# THE EFFECTS OF SOCIAL INTERACTIONS ON LEARNING AND MEMORY IN THE HONEY BEE *Apis mellifera*

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## Abstract

The honey bee *Apis mellifera* has been used to study the genetics of learning and memory for several decades. In Chapter 2, a review of the literature revealed that learning and memory phenotypes are highly heritable. Several quantitative trait loci and several specific genes which code for neurotransmitter receptors and transports have been identified. Whereas transcriptomic approaches showed that the process of learning and memory involves hundreds of genes. Although understanding the genetic components is crucial, it is also important to understand how environmental factors affect learning and memory. One environmental factor is social interactions. In Chapter 3, I investigate the effect of social interactions on discrimination learning by randomly assigning bees into three different social groups: 1 bee, 8 bees, and 32 bees. Using the proboscis extension conditioned response test, I found that the fewer social interactions a bee experiences, the more responsive she is to sucrose. Bees raised in groups of 32 had the best performance in discrimination learning.

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# Chapter One

# Overview

The study of learning and memory is an active area of research because it is a pivotal cognitive function in animals. Learning has been documented across a wide range of taxa, including mammals, birds, amphibians, reptiles, fish, and insects (e.g. Bisazza, et al. ; Capaldi, et al. 1999; Hoppitt and Laland 2008; Kieffer and Colgan ; Papaj and Prokopy 1989; Suboski 1992). It appears that learning is adaptive because it enables organisms to adjust their behaviour in response to cues from the environment, thus increasing their probability of survival (Alcock and Farley 2001). Learning and memory in honey bees is important as well and has been studied for several decades.

There is a substantial body of research on the genetics of learning and memory in honey bees. In Chapter 2, I present a review of the literature focused on the genetics of learning and memory in honey bees. Over the years, researchers have discovered that learning and memory is heritable in honey bees. They identified several important genes which affect different aspects of learning and memory. Transcriptomic approaches revealed that learning and memory is a process that involves hundreds of different genes. And population genetic studies showed that genes associated with cognitive processing are under positive selection.

Environmental factors affect learning and memory in honey bees as well. Food conditions (Fewell and Winston 1992) and exposure to pheromones (Pankiw, et al. 1998) are known to have an effect, for example. Interaction with nestmates is an important type of environmental stimulus. From the moment a honey bee emerges, it is surrounded by thousands of conspecifics from birth (Winston 1991). Workers interact with thousands of individuals and work cooperatively to feed the brood, maintain the hive, and forage for pollen and nectar. It is still unclear to what degree social interactions affect learning and memory in the honey bee. In

Chapter 3, I present an experiment exploring the effects of social interactions on learning and memory. I placed honey bees from emergence in groups of 1, 8, or 32 bees and tested them for sucrose responsiveness, discrimination learning, one hour memory, and 24 hour memory using the proboscis extension paradigm (PER). My experiment revealed several important insights on the relationship between social interactions, sucrose responsiveness, and learning and memory in honey bees.

# Chapter Two

# A Review of the Genetics of Learning and Memory in the Honey Bee *Apis mellifera*

## Introduction

Learning and memory are crucial cognitive functions in animals. Learning has been widely documented in mammals (Conway and Christiansen 2001; Hoppitt and Laland 2008; Koek 2011), birds (Capaldi, et al. 1999; Griffin 2004; Nowicki, et al. 2002), amphibians (Bisazza, et al. 1998; Hepper and Waldman 1992; Suboski 1992), reptiles (Bisazza, et al. 1998; Suboski 1992; Wilkinson, et al. 2010), fish (Brown and Laland 2003; Kieffer and Colgan 1992; Odling-Smee and Braithwaite 2003), and insects (Dukas 2008; Leadbeater and Chittka 2007; Papaj and Prokopy 1989). Even single-celled organisms have a basic form of learning (Armus, et al. 2006; Armus, et al. 2010). Learning is believed to be adaptive because it allows organisms to adjust their behaviour in response to cues from the environment, thereby increasing their probability of survival (Alcock and Farley 2001). Understanding the genetic basis of learning and memory is an active area of research in biology.

