. Instead my analysis is as follows. For each dependent variable I ran two ANOVAs one for day and one for night. The independent variables were time (repeated measure), sex (some experiments use only males so this factor is not included), genotype and treatment. There was an interaction terms sex*genotype*treatment.The B.S.E. was sex*treatment*genotype. Time in and of itself is not a very interesting variable and is just there as an ANOVA without it would fail to take in to consideration the correlation of each time point, that is

that it is a repeated measure. If the three-way interaction term was significant I then carried out 4 ANOVAs by day and sex. The independent variables here would be time (repeated measure), genotype and treatment. The important term here is an interaction term between genotype and treatment. If this was significant, I could say the genotypes responded differently to the treatments. But I still don't know which genotype's response is different from which. It is quite possible that the two control genotypes' responses to treatment are different from each other but not statistically different from that of the immune stimulated genotype. This would be uninteresting. Therefore if the interaction term was significant here, I repeated this analysis twice, once for genotype 1 and 3 and once for genotype 1 and 2. If the interaction terms in both these ANOVAs are significant I can say that genotype 1 (the immune stimulated genotype) responses differently to the control genotypes for that given sex and in either day or night. From looking at the respective means graph I can tell whether it increases or decreases relative to controls. Finally looking at the respective 2-way ANOVA with time (repeated) and treatment for the given day night, sex and genotype will tell me if that increase or decrease is statistically significant. Please see table 4-1. Although somewhat complicated this is much more powerful than the original analysis and only requires a bonferroni correction with α set to 0.025, compensating for the two ANOVAs, one for day and one for night. For the three way ANOVAs we must decrease α to 0.0083 (0.05/6) as we have Day/Night and three ANOVAs one for all the data and one each for genotype 1, 2 and 1, 3. See Table 4.1 for an outline.

ANOVA	**Potential Number of ANOVAs**	**Model**	**By**	**Critical term**
4-way	2	Time, Treatment, Genotype, Sex, Sex* Treatment* Genotype	Day/Night	Sex* Treatment* Genotype
3-way	4	Time, Treatment, Genotype, Treatment* Genotype	Day/Night, Sex	Treatment* Genotype
3-way (1vs2)	4	Time, Treatment, Genotype, Treatment* Genotype	Day/Night, Sex, just for genotype 1 vs 2	Treatment* Genotype
3-way (1vs3)	4	Time, Treatment, Genotype, Treatment* Genotype	Day/Night, Sex, just for genotype 1 vs 3	Treatment* Genotype
2-way (equivalent to original analysis)	12	Time, Treatment	Day/Night, Sex, Genotype	Treatment

Table 4-1

Outline of statistical analysis used in this chapter. The ANOVAs were repeated for each level of the variables shown in the 'By' column. After completing the ANOVA on a given row, I only did the next row if the critical term was significant.

To help interpretation of the mean graphs (Figures 4.4B and 4.5B), I also calculated the % relative change caused by RU486 for genotype 1.

relative change caused by RU486 for genotype 1. First I calculated the % change in the dependent variable caused by feeding on RU486, as the

$$\frac{value(treatment{:}yes) - value(treatment{:}no)}{value(treatment{:}no)} X100$$

% relative change for genotype 1 was

%change (genotype1) – mean (%change for genotype 2 and 3). Although useful when both the controls responded similarly, this measure is difficult to interpret when the controls move in opposite directions.

Results

All the analyses detailed in the Methods were carried out. However only the 3-way ANOVAs split over genotypes 1 and 2 and genotypes 1 and 3 (or the higher level analysis if this higher level was shown to be not significant) is reported. The final level (2-way ANOVA) was found to be uninformative as it didn't give any extra information, not provided higher up. At the end of the results is a summary table of all the results (Table 4.6).

3.1.Light:Dark Condition

I analyzed each dependent variable separately, sleep bins, mean waking and finally sleep bouts. Immune stimulated males sleep more during the day (see Table 4.2 and Figure 4.4) and less during the night (Table 4.2 and Figure 4.4). There was no equivalent effect on females (Table 4.2). Immune stimulated male flies during both the day and night and female flies during the day show decreased mean waking activity (Table 4.2 and Figure 4.5). Immune flies have a reduced number of sleep bouts during the night (Table 4.2)

Table 4.2 details the F-statistics for all variables including nonsignificant results not presented above.

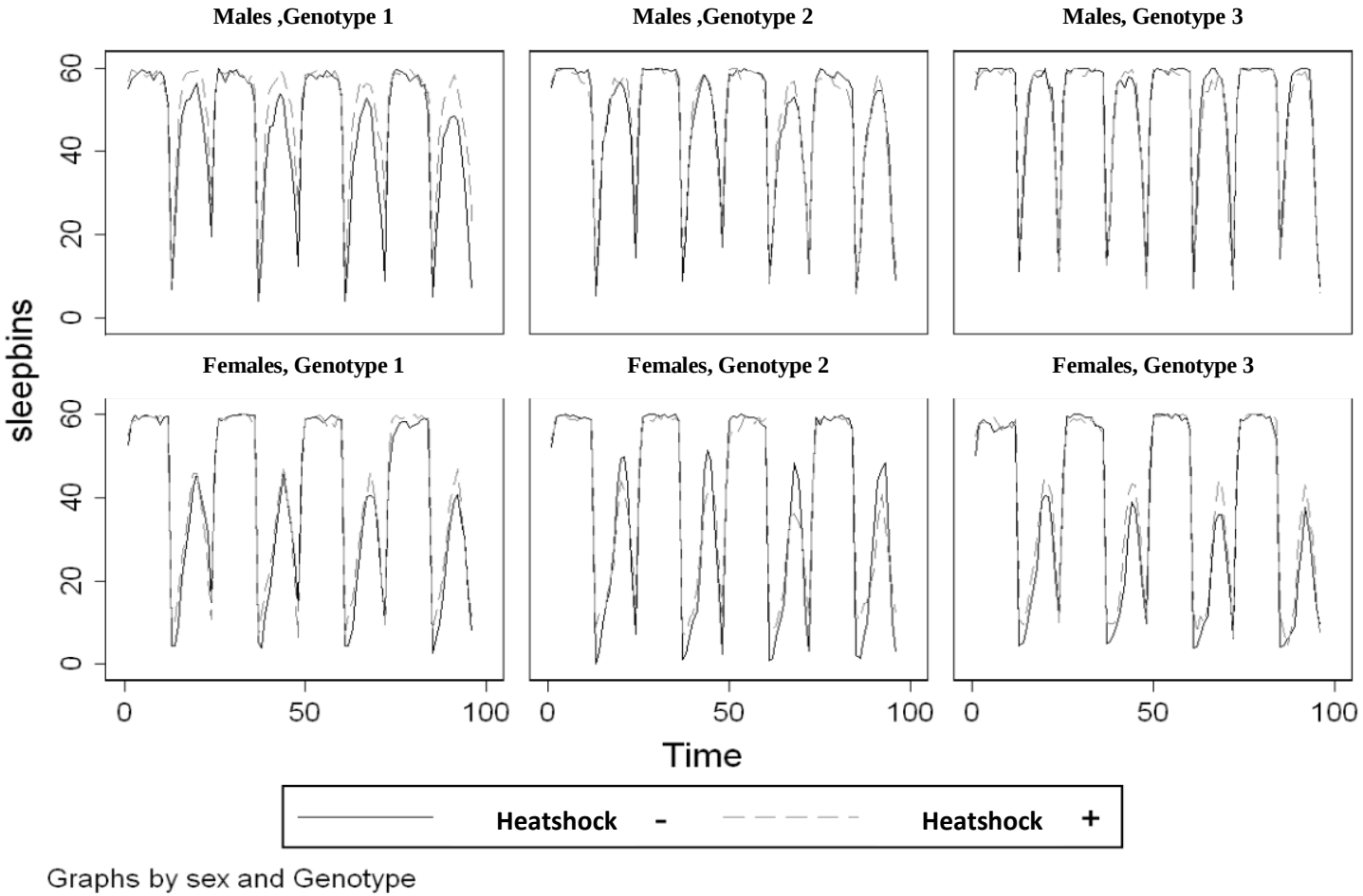


Figure 4.4A

Total sleep bins per hour for all three genotypes1,2 and 3. The total sleep bins for males and females of genotype 1 hsgal4 X UAS-PGRP, genotype 2 w^{1118} X UAS-PGRP, and the control genotype3 hsgal4 X w^{1118}. The experiment began in the dark, so time 1-12 is night and 13-24 is day and so on.

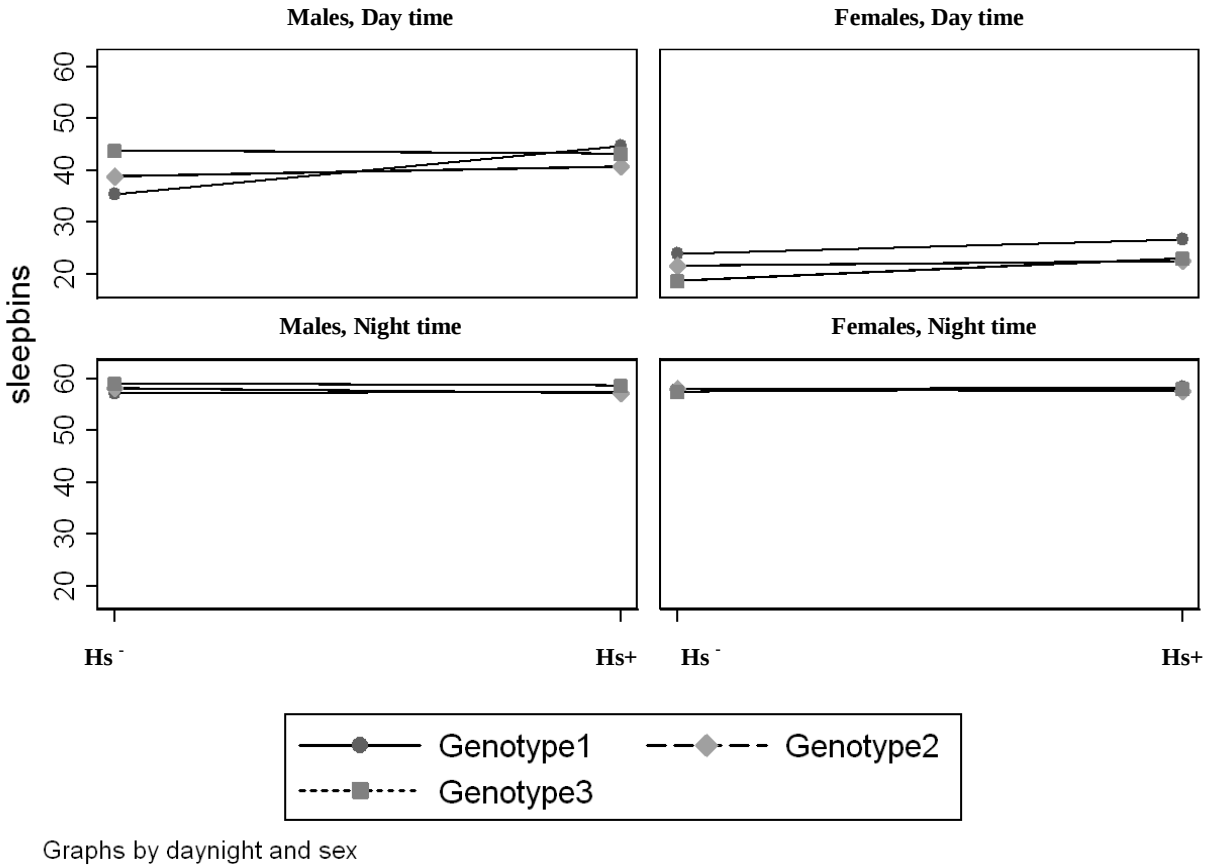


Figure 4.4B

Means of total sleep bins per hour for all three genotypes (1) hsgal4 X UAS-PGRP, (2) w^{1118} X UAS-PGRP and (3) hsgal4 X w^{1118} during the day and during the night, and the means of the total sleep bins for the females of the three genotypes 1, 2 and 3 during the day and during the night