The honey bee, *Apis mellifera*, has served as a model for the study of learning and memory in insects. Several life history traits make learning and memory crucial to a honey bee's life. Honey bee colonies typically contain 20,000 to 60,000 workers that must recognize and communicate with other nestmates (Getz and Smith 1983; Michelsen 2003). Indeed, social bees tend to have better learning abilities than solitary bees (Dukas and Real 1991). As with other nest-building animals, honey bees must learn the location of their nest, which they do so via a specific orientation behaviour (Capaldi, et al. 2000; Lutz and Robinson 2013; Zeil 1993; Zeil, et al. 1996). They also must explore their surroundings to find profitable flowers, create spatial maps (Menzel, et al. 2005), and communicate the learned information via the waggle dance (Von Frisch 1967). Finally, honey bees are generalists and must learn to forage and manipulate

different plants that vary in their resource availability over short (i.e. hours of day) and intermediate (i.e. days to weeks) timescales (Von Frisch 1967). These life history traits likely contributed to the development of the honey bee's remarkable capacity for learning, which include generalization (i.e. treating similar stimuli as equivalent) (Stach, et al. 2004), learning contextual information (Collett, et al. 1997), and learning concepts of 'sameness' and 'difference' (Giurfa, et al. 2001).

The honey bee has many useful attributes for studying the genetics of learning and memory. Honey bee colonies are large and can be easily manipulated. This allows researchers to study large cohorts of bees with known age in both the lab and the field. It is also easy to manipulate several factors that can affect learning and memory, such as different food conditions (Fewell and Winston 1992), social conditions (Kadowaki 2006), and exposure to pheromones (Pankiw, et al. 1998). The honey bee's mating system also facilitates estimates of heritability of learning and memory. Queen honey bees are diploid and they mate with 10 to 20 different haploid males (drones) (Estoup, et al. 1994; Haberl and Moritz 1994; TABER III 1954; Tarpy and Nielsen 2002), which results in 10-20 different families of workers within a colony. Half sisters (i.e. workers from different fathers) are 25% related, while full sisters (i.e. workers from the same father) are 75% related (Winston 1991). Workers in a colony experience the same social environment and maternal effects, but can vary with respect to their paternal alleles. Differences in learning and memory between different patrilines within a colony can thus be used to estimate broad-sense heritability of learning and memory phenotypes (Laloi and Pham-Delegue 2010). Finally, the fully sequenced honey bee genome (Weinstock, et al. 2006) led to the development of genomic tools that have greatly improved our understanding of learning and memory at the

molecular level (Chandrasekaran, et al. 2011; Harpur, et al. 2012; Harpur and Zayed 2013; Kent, et al. 2012; Sarma, et al. 2009; Weinstock, et al. 2006; Zayed, et al. 2012).

Here I provide a review of the genetics of learning and memory in honey bees. There are several reviews on the nature of learning and memory in honey bees (Giurfa 2007; Menzel 1990; Menzel and Muller 1996), therefore I will focus on the genetics. I will first review traditional forward genetic studies documenting the degree to which learning and memory is heritable in honey bees, and the genetic architecture of learning and memory phenotypes. I will then review recent studies employing candidate gene studies, reverse genetic approaches, and transcriptional profiling to characterize genes involved in learning and memory in honey bees.

**What is learning and memory, and how are these processes measured?**

Learning and memory refers to many distinct but interconnected cognitive processes. Learning and memory first involves sensory input, such as a visual or olfactory signal, then working memory, which lasts a few minutes, and then the information can either be lost or transferred into longer term memory (Atkinson and Shiffrin 1968; Baddeley and Hitch 1975; Cowan 2008). Longer term memory can also be subdivided into one that lasts a few hours or a few days, since they have different molecular mechanism of formation (Izquierdo, et al. 1999; Rosenzweig, et al. 1993). The process of learning and memory in honey bees can be divided into the following modes: acquisition, consolidation (memory formation), retention (memory storage), and recall (memory retrieval) (Menzel and Muller 1996).