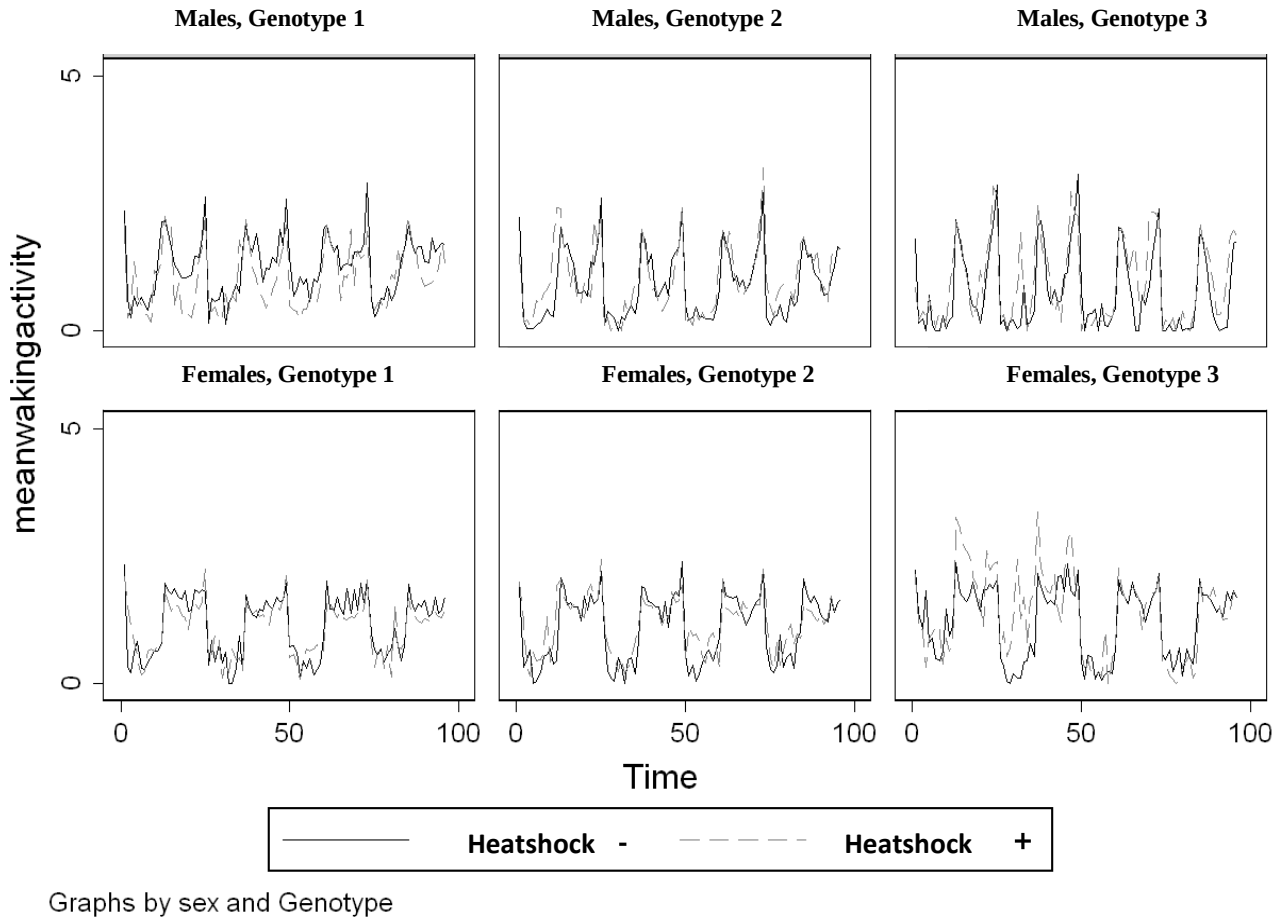


Figure 4.5A

Total mean waking activity per hour for all three genotypes 1, 2 and 3. The total mean waking activity for males and females of genotype 1 hsgal4 X UAS-PGRP, the control genotype 2 w^{1118} X UAS-PGRP, and the control genotype 3 hsgal4 X w^{1118}. The experiment began in the dark, so time 1-12 is night and 13-24 is day and so on.

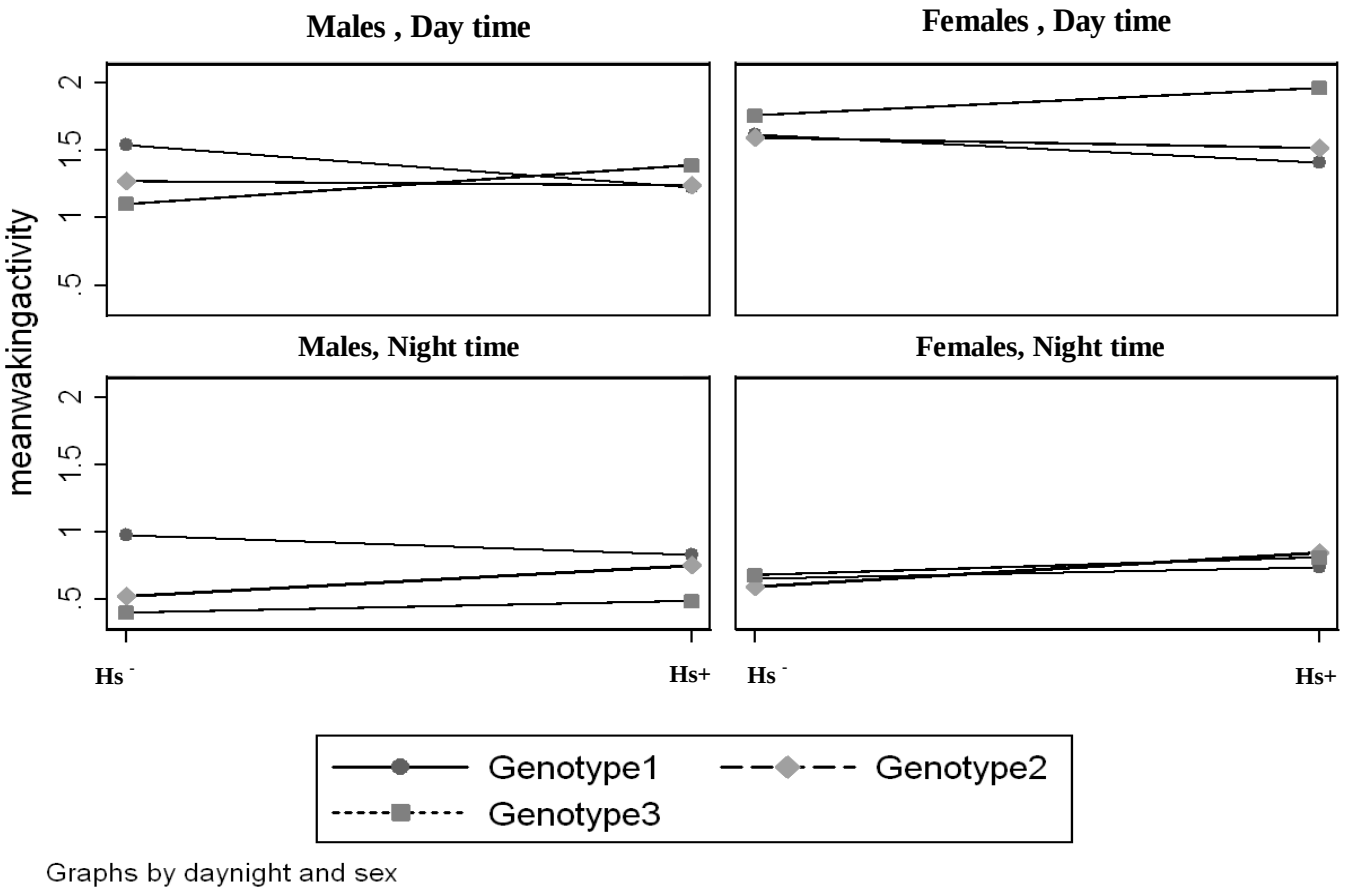


Figure 4.5B

Means of total mean waking activity per hour for all three genotypes1,2 and 3. The means of the total mean waking activity for males of the three genotypes 1 hsgal4 X UAS-PGRP, 2 w^{1118} X UAS-PGRP and 3 hsgal4 X w^{1118} during the day and during the night. And the means of the total mean waking activity for the females of the three genotypes 1, 2 and 3 during the day and night.

	Male						**Female**					
	Day			Night			Day			Night		
	Change	1,2	1,3	Change	1,2	1,3	Change	1,2	1,3	Change	1,2	1,3
Sleep bins	↑	1,3743 54.88 <0.00001	1,3791 121.17 <0.00001	↓	1,3743 14.32 0.0002	1,3791 8.04 0.0046	-	2,5951 4.80 0.0083		-	1,4301 6.79 0.0092	1,3791 0.29 0.5901
Waking activity	↓	1,3743 20.98 <0.00001	1,3791 60.79 <0.00001	↓	1,3743 18.01 <0.00001	1,3791 8.04 0.0046	↓	1,4031 7.51 0.0061	1,3791 33.36 <0.00001	-	2,5951 1.53 0.2160	
Sleep bouts	-	1,3743 0.54 0.4604	1,3791 17.21 <0.00001	↓	1,3743 16.60 <0.00001	1,3791 17.33 <0.00001	-	2,5951 1.31 0.2699		-	2,5951 4.38 0.0125	

Table 4-2

Heatshocked LD experiment: Summary of the of the effects of the dependent variables. First title row is sex of flies tested. Second title row is whether it was day or night. Third title row displays if there was a significant effect (Heading=Change), associated with this are the ANOVA outputs for genotype 1, 2 (Heading = 1, 2) or genotype 1, 3 (Heading = 1, 3). ↑ means there was a significant increase. ↓ means there was a significant decrease. – means there was no significant change. Within a statistics cell the first row is the degrees of freedom, the second is the F-statistic and the third row is the p value. If the null hypothesis was accepted at the 3-way ANOVA (see Table 4.1), I merged the appropriate statistic cells.

3.2.Light: Light Condition:

In Light: Light condition I also analyzed each dependent variable separately, sleep bins, mean waking and finally sleep bouts, at three different conditions, Light/Dark part, Light/Light for the first 24 hours part and whole Light/Light part.

The only significant result is an increase in mean waking activity in immune stimulated males when looked at over the whole of the LL phase (see Table 4.3).

Table 4.3 details the F-statistics for all variables including nonsignificant results not presented above.

	LD						24 LL						Whole LL					
	Day			Night			Day			Night			Day			Night		
	Change	1,2	1,3	Change	1,2	1,3	Change	1,2	1,3	Change	1,2	1,3	Change	1,2	1,3	Change	1,2	1,3
Sleep bins	-	2,10751 2.00 0.1358		-	1,7199 2.90 0.0885	1,13583 0.20 0.6574	-	1,1799 4.27 0.0388	1,10919 8.23 0.0041	-	1,1799 0.17 0.6797	1,10919 12.32 0.0005	-	1,1259 0.60 0.4395	1,1358 2.97 0.0848	-	1,1259 6.80 0.0091	1,1358 0.2 0.6574
Waking activity	-	1,6359 8.58 0.0034	1,1109 1.15 0.2838	↑	1,6359 14.93 0.4395	1,11099 31.50 <0.00001	-	2,2255 4.56 0.0106		-	1,1487 0.02 0.8999	1,8663 20.26 <0.00001	↑	1,9599 9.15 0.0025	1,12467 28.15 <0.00001	-	1,9599 0.51 0.4769	1,12467 40.88 <0.00001
Sleep bouts	-	2,10751 0.63 0.5326		↑	1,7199 12.78 <0.00001	1,1358 25.59 0.5326	-	1,1799 3.54 0.0601	1,10919 6.53 0.0601	-	1,1799 2.98 0.0844	1,10919 4.04 0.0445	-	1,12599 0.91 0.3399	1,16247 58.59 <0.00001	-	1,12599 0.97 0.3252	1,16247 31.85 <0.00001

Table 4-3

Heatshocked LL experiment: Summary of the effect of the dependent variables. First title row is the condition status of flies tested. Second title row is whether it was day or night. Third title row displays if there was a significant effect (Heading=Change), associated with this are the ANOVA outputs for genotype 1, 2 (Heading = 1, 2) or genotype 1, 3 (Heading = 1, 3). ↑ means there was a significant increase. ↓ means there was a significant decrease. – means there was no significant change. Within a statistics cell the first row is the degrees of freedom, the second is the F-statistic and the third row is the p value. If the null hypothesis was accepted at the 3-way ANOVA (see Table 4.1), I merged the appropriate statistic cells.

3.3.Dark: Dark Condition:

In Dark: Dark condition I did the same as previously in the Light/ Light part. I analyzed each dependent variable separately, sleep bins, mean waking and finally sleep bouts, at three different conditions, Light/Dark part, Dark/Dark for the first 24 hours part and whole Dark/Dark part. There were no significant effects in Dark/Dark condition. Table 4.4 details the effects of all variables, and the statistics for all the dependent variable.

	LD						24 DD						Whole DD					
	Day			Night			Day			Night			Day			Night		
	Change	1,2	1,3	Change	1,2	1,3	Change	1,2	1,3	Change	1,2	1,3	Change	1,2	1,3	Change	1,2	1,3
Sleep bins	-	1,5759 53.77 <.00001	1,13679 6.31 0.0120	-	1,5759 12.40 0.0004	1,13679 5.44 0.0197	-	1,1799 4.27 0.0388	1,10919 8.23 0.0041	-	1,1799 0.17 0.6797	1,10919 12.32 0.0005	-	1,12599 0.60 0.4395	1,13583 2.97 0.0848	-	1,12599 6.80 0.0091	1,13583 0.2 0.6574
Waking activity	-	1,6359 8.58 0.0034	1,11099 1.15 0.2838	↑	1,6259 14.93 0.4395	1,11099 2.97 <.00001	-	2,2255 4.56 0.0106		-	1,1487 0.02 0.8999	1,8663 20.26 <0.00001	-	1,9599 9.15 0.0025	1.12467 28.15 <0.00001	-	1,9599 0.51 0.4769	1,12467 40.88 <0.00001
Sleep bouts	-	2,10751 0.63 0.5326		↑	1,7199 12.78 =0.0004	1,13583 25.59 <.00001	-	1,1799 3.54 0.0601	1.10919 6.53 0.0106	-	1,1799 2.98 0.0844	1,10919 4.04 0.0445	-	1,12599 0.91 0.3399	1,16247 31.85 <0.00001	-	1,12599 0.97 0.3252	1,16247 31.85 <0.00001

Table 4-4

Heatshocked DD experiment: Summary of the effect of the dependent variables. First title row is the condition status of flies tested. Second title row is whether it was day or night. Third title row displays if there was a significant effect (Heading=Change), associated with this are the ANOVA outputs for genotype 1, 2 (Heading = 1, 2) or genotype 1, 3 (Heading = 1, 3). ↑ means there was a significant increase. ↓ means there was a significant decrease. – means there was no significant change. Within a statistics cell the first row is the degrees of freedom, the second is the F-statistic and the third row is the p value. If the null hypothesis was accepted at the 3-way ANOVA (see Table 4.1), I merged the appropriate statistic cells.

3.4. Geneswitch:

In the Geneswitch part I analyzed each dependent variable separately, sleep bins, mean waking and finally sleep bouts for both males and females at only Light/ Dark condition only. I also analysed females during the first 24 hours to try to compensate for nonvirginity. There is a decrease in sleep both during day and night in immune stimulated male flies (Table 4.5 and Figure 4.6). In immune stimulated females there is an increase in sleep during the day when examined over the whole of the LD phase (Table 4.5 and Figure 4.6). There was no change in mean making activity in immune stimulated males (Table 4.5). Table 4.5 details the effects of all variables including nonsignificant ones not presented above.

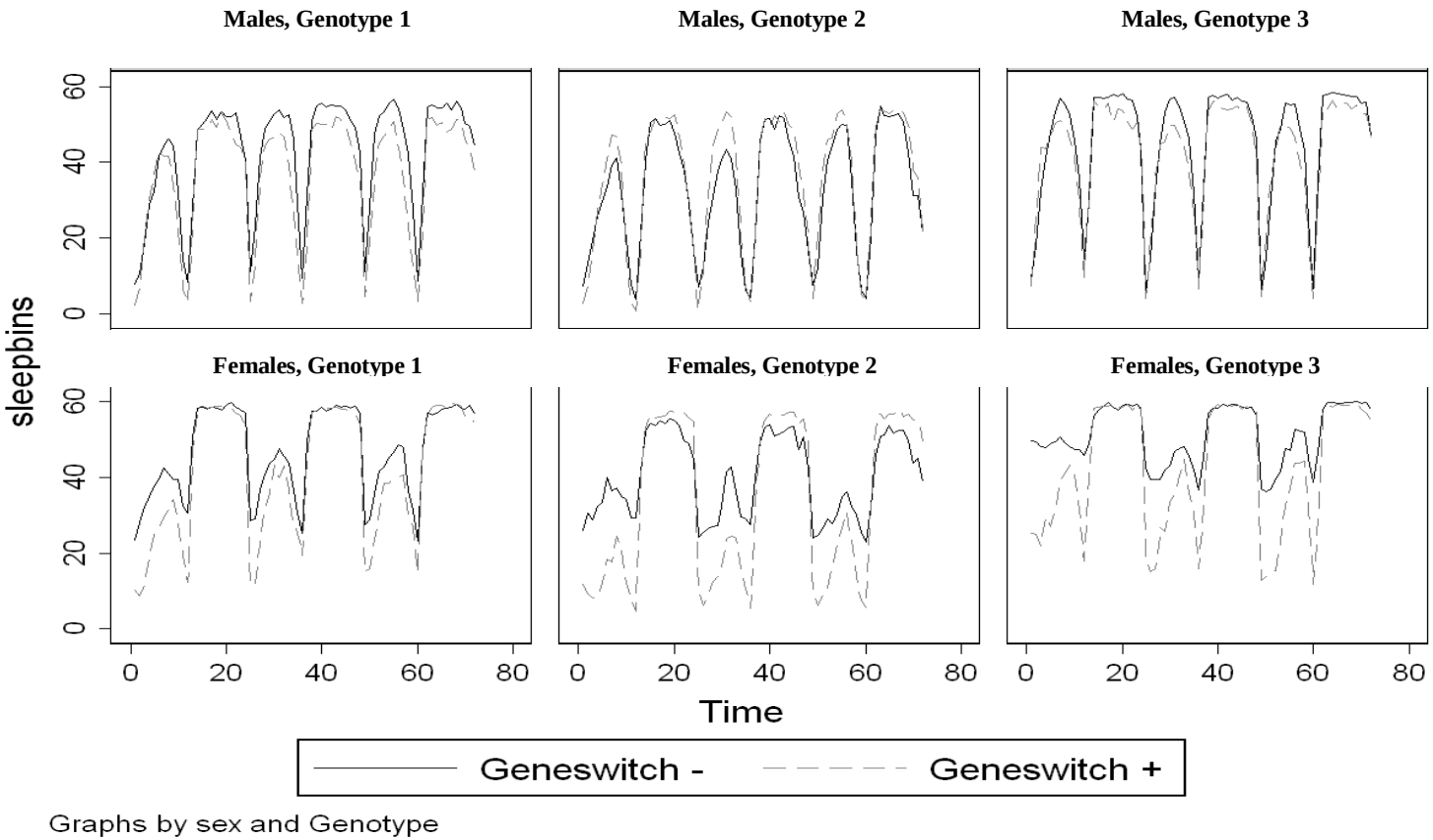


Figure 4.6A

Total sleep bins per hour for all three genotypes. The total sleep bins for males and females of genotype 1 gene switch gal4 X UAS-PGRP. and the total sleep bins for males and females of the control genotype 2 w^{1118} X UAS-PGRP. And, the total sleep bins for the control genotype 3 gene switch gal4 X w^{1118}. The experiment began in the dark, so time 1-12 is night and 13-24 is day and so on.

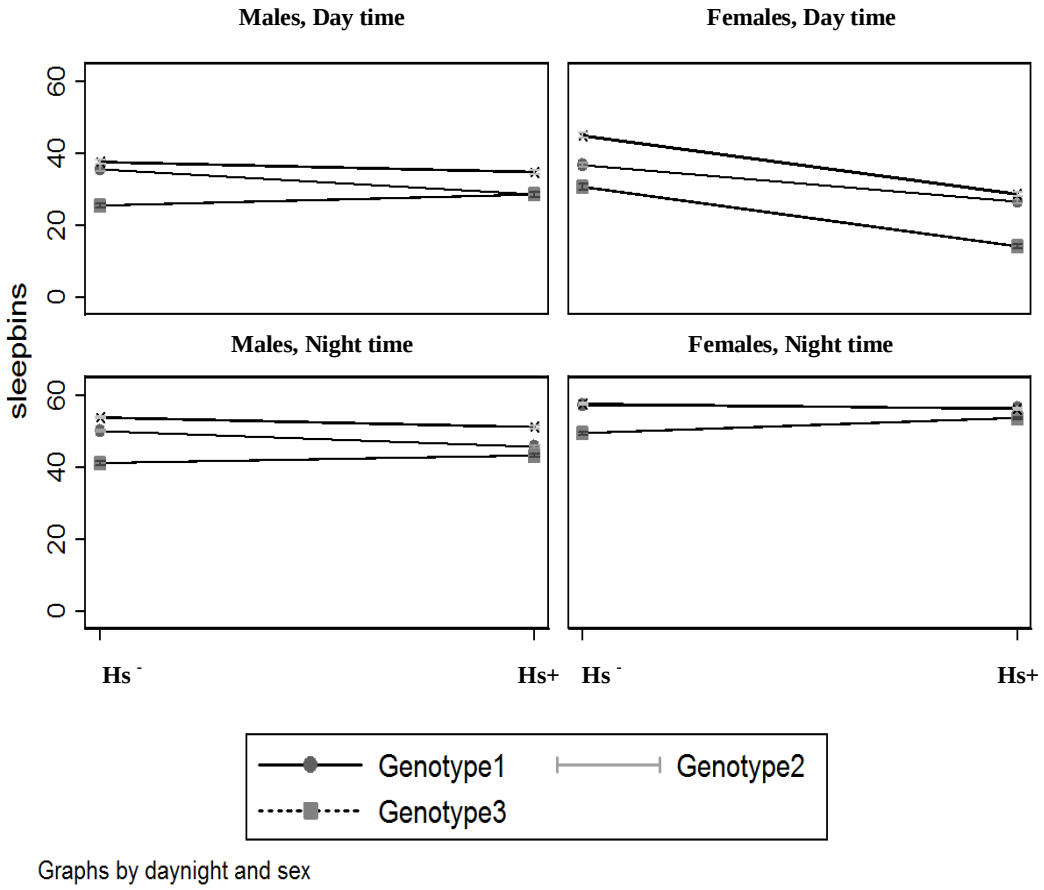


Figure 4.6 B

Means of total sleep bins per hour for all three genotypes 1, 2 and 3. the means of the total sleep bins for males of the three genotypes 1 gene switch gal4 X UAS-PGRP, 2 w^{1118} X UAS-PGRP and 3 gene switch gal4 X w^{1118} during the day. and during the night and the means of the total sleep bins for the females of the three genotypes 1, 2 and 3 during the day and during the night .