Studies of learning and memory in honey bees have predominantly used classical conditioning using the proboscis extension reflex (PER) (Fig. 2-1). In the basic form of the method, a neutral stimulus such as an odour (i.e. conditioned stimulus), is paired with a

biologically significant stimulus such as sucrose (i.e. unconditioned stimulus) that elicits the proboscis extension reflex (i.e. unconditioned response) (see Table 2-1 for definitions). After several such pairings, the insect extends its proboscis only after the presentation of the odour, without the sucrose, indicating that it learned to associate the odour with the reward; at that point, the proboscis extension reflex represents a conditioned response to the conditioned stimulus. The PER assay also allows for quantifying aversive learning by using non-rewarding unconditioned stimuli during testing (e.g. salt solution). By pairing one odor with sugar and another with salt, the bee can learn to discriminate between the two scents. Extinction of the response can be measured by looking at how many trials it takes for the insect to stop extending its proboscis when the scent is no longer followed by sugar reward; latent inhibition is measured by first exposing the bee to an odour (without a sugar pairing) and then measuring the lag in acquisition through the regular procedure; and habituation is measured by simply repeatedly stimulating the bee's antenna with a sugar solution and measuring when it stops to extend the proboscis. Using an easily observable response like the PER allows researchers to resolve some of the problems of studying complex processes like learning and memory.

**Heritability of learning and memory in honey bees**

Heritability represents that proportion of variance in a phenotype ($V_P$) that is caused by genetic factors, which can occur because of alleles with additive or dominance effects. Narrow sense heritability ($h^2$) represents the proportion of phenotypic variance that is caused by additive genetic variance ($V_A/V_P$) and is typically measured by quantifying resemblance between relatives. On the other hand, broad sense heritability ($H^2$) represents the contribution of both additive and dominance genetic variance to phenotypic variance ($[V_A+ V_D] /V_P$), and is

commonly measured by quantifying the proportion of within colony phenotypic variation that is caused by patriline variation. Several studies have estimated the heritability of learning and memory phenotypes in honey bees (Table 2-2), as summarized below.

*Heritability of learning and memory*

At first, studies focused on establishing whether learning and memory are heritable in honey bees. Several studies have reported large natural variation in worker bee's learning and memory abilities for the following processes: acquisition (learning to associate scent with reward), sensitization (repeated exposure to stimulus results in an increased response) discrimination (learning to associate one scent with a reward and another with a punishment), and reversal (learning that the previously rewarding scent is now followed by punishment) (Menzel and Bitterman 1983; Menzel, et al. 1974). In order to determine what leads to this variation in learning and memory, Bhagavan, et al. (1994) used the PER assay to study differences in learning and memory associated with worker age, social role, and genotype. Genotype was determined by selecting drones with different PER scores and then testing their worker progeny. Genotype was found to be the only significant factor affecting learning and memory in this study. However, later studies found that nurses, bees that mainly focus on larvae and brood care, have faster extinction rates than foragers, bees that mainly focus on food collection, in olfactory PER (Ben-Shahar, et al. 2000) and that really old foragers (15-57 day old) have a lower rate of acquisition than same aged nurses or younger foragers and nurses (Behrends, et al. 2007). Benatar, et al. (1995) selected drones based on discrimination PER performances and corroborated that only one generation of selection was sufficient to produce significant differences between the selected lines, suggest that contributes greatly to differences

in learning and memory between individuals. The Authors found large variation in learning and memory within the selected lines, suggesting that several to many alleles with small effects on learning and memory were still segregating within the selected lines.

After establishing that learning and memory has a considerable genetic component, several researchers focused on quantifying the heritability (Table 2-2). The first experiments to quantify the genetic nature of learning and memory in honey bees used *Apis mellifera capensis*, also known as the Cape bee. Workers from this subspecies are capable of thelytokous parthenogenesis, which produces practically identical female offspring (Verma and Ruttner 1983). Brandes (1988) used the unique genetics of Cape honey bee workers to estimate the heritability of learning and memory by selecting for olfactory PER performance. Using different quantitative genetic methods, he calculated several estimators of broad sense heritability utilizing a parent-offspring regression $H^2$=0.45, a sibling analysis $H^2$=0.54, and a realized heritability from response to selection $H^2$=0.39. Estimates of the narrow sense heritability produced a similar value ($h^2$=0.43), suggesting that alleles which affect learning and memory rarely exhibit dominance. In a follow-up study, Brandes (1991) found that only one generation of selection on the Cape honey bee results in lines with significantly different learning performance. Using partiline analysis, Laloi and Pham-Delegue (2010) estimated significant broad sense heritablities for learning acquisition and extinction ($H^2$=0.22 and $H^2$=0.13 respectively). Brandes and Menzel (1990) tested the genetic relationship between PER and visual learning of free-flying bees by creating ‘good’ and ‘poor’ learning lines using olfactory PER, and evaluating the performance of these lines on visual discrimination tasks. Bees from ‘good’ lines performed better on visual discrimination relative to bees from ‘poor’ lines, indicating that olfactory and visual learning are influenced – at least in part – by pleiotropic mutations. The

results above demonstrate that learning and memory has a substantial heritable component in honey bees, and that up to 50% of the phenotypic variance in learning and memory can be mostly explained by the segregation of alleles with additive effects.