	Male						**24 Female**						**Whole Female**					
	Day			Night			Day			Night			Day			Night		
	Change	1,2	1,3	Change	1,2	1,3	Change	1,2	1,3	Change	1,2	1,3	Change	1,2	1,3	Change	1,2	1,3
Sleep bins	↓	1,4607 136.29 <0.00001	1,4533 26.87 <0.00001	↓	1,4607 85.53 <0.00001	1,4535 8.49 0.0036	-	2,2327 4.03 0.018		-	1,1545 0.1 0.7515		↑	1,4679 26.89 <0.00001	1,4679 24.34 <0.00001	-	1,4679 73.11 <0.00001	1,4679 3.63 0.0569
Waking activity	-	2,6839 0.5 0.6044		-	1,4607 63.34 <0.00001	1,45 35 1.96	↓	1,1559 65.51 <0.00001	1,1559 58.33 <0.00001	-	2,2311 1.96 0.1407		↓	1,4679 225.56 <0.00001	1,4641 203.89 <0.00001	-	2,6839 1.14 0.3192	
Sleep bouts	-	1,4607 6.42 0.0113	1,4535 10.43 0.0012	↑	1,4607 16.38 <0.00001	1,4535 7.56 0.0060	-	1,1559 0.48 0.4878	1,1559 55.91 <0.00001	-	2,2327 1.45 0.2341		-	1,4679 6.97 0.0083	1.4679 108.99 <0.00001	-	2,6839 2.83 0.0591	

Table 4-5

LD Geneswitch experiment: Summary of the effect of the dependent variables. First title row is sex of flies tested. Second title row is whether it was day or night. Third title row displays if there was a significant effect (Heading=Change), associated with this are the ANOVA outputs for genotype 1, 2 (Heading = 1, 2) or genotype 1, 3 (Heading = 1, 3). ↑ means there was a significant increase. ↓ means there was a significant decrease. – means there was no significant change. Within a statistics cell the first row is the degrees of freedom, the second is the F-statistic and the third row is the p value. If the null hypothesis was accepted at the 3-way ANOVA (see Table 4.1), I merged the appropriate statistic cells.

	Heatshock																***Geneswitch***					
	M														*F*		*M*		*F*			
	LD		*LL*						*DD*						*LD*		*LD*		*LD*			
	LD		LD		First 24h LL		Whole LL		LD		First 24h DD		Whole DD		LD		LD		First 24h LD		LD	
	D	N	D	N	D	N	D	N	D	N	D	N	D	N	D	N	D	N	D	N	D	N
Sleep bins	↑	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓	↓	-	-	↑	-
Waking activity	↓	↓	-	↑	-	-	↑	-	-	↑	-	-	-	-	↓	-	-	-	↓	-	↓	-
Sleep bouts	-	↓	-	↑	-	-	-	-	-	↑	-	-	-	-	-	-	-	↑	-	-	-	-

Table 4-6

Summary of results for all sleep experiments. The first title row is whether it was a heatshock or geneswitch experiment. The second title row is the sex. Next is what lighting regime experiment it was. The fourth title row is what proportion of the data is being analysed. The final title row is whether it was day or night. ↑ means there was a significant increase due to the immune response. ↓ means there was a significant decrease. – means there was no change.

Discussion

My initial hypothesis was that an immune response would lead to a reduction in sleep, presumably followed by a decrease in learning ability. I found this reduction in sleep when the immune system was stimulated using the geneswitch system in males. Using the heatshock system, it seemed rather that males slept more during the day. The increase in sleep in the heatshocked males was accompanied by a decrease in waking activity, suggesting that the effect was not specific to sleep. This was not the case in males stimulated using the geneswitch system, there was no change in activity. This is strong evidence that stimulating the immune system using the geneswitch system lead to a specific effect on sleep.

Heatshock causes genotype 1 males to sleep more during the day. Although the LD phases of the LL and DD experiment are not significant, this is only because of the conservative α value of 0.0083. If I combine the probabilities from all these tests using Edgington's additive method (Edgington 1972), again I find that heatshocked genotype 1 males sleep more during the day (Genotype1,2: no. of studies =3, $p = 0.00042$, Genotype1,3: no. of studies =3, $p = 0.00054$) but not at night (Genotype1,2: no. of studies =3, $p = 0.00012$, Genotype1,3: no. of studies =3, $p = 0.05280$). Equally the reduction in mean waking activity during both the day and night comes out of the Edgington analysis (Day: Genotype1, 2: no. of studies =3, $p < 0.00001$, Genotype1,3: no. of studies =3, $p = 0.00381$, Night:Genotype1,2: no. of studies =3, $p < 0.00001$, Genotype1,3: no. of studies =3, $p < 0.00001$). The number of sleep bouts was decreased, that is sleep was more consolidated, in the LD experiment during the night for heatshocked Genotype 1 males and during the day

of the LD phase of the DD experiment. However both of these results become nonsignificant when the Edgington method is applied (Day: Genotype1, 2: no. of studies =3, $p = 0.0235$, Genotype1,3: no. of studies =3, $p < 0.00001$, Night:Genotype1,2: no. of studies =3, $p = 0.0004$, Genotype1,3: no. of studies =3, $p = 0.0208$). Even if significant, these results just concur with the general reduction in activity of heatshocked Genotype 1 flies. For heatshocked females I only have the LD experiment. Genotype 1 females seem largely unaffected by heatshock, with only daytime activity showing a 16% decrease.

With the previous analysis in my first thesis, this result was not so clear cut, but it was clear that immune stimulated flies slept more due to reduced activity. I next carried out the continuous lighting regime experiments. I chose to look just at males as they gave the clearest results. Constant darkness will allow the flies to free-run, meaning any effect I see should be due to the effect of the immune response on the circadian control of activity. The constant light experiment will tell me if the effect is on the light arousal level of the flies. I analysed this data in two ways, using only the first 24 hours of the continuous regime and using the whole of the continuous lighting time. This is more important in the DD experiment as the flies begin to free-run they will eventually be out of sync with both each other and what I consider the subjective day and night. This has the potential to confuse my statistical analysis. I suggest this is the case with the whole DD results (Table 4.4). As well as showing a 7.5% decrease in number of sleep bins they also show a reduction in waking activity and sleep bouts. The only significant result in the LL experiment was an increase in mean waking activity during the day.

Overall, I would say the continuous lighting experiments were uninformative. I would have predicted one of two results. 1) In the LL experiment I would have expected genotype 1

flies to show increased sleep bins and decreased activity during both subjective day and night when heatshocked. Thereby replicating the effect of heatshock during LD. This would suggest that heatshocked genotype 1 flies have a poor arousal response to light when heatshocked. Large ventral lateral neurons (ILNvs), a subset of the *Drosophila* clock circuit, have been shown to have a role in arousal to light (Franken and Tafti, 2003). They only promote wakefulness during the light phase of standard light/dark conditions and have no effect in constant darkness (Shang et al., 2011). 2) That the effect seen in the LD lighting regime would be replicated in the DD lighting regime. That is, I would have expected to see heatshocked genotype 1 flies with increased sleep and decreased activity during the subjective day and no difference at subjective night. This would have shown that what is being affected by the immune response is the circadian control of sleep. Kuo et al., (2010) show that the increase in sleep is restricted to the morning hours after inducing the immune system by infection with Gram-negative bacteria, and this is dependent on the time of day of treatment such that night time treatment produced stronger effects than those in the daytime. This pattern persists in constant darkness, which strongly suggests a role of the circadian clock in regulating this response.

The geneswitch results are much clearer and show exactly the pattern I predicted. The females show an increase in sleep bins and a decrease in activity depending on whether we look at the first 24 hours or the whole period. This was an attempt to compensate for using nonvirgin females, the logic being that whatever the effect of mating, it would be less pronounced in the beginning. Previously, it has been shown that females sleep only 40 per cent of the time that male flies sleep (Huber et al. 2004; Andretic & Shaw 2005), so males spend more time conserving energy and avoiding physical and predatory dangers, while

females stay most of the time awake to forage for food and selecting sites for laying eggs, this wakefulness is greatly reduced in virgin females, who display a male-like tendency for robust day-time sleep (Isaac et al., 2010). This does not distract from the male results as males are often preferred in fly sleep research due to their more extreme phenotypes (Isaac et al., 2010).

The immune stimulated males in this experiment show a 23% decrease in sleep during the day and a 9% decrease at night. There was no corresponding change in mean waking activity. There was a significant but very small increase (0.5%) in the number of sleep bouts during the night. This suggests that immune stimulated males had slightly more disturbed sleep at night. The important result though is the found decrease in sleep with no associated effect on mean waking activity. This is exactly what I predicted and fits in nicely with my proposed immune-sleep-memory axis. However before I discuss this I must first explain why immune stimulation by geneswitch causes a decrease in sleep whereas an apparent immune response by heatshock causes a decrease in activity, with a corresponding increase in sleep.

Both the Geneswitch and heatshock experiments use 3 genotypes each. The first genotype is the GAL4-UAS-PGRP cross. Heatshocking or feeding RU486 (Geneswitch) to this genotype will lead to the expression of peptioglycan receptor protein LC (PGRP-LC), which is a pattern recognition protein for the ImD pathway. That is, its expression will lead to the upregulation of several antimicrobial peptides (Gottar et al., 2002).” The AMPS are the end point of an immune response to bacteria. However, whether or not these approaches mimic immune responses observed in natural infection remains to be

determined. This would necessitate detailed microarray studies that are beyond the scope of this thesis.

The HsGal4 system is leaky. That is even without the actual heatshock, a low level of expression of the construct is usually found. This is often used to explain residual expression and effects in heatshock experiments (McGuire et al., 2005). In our system this would result in lines of hsGal4-UAS-PGRP (Genotype 1) having a chronic upregulation of the immune response, although still lower than when heatshocked. This has in fact been shown to be the case by Gottar et al., (2002), see Figure 4.7. The unheatshocked genotype 1 (G) has approximately three times the expression of PGRP than the control flies (A).

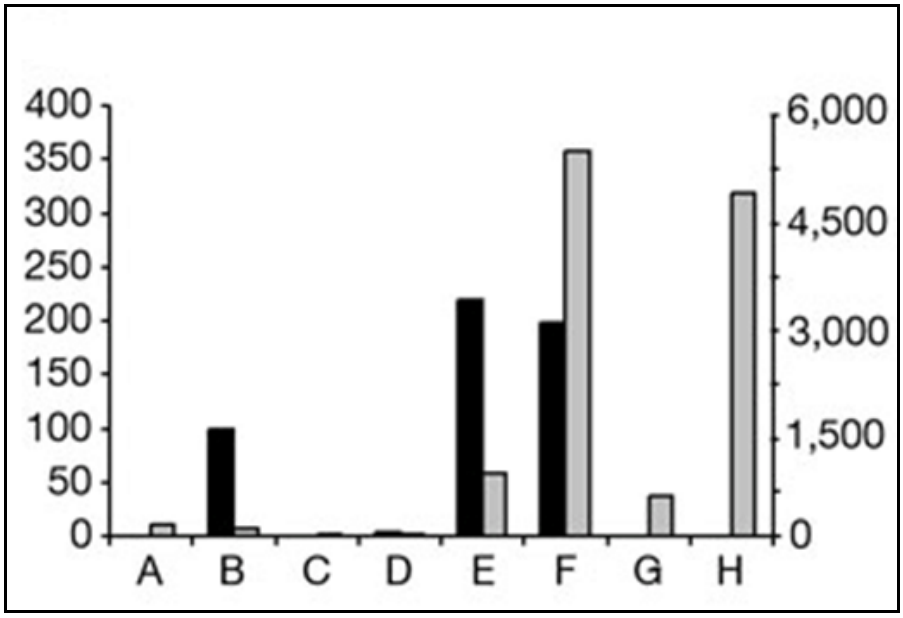


Figure 4.7

Taken from Gottar et al 2002. Our genotype 1 (here called *hs-GAL4/PGRP-LC*^{7454}) is leaky. Only A,B,G and H are of interest for this thesis. A, non-challenged wild-type males; B, challenged wild-type males (*E. coli*); G, non-heat-shocked *imd/imd; hs-GAL4/PGRP-LC*^{7454} non-challenged females; H, heat-shocked *imd/imd; hs-GALl4/PGRP-LC*^{7454} non-challenged females. *Diptericin* (black bars) and *PGRP-LC* (grey bars) transcript levels were measured by quantitative RT-PCR. Results were first standardized against *Rp49* and then the value obtained for induced males or females was taken to be 100 as a reference. Left *y*-axis scale, *Diptericin*; right *y*-axis scale *PGRP.*

From this I suggest that genotype 1 flies will be chronically immune stimulated. What effect will this have on their general biology? This whole thesis is concerned with one trade-off with immunity, that of memory and learning. However throughout the thesis I have presented many more trade-offs with immunity. A chronic immune response could be seen as a form of stressor for the fly. The physical act of heating, the heatshock, is a well known stressor in *Drosophila* (Schlesinger et al. 1982). So one hypothesis of what is happening here is that genotype 1 flies have a chronic immune response, which neither of the control lines has. When heatshocked this stresses them which leads to a decreased activity and increased sleep. Their immune system is stimulated as well, but this response is overwhelmed by the stress response.

Having given at least a viable explanation of why my heatshock and geneswitch experiments differ, I will know focus on my geneswitch result. Males sleep less during the day and night, with no associated decrease in activity. That is, the immune response is associated with a reduction in sleep. This fits perfectly with my immune-sleep-memory hypothesis to explain the immune associated reduction in learning and memory. Three questions remain: 1) Why does my result agree with Shirasu-Hiza et al (2007) but not Kuo et al (2010). 2) What is the molecular link which causes the immune response to decrease sleep and 3) what is the molecular link which causes reduction in sleep to lead to a reduction in learning and memory?

We have three results looking at the effect of immune response on sleep. Shirasu-Hiza et al (2007) found that when they infected males with gram-positive bacteria they found a reduction in sleep. Kuo et al. (2010) found that when they infected females with gram-negative bacteria, the flies slept more. I found that when I stimulated the immune response

by a geneswitch activation of PGRP, males slept less. The discrepancy between the first two results Kuo et al explain by the different methods of infection. This could be the case, although Kuo et al then go on to claim generality for their result, with no consideration of the previous discrepancy. My experiment should have stimulated the imd pathway, the same as responds to gram-ve bacteria, suggesting that my result should follow Kuo et al. But that ignores the fact that Kuo et al (or for that matter Shirasu-Hiza et al) did not stimulate the immune response but rather infected the flies. Only my result shows the effect of immunity without the complications of infections. I have already discussed the difference between males and females with regards sleep. It is entirely possible that males and females also differ in their immune response effect on sleep. There is some tantalizing evidence of this with my geneswitch female data. They do indeed sleep more when immune stimulated (one result is classed as not significant but the $p = 0.018$). However taking in to account the lack of virginity in these flies, perhaps the safest route here is to prescribe a repeat of the female experiment with virgin females this time. If it did indeed find an increase in sleep in immune stimulated females, this would explain the discrendancy between published results.

Kuo et al (2010) provide evidence that *Relish* is necessary for immune modulated change in sleep. However they do not show it is sufficient. That is, it is Relish itself that is interacting with the immune response. To do this they would have to test the sleep of immune stimulated fly mutants that are null for components of the *imd* pathway downstream of *Relish*. If they still showed the immune modulated change in sleep behavior, then we could be confident Relish was the culprit.

Seugnet et al (2008) showed that dopamine agonists rescued reduction in learning and memory caused by sleep deprivation. Dopamine had no effect on memory without sleep deprivation. Importantly, sleep-deprivation-induced learning impairments could be rescued by targeted gene expression of the dopamine receptor to the mushroom bodies. A first experiment here would be to see if this treatment would also rescue my immune-sleep-memory flies. Of course, I still haven't shown that immunity affects learning and memory in flies. This is the subject of my next chapter.

Chapter Five: Interaction between immune system And memory in *Drosophila melanogaster*

1. Introduction

Previously it has been shown that an immune response affects learning in insects (Mallon et al., 2003; Gegear et al. 2006; Iqbal & Mueller 2007). The fruit fly, Drosophila melanogaster, has been tremendously helpful to the analysis of molecular components and cellular pathways that mediate associative learning (Kim et al., 2007) and immunity (Lemaitre & Hoffman, 2007) because it provides an extensive resource for genetic and transgenic mutants, tools and information. Furthermore, its relatively short life cycle (12 days) makes it quite feasible to investigate a large number of genetically homogeneous animals, avoiding differences caused by an individual's genetic background and thus correlating behavioral phenotypes with the function of a gene or the manipulation under examination. The Mallon's lab work on immune-mediated changes in memory, has been done on bees. Although there are an increasing number of genetic tools for both honeybees (RNAi, genome published) and bumblebees (RNAi, full genome to be published this year), they are unlikely to ever offer the same level of tractability as Drosophila. Also, as per Chapter 4, I have shown that, in Drosophila, sleep is a potential intermediary between memory and immunity. To further develop research into insect immune-memory interactions, this chapter aims is to develop Drosophila as a model for this interaction.

1.1.Fly memory paradigms

Drosophila is a well-used model in the study of learning and memory. In this section I detail some of the conditioning paradigms used in this research.One of the well-established conditioning paradigms in Drosophila is classical olfactory conditioning, which tests flies' capacity to associate olfactory (a specific odor, the conditioned stimulus (CS) and aversive mechanosensory (electric shock, the unconditioned stimulus

(US)) inputs. Quinn et al., (1974) were the first to demonstrate that flies learn to avoid the odor, previously presented with aversive electric shock. Later, Tully and Quinn (1985) improved the paradigm by setting up critical training and testing parameters, which have been widely adopted by many investigators. To train Drosophila, flies, placed in a small plastic tube containing an electrifiable grid, are exposed to a first odor (CS+) while also undergoing an electric shock (US) followed by a second odor (CS) without any shock.

In Drosophila, an olfactory conditioning paradiagm utilizing positive reinforcement has also been described (Tempel et al., 1983). They allowed flies to run into a tube containing a copper grid coated with CS+ odour and sucrose (US), and then are moved to a tube coated with CS- odour without sucrose. Flies are transferred to a T-maze for measuring preference to the CS+ as a measure of learning or memory retention. This model demonstrates operant conditioning, as flies run freely inside the tubes, experience CS and US inputs and learn their consequences. A modified version of this was used by Kim et al., (2007) (Figure 5.1).

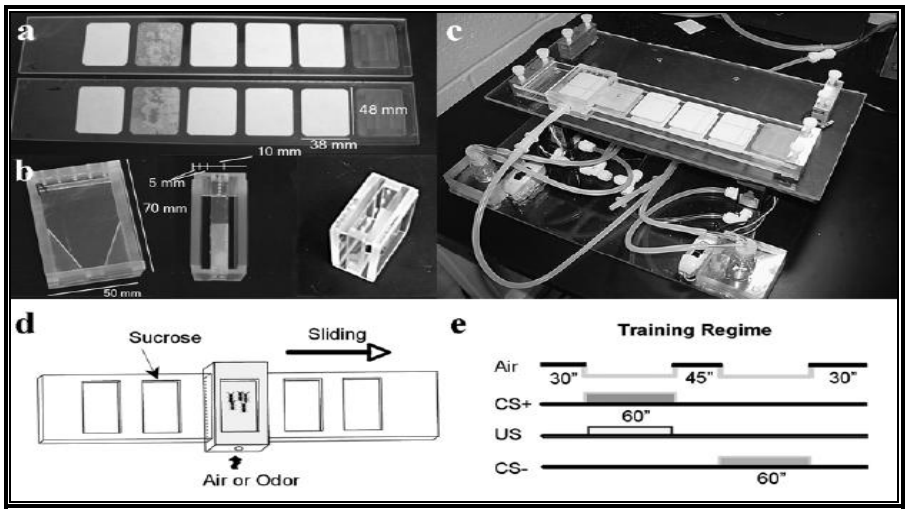


Figure 5.1

Sliding box conditioning apparatus and training regime used by Kim et al., (Adapted from Kim et al., 2007)

Another type of learning paradigm involves species-typical, reinforcement-appropriate responses, elicited by environmental stimuli. Such responses, when reinforced, become more likely to recur in the particular situation (Thorndike, 1911). This type of learning behavior has been demonstrated in flies using the heat box (Wustmann & Heisenberg, 1997) and flight simulator (Wolf & Heisenberg, 1991) assays. The heat-box is typically used in a place memory paradigm in which flies are punished by heat when entering a particular side of the box creating an association between walking in the punished side with heat (Figure 5.2). Single flies, walking freely back and forth in a narrow alley in complete darkness, are conditioned to avoid one half of the length of the alley by being heated instantaneously on entering that half. The temporal scheme of heating and cooling simulates a spatial temperature gradient in the chamber for the fly. The training is followed by a test period without any heat. During the whole experiment, the position of the fly in the chamber is monitored, and the fraction of time the flies spent on the "unpunished" side is calculated. Besides temperature, the fly can use only tactile information and path integration for orientation (ideothetic orientation, i.e., the accumulation of the internal representations of the fly's turns and steps). This allows measurement of individual flies behaviour in a way analogous to the DAMS equipment in circadian behaviour. See Figure 5.2.

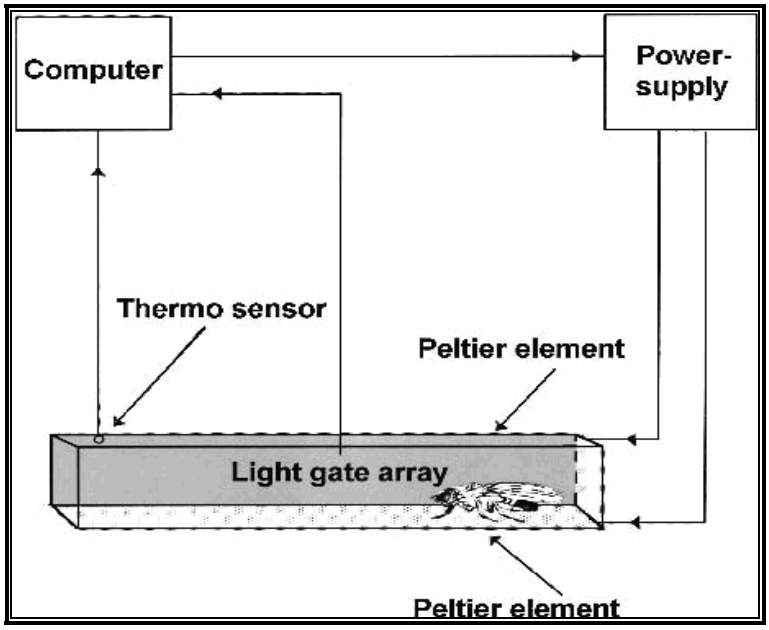


Figure 5.2

Schematic diagram of heat-box paradigm.