*Sucrose responsiveness drives heritability of some learning and memory phenotypes*

Differences in learning and memory abilities could stem from either differences in perception, differences in processing, differences in storage, or differences in retrieval. The genetic and molecular nature of these processes could also be different and affected by different genes. Sucrose responsiveness tests the lowest sucrose concentration a bee will respond to with a proboscis extension (Scheiner, et al. 1999) and it is associated with some aspects of learning and memory. Scheiner, et al. (2001) found that the higher the sucrose responsiveness in foraging honey bees, the higher the tactile and olfactory acquisition scores. Sucrose responsiveness does not, however, appear to influence extinction. The extinction scores for tactile learning did not correlate with sucrose responsiveness in 75% cases. Pollen foragers are more responsive to sucrose than nectar foragers (Pankiw and Page Jr 1999) and the differences between pollen and non-pollen foragers in sucrose responsiveness found throughout the season correlates with differences in tactile and olfactory acquisition; and sucrose responsiveness positively correlates with tactile and olfactory acquisition (Scheiner, et al. 2003). Using nectar foragers from a naturally mated colony, Scheiner (2004) found that habitation and dishabituation scores were correlated with both sucrose responsiveness and sucrose concentration. Sucrose responsiveness is associated with acquisition, habituation and dishabituation, but not extinction.

Selection experiments on pollen hoarding determined that sucrose responsiveness has a genetic component. Page and Fondrk (1995) performed an artificial selection experiment by

selecting colonies with high and low pollen stores respectively for several generations. Differences in pollen load in foraging bees were seen at the first generation, and differences in the number of pollen and non-pollen foragers were observed by the third generation of selection. Hunt, et al. (1995) crossed these lines and mapped two major quantitative trait loci (QTL) affecting pollen hording, *pln1* and *pln2*. The high pollen lines bees were more responsive to sucrose than the low pollen lines (Page Jr, et al. 1998; Pankiw and Page Jr 1999) and it was subsequently determined that the genetic loci that affect pollen hoarding also affect sucrose responsiveness (Rueppell, et al. 2006).

Sucrose responsiveness appears to affect learning through reward perception, therefore it affect the aspects of learning that most depend on reward (sucrose) concentration, such as acquisition, reversal learning (Scheiner, et al. 1999) and habituation (Scheiner 2004). Extinction does not always correlate with sucrose responsiveness (Scheiner, et al. 2001): it correlates with a stimulus that was previously rewarded, but not with stimuli that was previously unrewarded. Sucrose responsiveness is affected by environmental conditions (Pankiw and Page Jr 2003; Pankiw and Page 2001), but has a definite genetic component (Rueppell, et al. 2006). Unfortunately, there are no selection studies linking the genetics of sucrose responsiveness to learning and memory, therefore the evidence presented here is only indirect: when pollen hoarding is selected for, sucrose responsiveness is affected, which in turn affects some aspects of learning and memory.

*Quantitative Trait Loci affecting learning and memory*

Quantitative Trait Loci (QTLs) are regions of the DNA in which a gene (or a transcription factor) is present that affects a certain phenotypes. Thus, identifying a QTL that

affects learning and memory is the first step in identifying the gene that affects the phenotype. Latent inhibition and reversal learning abilities are heritable and seem to correlate in honey bees (Chandra, et al. 2000; Ferguson, et al. 2001). Queens and drones from fast and slow reversal lines (Ferguson, et al. 2001) were tested for reversal learning after discrimination testing. Fast reverser queens were inseminated by a single slow-reverser drone. The resulting offspring were reared into queens and allowed to mate naturally (Chandra, et al. 2001). The drone offspring were tested for reversal learning and latent inhibition. The authors identified two QTLs (*lrn2* and *lrn3*) that explained a total of 27% of the phenotypic variance for reversal learning, and a QTL (*lrn1*) that explained 14.1% of the phenotypic variance for latent inhibition (Chandra, et al. 2001). The latent inhibition QTL did not map onto either of the reversal learning QTLs, indicating that they are not genetically linked. Unfortunately this study was performed prior to the sequencing of the honey bee genome and used anonymous genetic markers for mapping; the study cannot be used to identify the candidate genes underlying these QTLs.