In this chapter I wanted to develop a method allowing me to study the immune-memory interactions, previously found in bees, in Drosophila. I choose the aversive olfactory conditioning pioneered by Quinn et al. (1974). This have two main advantages, it was long established and due to a modification by Mery (Mery and Kawecki, 2005), see Methods, was also the simplest to set up.

1.2.Fly innate immunity

Due to genetic tractability Drosophila melanogaster makes an excellent model organism for studying innate immunity (Lemaitre and Hoffman, 2007). In addition due to the lack of adaptive immune system, components of innate immune system can be studied that might otherwise be obscured by the adaptive response. Details of the immune response can be found in the thesis introduction. Briefly, the immune response in Drosophila melanogaster can be divided into two main categories, passive barriers and active responses. The passive barriers consist of physical barriers such as the Drosophila

exoskeleton, peritrophic membrane, and tracheal lining. The active responses include production of antimicrobial peptides and reactive oxygen species. In addition, specialized haemocytes participate in phagocytosis and encapsulation of foreign intruders in the haemolymph (Lemaitre and Hoffman, 2007).

1.3.Interaction between immunity and memory in fly

Kolss et al. (2006) did investigate if there was an evolutionary trade-off between immunity and memory. They tested the ability to defend against parasitoids in lines selected for increased memory scores and tested the memory scores in lines selected for an ability to defend against parasitoids. They found no evidence for a relationship between these two attributes and concluded that there was no evolutionary trade-off between memory and immunity in Drosophila. However this does not mean a physiological link does not exist. For the difference between a physiological and an evolutionary trade-off, please see chapter 3. Recently, Copf et al (2011) showed knocking out (RNAi) homologs of members of the JAK/STAT pathway affected Drosophila ability to form odor aversive long term memories. The JAK/STAT pathway also functions in the immune response.

I need to find a way to stimulate the immune system. Other studies have done this by infecting the animals. For example, in honeybees deformed wing virus (DWV) infections at high doses can lead to their definitive pathology and appear to generate negative effects on behavior and learning at lower doses (Iqbal and Mueller, 2007). Shirasu-Hiza et al. (2007) reported that mutant flies per^{01} were more susceptible to *Streptococcus pneumoniae* and *Listeria monocytogenes* compared to wild type flies to display altered survival rates following infection. As discussed previously this confounds the effects of the immune response with the effects of the parasite. With the

bumblebee I could simply inject with LPS (see Chapter 2). Injection of Drosophila adults is carried out. Dzitoyeva et al. (2001) have shown that RNAi can be induced in adult fruit flies by injecting dsRNA into the abdomen of anesthetized Drosophila, and that this method can also target genes expressed in the central nervous system (CNS). However, my supervisor attempted and was unable to establish this system in the lab. Rather than stimulating the fly immune system with an external antigen I will use a previously developed UAS system. Peptioglycan receptor protein LC (PGRP-LC) is known to lead to expression of the constituent parts of the IMD pathway. The IMD pathway leads to the production of antimicrobial peptides that defend against gram negative bacteria (Gottar et al., 2002). Here, the same two systems I used in chapter four to stimulate the immune system of Drosophila are also used in this chapter. I use transgenic fly lines (hs-Gal4 and UAS-PGRP) and the Geneswitch system (Geneswitch Gal4 and UAS-PGRP) to stimulate an immune response However, whether or not these approaches mimic immune responses observed in natural infection remains to be determined. This would necessitate detailed microarray studies that are beyond the scope of this thesis. To test for alteration in memory I will use a modified aversive learning paradigm (Mery and Kawecki 2005).

2. Methods and Materials

2.1. Activation of the immune system

The methods used here were the same as in chapter four. Briefly, the immune system was activated by either heatshocking or feeding RU486 to the correct genotype. Flies were raised in standard conditions and were 3-5 days old during the experiment. (Two days feeding the flies with RU486 and leave them one day after training and before testing them into the T maze).The genotypes used were

Heatshock:

(4) ♀ **(vg)** UAS-PGRP **x** ♂ hsGal4 (immune activated)

(5) ♀ **(vg)** UAS-PGRP **x** ♂ w^{1118} (control)

(6) ♀ **(vg)** W^{1118} **x** ♂ hsGal4 (control)

Or

Geneswitch:

(4) ♀ **(vg)** UAS-PGRP **x** ♂ Geneswitch gal4 (immune activated)

(5) ♀ **(vg)** W^{1118} **x** ♂ Geneswitch gal4 (control)

(6) ♀ **(vg)** UAS-PGRP **x** ♂ w^{1118} (control).

2.2. Conditioning procedure

Conditioning began next day after the application of heatshock or RU486. In heatshock experiment conditioning and memory tests were performed on samples of (mean = 65) adult flies (sexes mixed). In the Geneswitch experiment each sample consisted of 50 adult flies of one sex only. The conditioning procedure consisted of 5 training sessions separated by 20 min intervals (spaced protocol). In each training session flies were first exposed for 30 second to one odorant simultaneously with mechanical shock delivered

every 5 second by a test tube shaker. This period was followed by a 60 second rest period (no odor and no shock). Then, for 30 second another odorant was delivered, without shock. Odorants used for conditioning are 3-octanol and 4-methylcyclohexanol (both 0.6ml/l of paraffin).

2.3. Memory assay

24 hours after the conditioning period flies were transported to the choice point of a T-maze, in which they were exposed to two converging currents of air, one carrying 3-octanol and the other 4-methylcyclohexanol, and allowed to choose between the two odors for 60s, see Figure 5.3. The memory score was calculated as the difference in the proportion of individuals choosing octanol between flies conditioned to avoid methylcyclohexanol and those conditioned to avoid octanol. In heatshock experiments for each genotype nine paired for odor replicates were carried out for each treatment, And in Geneswitch experiments ten paired replicates were carried out for each genotype, five for males and the same for females.

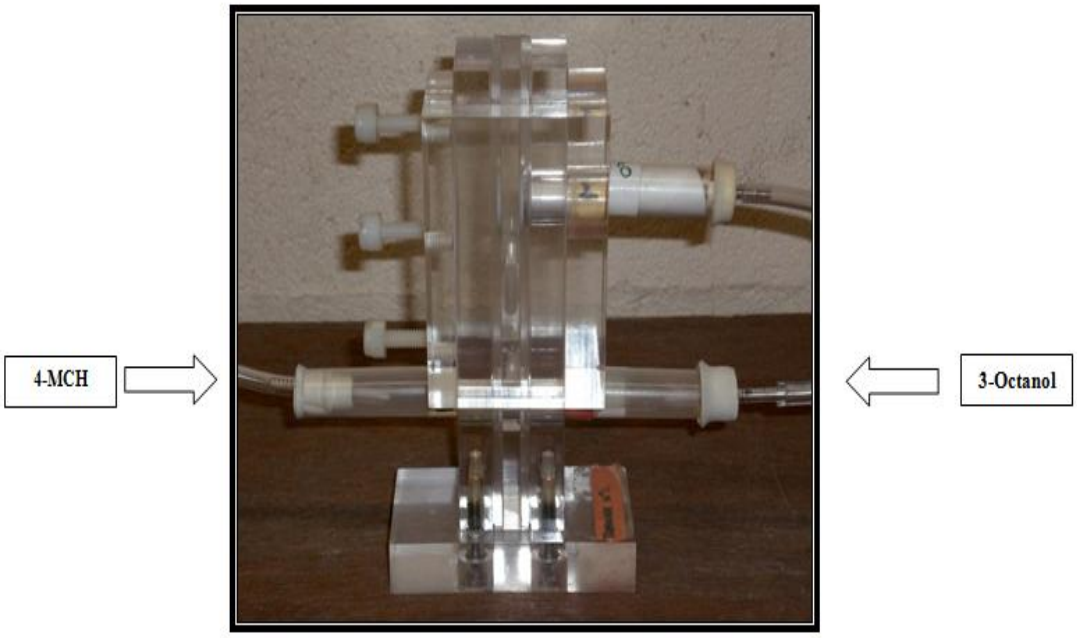


Figure 5.3

This picture represents the T maze system, where flies were exposed to two currents of air, one carrying octanol(3-OCT) and the other methylcyclohexanol(4-MCH), and allowed to choose between the two odors for 60s.

2.4. Statistical analysis

The dependent variable was memory score. First I checked if the data was normally distributed using a Shapiro-Wilks test. If it was not, the data was transformed using a box-cox transformation. The independent variables were genotype, treatment (heatshock yes/no OR Geneswitch yes/no) and sex (only for Geneswitch experiment.) ANOVAs were carried out in Stata 11 with all dependent variables plus the three way interaction (for Geneswitch) and two way interaction terms. Sex is included as there may be differences between males and females in memory score and we used both males and females to increase number of replicates. It is good analytic practice to include all variables that may lead to variation. These can be removed from the ANOVA if these are shown to be nonsignificant. Both genotype and treatment are main

effect variables, however what is really of interest is their interaction. I expect genotype 1 when either heatshocked or fed RU486 to produce an immune response, I do not expect that of the two control genotypes. If this happens it will lead to a significant interaction term.

3. Results

3.1. Heatshock

The memory scores were distributed normally (Shapiro WIlks normality test: W = 0.98106, n = 41, p = 0.71549).

Both heat shock ($F_{1,40} = 9.58$, p = 0.0039) and genotype ($F_{2,40} = 9.77$, p = 0.0004) had an effect on memory scorers, see figure 3.5. There was no significant interaction effect between heat shock and genotype, that is heatshocking flies had the same effect on each genotype ($F_{2,40} = 0.16$, p = 0.8511). See Figure 5.4.

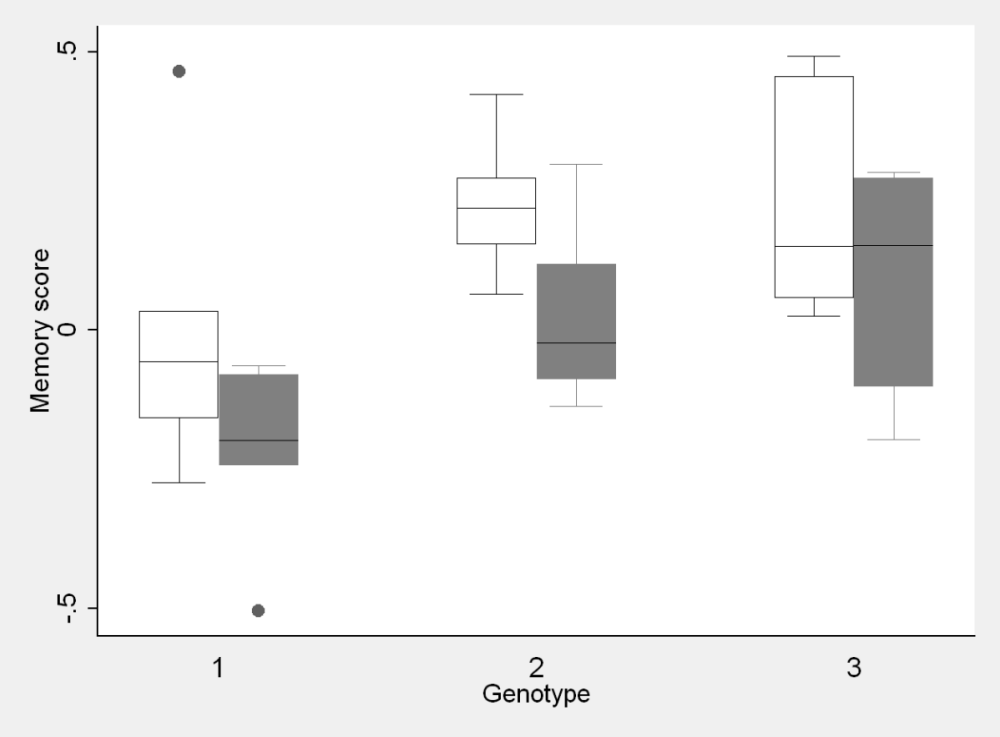


Figure 5.4

Boxplot of the memory score of the three genotypes. (1) hsGal4 UAS-PGRP (immune activated genotype). (2) W^{1118} UAS-PGRP and (3) hsGAL4 $w^{1118.}$ Genotypes 2 and 3 are the two control genotypes. White boxes are no heatshock treatments and the grey boxes are the heatshock treatments.

3.2. Geneswitch

The memory scores were box-cox transformed using the following equation:

$$\frac{(Memoryscore+1)^{3.024752}}{4.024752}$$

The transformed scores were distributed normally (Shapiro-Wilk normality test: W =0.98425, n = 60, p = 0.63119). The untransformed data was used in Figure 5.5.

Of the 3 main factors, only Genotype had an effect on the memory score (3 way ANOVA: Genotype: $F_{2,59}$ =8.25, p = 0.0008, Geneswitch: $F_{1,59}$ =1.29, p = 0.2617 Sex: $F_{1,59}$ =3.08, p = 0.0857). There was a significant 3- way interaction term (Genotype, Sex and Geneswitch) (3 way ANOVA: $F_{5,59}$ =3.54, p = 0.0084).

Due to this significant interaction term, I then carried out two way ANOVAs on male and female data separately. The 2 way interaction term (Genotype by Geneswitch) was only significant for males (Males: 2 way ANOVA: $F_{2,29}$ = 4.81, p = 0.0175, Females: 2 way ANOVA: $F_{2,29}$ = 1.52, p = 0.2391). When I checked to see which genotype was different from which, by restricting the analysis to only pairs of genotypes, I found that the effect of geneswitch on genotype one was no different than on the two controls (2 way ANOVA for genotype1, 2: $F_{1,19}$ = 1.81, p = 0.1977 and: 2 way ANOVA for genotype 1, 3: $F_{1,19}$ = 2.61, p = 0.126 and 2 way ANOVA for genotype2, 3: $F_{1,19}$ = 12.26, p = 0.0030). See figure 5.5.

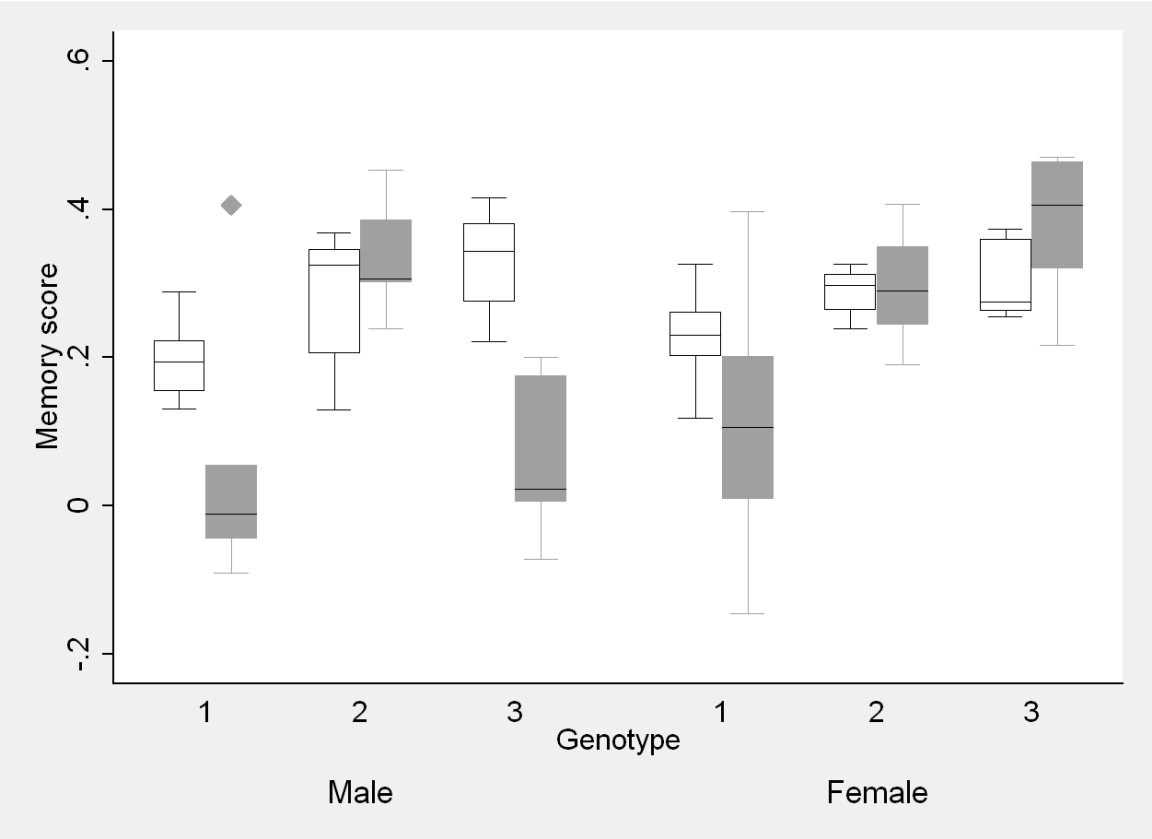


Figure 5.5

Boxplot of the memory score of the three genotypes. (1) GeneswitchGal4 UAS-PGRP (immune activated genotype). (2) GeneswitchGal4 w^{1118} and (3) w^{1118} UAS-PGRP (are the two control genotypes). Genotypes 2 and 3 are the two control genotypes. White boxes are no geneswitch treatments, and the grey boxes are the geneswitch treatments.

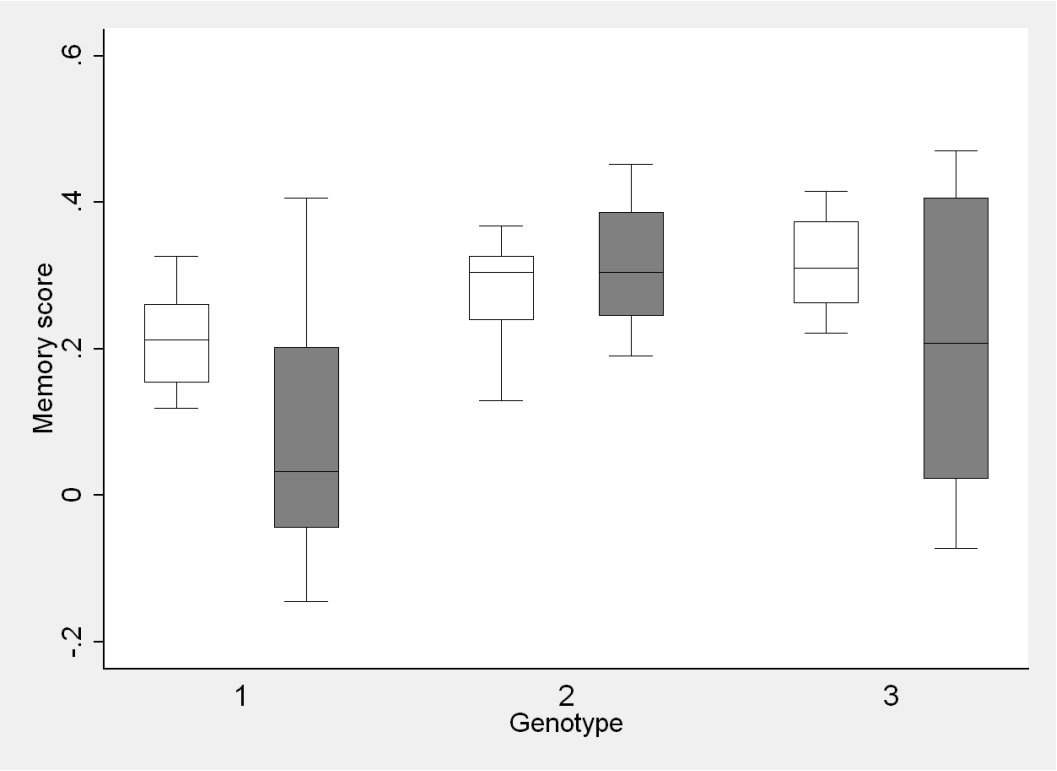


Figure 5.6

Boxplot of the memory score of the three genotypes combined for both sexes. (1) GeneswitchGal4 UAS-PGRP (immune activated genotype). (2) GeneswitchGal4 w^{1118} and (3) w^{1118} UAS-PGRP (are the two control genotypes). Genotypes 2 and 3 are the two control genotypes. White boxes are no geneswitch treatments and the grey boxes are the geneswitch treatments.

4. Discussion

Although I found that heatshock decreases memory scores in flies and the individual genotypes have significantly different memory scores, I found no evidence for an interaction between heatshock and genotype. Equally, although genotype again had an effect on memory scores in the Geneswitch experiments and there was an interaction between genotype and Geneswitch in male flies, this difference was not due to genotype 1 (the experimental genotype) but rather due to differences between the two control genotypes. If immunity affected memory in flies, I would have expected an interaction, specifically the difference between memories scores of immune-stimulated (heatshock or Geneswitch) genotype 1 and non-stimulated genotype 1 should have been larger than the differences due to treated in the other two genotypes.

Although my results suggest that immunity does not affect memory in flies, this seems unlikely. Why should flies be different from other insects (Mallon et al., 2003)? This is especially puzzling given the recent evidence of the role of immune cytokines in Drosophila long-term memory (Copf et al., 2011). To muddy the waters further, a recent unpublished report claimed that immune challenged flies have increased memory abilities (Babin, 2009).

From these confusing results one thing is clear, heatshock has a large effect on memory. One possibility is that the effect of immunity on memory is small compared to the general effect of heatshock. Mallon et al. (2003) reported a decrease in learning due to immunity of about 40%. The effect of heatshock on genotype 2 was an 86% decrease in memory. It seems possible that any significant effect was swamped by the effect of heatshock itself.

This effect could be made up of two parts, the physical effect of heatshock and the effect due to non-specific heatshock proteins on memory. The cellular response to heatshock (body temperature elevated at least 2-5°C above the optimal level) was first observed in Drosophila (Schlesinger et al., 1982) and has since been described in every system examined, from bacteria to humans (Schlesinger et al., 1982; Lindquist 1986; Nowak 1993). It was found that memory consolidation was disrupted by heatshock during training but not before or after training in *Caenorhabditis elegans* (Beck and Rankin, 1995). In addition, heatshock before training failed to induce thermal tolerance to the effects of heatshock during training on long-term memory formation in *Caenorhabditis elegans*. This finding demonstrates that heatshock can disturb memory.