Sucrose responsiveness correlates with both pollen hoarding behaviour and learning and memory (see above). Using bees from different pollen hoarding lines, Rueppell, et al. (2006) measured sucrose responsiveness and conducted genetic analysis in search for QTLs. A queen from a low pollen hoarding line (Page and Fondrk 1995) was instrumentally inseminated by a drone from a high pollen hoarding line (Rueppell, et al. 2006). The resulting offspring were reared into queens and were inseminated with either low-line or high-line drones. The progeny were tested for sucrose responsiveness and then genetically analyzed for QTLs that are involved in pollen hoarding (Hunt, et al. 1995; Page, et al. 2000; Rüppell, et al. 2004) and new QTLs. In the high backcross, 11.7% of the phenotype variance could be explained by a putative QTL that maps onto *pln1* and in the low backcross 69.3% of the variance could be explained by a single

QTL and another 14.0% of variance by a second, putative QTL. Later, Hunt, et al. (2007) identified the potential genes that are present in the QTLs affecting sucrose responsiveness. These genes include a tyramine receptor (see below), as well as *Amfor*, a gene associated with the onset of foraging (Ben-Shahar, et al. 2002) and sucrose responsiveness (Thamm and Scheiner 2014).

**Transcriptomics**

Analysis of global brain gene expression can provide insights about the molecular and genetic basis of learning and memory, and several transcriptomic studies have highlighted genes that may be associated with learning and memory in honey bee workers. Worker bees perform in-hive duties, such as nursing, then transition to foraging (Winston 1991). The nurse to forager transition is associated with large changes in the bee physiology (Robinson 2002) and an expansion in the volume of the mushroom bodies (Withers, et al. 1993) – the brain regions associated with learning and memory in insects. Whitfield, et al. (2003) compared brain gene expression in nurses and foragers, and discovered gene expression differences at more than 2000 genes (out of the 5500 studied). These included *foraging,* which is important for visual memory in *Drosophila* (Kuntz, et al. 2012), and a carbonic anhydrase gene, which functions in learning and memory in mammals (Sun and Alkon 2002). A follow up study contrasted young and old workers and drones; both drones and workers undergo a process of behavioural maturation, and the microarray experiments allowed the researchers to identify genes that were uniquely associated with worker behavioural maturation (Zayed, et al. 2012). The authors were able to identify 565 genes that were specifically associated with worker maturation. These genes were enriched for learning and memory associated processes and included genes involved in Notch

signalling, which is involved in spatial learning and memory in mammals (Yoon and Gaiano 2005) and long term memory in *Drosophila* (Ge, et al. 2004; Matsuno, et al. 2009). In addition to the general contrasts between nurses and foragers, Naeger, et al. (2011) directly examined changes in brain gene expression associated with learning rewards over space and time. The authors trained two groups of honey bees to feed at two artificial feeders at different locations, one had sucrose solution in the morning, while the other in the afternoon. The trained bees were collected both in the morning and afternoon and a brain microarray analyses was performed, allowing the authors to explore the transcriptional differences related to the formation of spatiotemporal memories. After removing the genes that were expressed differently due to time of the day, time of training, and food anticipation, 352 out of the 1329 differently expressed genes were associated with either formation or existence of spatiotemporal memories. Those genes were involved in synaptogenesis, synapse organization and biogenesis, based on gene ontology (GO) analysis. Further, Wang, et al. (2013) trained honey bees with olfactory PER conditioning and compared their brain gene expression to bees which underwent similar handling, but did not receive any training. 259 genes were differentially expressed after olfactory learning, including the octopamine receptor, muscarinic acetylcholine receptor, and nicotinic acetylcholine receptor alpha6 subunit.