The Geneswitch data is perhaps slightly easier to understand. The female pattern looks about right, although not significant. The male pattern is complicated by the large decrease of Genotype 3 when Geneswitch is activated. In the previous version of the thesis I suggested using an injection of lipopolysaccharisde, as an alternative method of generating an immune response (Mallon et al., 2003). I no longer feel this is the correct way forward. Although LPS would not have the unwanted strong effects of heatshock, it would I feel give similar results to the Geneswitch experiments. Overall I feel that increased statistical or methodical power is required. For example, the effect size of the genotype*Geneswitch interaction for males of genotype 1 and 2 is about 7% (eta^2 = 0.069). The equivalent value for the genotype effect was 32% (eta^2 = 0.319). This shows the effect due to the interaction is quite subtle, requiring a larger sample size to increase statistical power. To increase sample size I combined male and female Geneswitch data (see Figure 5.6). This is the equivalent analysis used in the heatshock experiments.

Although still not significant (2 way ANOVA, Genotype*Geneswitch $F_{2,59} = 1.41$, $p = 0.2522$), it does suggest a larger sample size may lead to it being statistically significant. One other option would be to change the experimental paradigm. The heat box paradigm would have all the advantages of the DAMS system, namely automation and individual data. Three thousand flies were used to produce Figure 5.6, if the heatbox paradigm was used we would have had 3000 data points as opposed to 30 paired replicates.

Learning is a subtle phenotype in flies, depending on multiple factors. Previously, it has been shown that flies exhibit better performance with more training in electric shock-mediated olfactory conditioning, and the degree of improvement depends on inter-training intervals (Tully and Quinn, 1985; Beck et al., 2000). Flies exhibit a substantial level of memory score after one training session, which was enhanced after more additional training sessions with one minute inter-training interval, four cycles of training also enhanced performance, but the difference in the performance scores obtained after two and four trainings was not statistically significant. The associative strength of Unconditioned Stimulus and Conditioned Stimulus for memory depends on the importance of stimuli (Tully and Quinn, 1985; Beck et al., 2000) and the context of their presentation (Tully and Quinn, 1985).

Given all this I suggest that the experiment detailed in this chapter was inconclusive. Heatshock seems an inappropriately harsh treatment for a subtle phenotype like learning. It will be very interesting to see what immune stimulator the project that found the memory increase used (Babin, 2009). If she used a pathogen she risks confounding the effect of the immune system with the effect of the parasite. Geneswitch probably gave a truer representation of the effect of the immune system on memory, showing it is a subtle effect that requires a larger sample size to detect.

Chapter Six: Discussion

General Discussion

Recognition of the interaction between the immune and the nervous system is an important advance in understanding the biological basis of behaviour. The negative effect of immune response on the nervous system relies on a network of bi-directional cross-talk pathways (neural and chemical pathways) between the two systems. Previously, several studies found a negative impact of an immune challenge on learning and memory performance in social insects. My project extended these studies and began the attempt to understand them mechanistically and evolutionarily

A good correlation was found between the performance of honeybees in proboscis extension reflex (PER) assays and in free flying experiments (Laloi, Bailez et al. 2000). In both honeybees (Mallon et al. 2003) and bumblebees (Riddell and Mallon 2006), it has been found that immune-induced PER impairment was only recorded when protein consumption was restricted. I used a free flying floral choice assay for testing bumblebees' memory after inducing immune system non-pathogenically by injection of lipopolyscharides (LPS), and I found impairment in the learning and memory of the bumblebees. This is similar to the effect that was found both in PER assays using LPS (Riddell & Mallon 2006) and how the bumblebees' learning performance was affected when infected by *Crithidia* (Gegear et al., 2006). These results indicate clearly that before it was assumed that a parasite has a direct effect on a host's behaviour, the effect of the immune response stimulated by the parasite must first be quantified.

Previous work focused on the physiological interactions between immunity and memory in insects. Chapter three looked for a possible evolutionary relationship between them. This was prompted by the work of Kolss et al. (2006), who found no such relationship in *Drosophila*. They tested the ability to defend against parasitoids in *Drosophila* lines selected for increased learning ability and tested the learning ability in lines selected for an ability to defend against parasitoids. I felt it was worth examining this in a system that had previously shown a physiological link between immunity and memory, namely the bumblebee. I have found positive correlations between the learning ability of colonies' workers and their immune system.

Previously this cross talk between immune response and memory was treated as a black box. What pathways are actually involved? I felt that sleep was a potential candidate as an intermediary. Both immunity and sleep use Relish (Williams et al., 2007) and sleep is known to have an effect on memory. In my research I triggered the flies' immune system directly instead of using parasites. I used the heatshock UAS-PGRP system in three different conditions (light/dark, light/light and dark/dark condition) and a Geneswitch UAS-PGRP system in light /dark only. Peptidoglycan receptor protein LC (PGRP-LC) is known to lead to expression of the constituent parts of the *imd* pathway. The *imd* pathway leads to the production of antimicrobial peptides that defend against gram negative bacteria (Gottar et al., 2002). Transgenic flies were used to avoid the confounding effects of parasitic infection. The two experiments differ in their results, with the heatshock system leading to increased sleep, but the geneswitch system producing reduced sleep. The latter agrees with my model to explain immune memory interactions, namely that the immune response uses up Relish that is required for sleep,

thereby reducing sleep. This in turn leads to a reduction in learning and memory modulated by dopamine. The difference can be explained by the leakiness of the heatshock system.

My final data chapter took a step back and tried to establish an immune-memory interaction paradigm in Drosophila. I continued to use the UAS-PGRP system. Flies can learn in a variety of tasks but, for more than two decades, the focus has been on olfactory learning (Davis, 1996, Dubnau and Tully 1998). Previous studies utilized olfactory avoidance learning in which flies learned to avoid running into a tube containing a certain odor after a training session that paired the odor with electrical shock as a negative reinforce (Quinn et al., 1974). I used transgenic flies to activate immune system in *Drosophila melanogaster* (UAS-PGRP X hsGal4 or UAS-PGRP X Geneswitch) system. An aversive learning paradigm modified from Mery and Kawecki (2005) was used to check the performance index of the memory scores in flies. A negative result was recorded so there was no significant interaction effect between heat shock and genotype. However I suggest that this is just an effect of sample size as the effect size if the interaction of genotype and treatment is quite small in the geneswitch experiment. However, the fact that I have not yet established a link between immunity and memory in *Drosophila* reduces the utility of my discovery of a link between immunity and sleep in *Drosophila.* It weakens the argument that sleep can be seen as an intermediary between immunity and memory. Establishing the link between immunity and memory in *Drosophila* must be the number one priority of future research.

These are the results of this thesis. Where should the work go now? There are three avenues down which the research could go; 1) evolution, 2) ecology and natural behaviours and 3) physiological/molecular mechanisms. The evolutionary route, by which I mean understanding the trade-off or lack there of between immunity and learning/memory, is I think a dead end. The way to look for a trade-off is to do as in Kolss et al. (2006), select lines for one trait and then measure the trait that you think has traded-off. Kolss et al. (2006) did not find a trade-off in fruit flies. I still believe that *Drosophila* is not the system to look for a trade-off. As stated in chapter three this is due to their natural biology not especially valuing these two traits relative to a bee. But bumblebees are definitely not the system to attempt a breeding experiment. The type of correlational study I carried out in chapter three is frowned upon in this trade-off research. This is mainly due to the many unknowns that may control the interaction of two traits in uncontrolled situations. For example, resource acquisition, how well resourced the colony is, will distort any natural trade-off.

Future experiments could embed this immune-memory interaction in the actual ecology of the animal. One experiment, for example, could be to look at the immune effect on foraging. Raine & Chittka (2008) found that the learning score of the colony was strongly associated with its ability to forage. A future experiment could inject half the workers in the colony with LPS and the other with ringer, and then allow them to forage in the field. By weighing the ingoing and outgoing foragers you would get an idea of how much resources a forager is taking in. I would predict that LPS bees would be poorer foragers. This would show that the immune reduction in learning and memory has a direct effect on the fitness of the colony. Virulence is the parasite-

mediated loss of fitness in the host. Therefore this experiment would show a different kind of virulence, the fitness cost of the immune response in respect to the parasite. This data could be plugged into models looking at the evolution of the immune response and parasite load to get a better understanding of optimal disease levels in social foragers.

What about the molecular basis of this interaction. Strong contenders are Relish as a link between immunity and sleep and dopamine as a link between sleep and learning and memory. In a sense the role of dopamine has already been discovered. Seugnet et al (2008) found that adding dopamine rescued sleep deprived flies memory loss. Would adding dopamine restore the memory of immune stimulated insects. With regards the role of Relish, it would just require an extension of the work of Kuo et al. (2010). They showed that Relish was required for the immune memory-sleep interaction, but not that it was sufficient. Imagine if it was not *Relish* but rather a gene downstream in the imd pathway that was responsible. Switching off *Relish* would abolish the interaction but not due to the direct effect of *Relish* but rather its effect on the actual culprit. So future work must knock off these genes, if they find that the interaction between immunity and sleep still persists, then we would know that *Relish* was indeed the link. Of course to do any of this, it would have to be confirmed that immunity affects memory in flies. As described in chapter five, this would require a refinement of my experimental paradigm to allow for more statistical power.

Which phase of learning is affected? My previous results showed that memory, defined in the evolutionary sense, was affected by an immune response. But is it the formation of the memory or its recall that is affected. This is important as the physiology of

memory formation and recall are quite different and lead the investigations down different routes. Using the PER set up, I could distinguish which phase of memory is affected. Normally to discover a substance's specific effect on memory, it must be injected at different times, however LPS effects are long lasting (up to a week), so a slightly different approach could be used. One group of bees would be injected with LPS as before. Another would be injected several hours after training and left for three days before testing. If memory formation is affected then only the first group will show a decrease in PER ability, as the immune response will be generated too late to affect the second group. If it is recall of the memory that is affected then both the group injected after training and the first group should show an effect. Both groups will have their respective control groups. Of course, all groups would have their memory tested 3 days after initial training, to allow for meaningful comparisons.

Why is this work important? I believe it has two sources of interest. The first is as an attempt to understand the evolution of complex systems and the second as the beginning of creating an insect model for brain-immune interactions. Convergence of biological traits in disparate species is a fundamental aspect of biology. Although all organisms must answer the same questions, and should therefore develop similar responses, the extreme similarity of evolutionary solutions provides important insight into the deeper structure of the evolutionary process. The links found between the nervous system and the immune system in many varied animal systems suggests there is something deeper going on. Is there something in the very basic structure of these two systems that means they much be connected? It could be simply that two complex systems such as the

nervous and immune systems must interact due to the limited number of components that a body can provide. This would be a fruitful area for future comparative research.

Psychoneuroimmunology (PNI) has grown dramatically in the past three decades. Ader, Felten, and Cohen (1981) introduced the term psychoneuroimmunogy as the title for their book, which included reviews of the state of the art regarding the role of the central nervous system (CNS) in the complicated interplay of behavior and the immune system. The term neuro acknowledged the brain as an important aspect of this system. The journal Brain, Behavior, and Immunity was started in the late eighties by the same group as editors. The first issue covered topics ranging from conditioning the immune response and stress effects on immune measures in animal models to stress, health, and immune relationships in humans. Brain, Behavior, and Immunity is now the official journal of the PsychoNeuroImmunology Research Society, which was chartered in 1993. For a fuller history of the field of PNI see Fleshner & Laudenslager (2004). For me, my research begins the search for an insect model of PNI. Most of the effects found in PNI are to do with stress responses and the innate immune system. Both of these systems are very similar in insects and mammals. The development of an insect model of these important health concerns would be an enormous boost to the research. This thesis is my contribution.

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