**Reverse genetic approaches for studying learning and memory in honey bees**

The reverse genetic approach involves studies of genes and their products in an attempt to discover the phenotypes they influence. In the honey bee, studies of gene function are greatly aided by the wealth of knowledge borrowed from genetic studies of *Drosophila* (Eisenhardt, et al. 2001; Wachten, et al. 2006) and model vertebrates (Knapska and Kaczmarek 2004).

Molecular studies on candidate learning and memory genes have greatly enhanced our knowledge of the genetic of learning and memory in honey bees, as summarized below.

*cAMP signalling*: The cAMP signalling pathway is crucial for learning and memory in vertebrates and invertebrates (Kandel 2012). cAMP-dependent protein kinase (PKA), cAMP response element binding protein (CREB), and the messenger adenylyl cyclase are central parts of this pathway and these components were identified in the honey bee (Eisenhardt, et al. 2001; Eisenhardt, et al. 2003; Wachten, et al. 2006). Wachten, et al. (2006) used cDNA, protein analysis, and immunocytochemistry to identify a membrane-bound adenylyl cyclase gene, *Amac3*, which shares 62% amino acid identity with *Drosophila ac39E*. Another study characterized the honey bee's CREB protein, which is encoded by the *AmCREB* gene (Eisenhardt, et al. 2003). *AmCREB* is expressed as eight different transcripts in the honey bee mushroom bodies, among other locations, and shares between 32%-86% amino acid identity with a variety of different mammalian CREB and CREM (cAMP response element modulator) proteins' functional domains (Eisenhardt, et al. 2003). Eisenhardt, et al. (2001) identified a PKA catalytic subunit, which shares 80-94% amino acid identity with *Drosophila melanogaster* and mammals, and is expressed in the mushroom bodies of the honey bee. Fiala, et al. (1999) used RNAi to showed that the downregulation of PKA activity resulted in the impairment of 24 hour and longer term memory, but not impairment of acquisition or shorter term (3 or 6 hr) memory. Later, Müller (2000) showed that PKA inhibition during training, but not after training, leads to longer term (3 days) memory impairment, implicating the pathway in long term memory consolidation.

*Neurotransmitters*: Several neurotransmitters and their receptors are involved in learning and memory in honey bees (reviewed by Giurfa 2007), including cholinergic receptors, such as octopamine and dopamine (Menzel, et al. 1999), glutamate receptors (Maleszka, et al. 2000), and nicotinic receptors (Lozano, et al. 1996; Thany and Gauthier 2005). Specific genes that code for these receptors have been identified (Table 2-3) (Beggs, et al. 2005; Grohmann, et al. 2003; Jones, et al. 2006; Kucharski, et al. 2000; Wachten, et al. 2006) and some have been directly implicated in learning and memory in the honey bee (Farooqui, et al. 2003; Kucharski, et al. 2007). Here I present information on the genes coding for particular receptors and transporters of those neurotransmitters that have been shown to affect learning and memory in honey bees.

Glutamate: Glutamate is an excitatory neurotransmitter implicated in learning and memory in animals (reviewed by Siegel, et al. 1999). Kucharski, et al. (2000) found *AmEAAT*, a putative glutamate transporter gene, using cDNA, *in situ* hybraidization, and northern blotting to be primarily expressed in the optic lobes and Kenyon cells (local neurons) of the mushroom bodies. It shares 54% amino acid identity with a human EAAT2 subtype. Glutamate is one of the most abundant neurotransmitters in the honey bee adult brain (Bicker 1999; Fuchs, et al. 1989) and glutamate transporter inhibitor injections into the brain impaired 24 hour memory, but not acquisition or 1 hour memory, in an olfactory PER procedure in a dose dependent manner (Maleszka, et al. 2000). The impairment can be rescued by memantine, a non-competitive NMDA receptor (see below) antagonist (Parsons, et al. 1999), when injected before training with the inhibitor or before testing, indicating that it is rescuing recall (Si, et al. 2004). This rescuing effect adds evidence that glutamate transport is important for learning and memory.

Glutamate receptors separate into 2 main categories: Metabotropic glutamate receptors (mGLuRs) and ionotropic glutamate receptors (iGluR) (Vandenberg 1998). In mammals, mGluRs are essential for memory formation, but not for learning new information, while *N*-methyl-D-aspartate (NMDA) receptors, a family of iGluR, are involved in acquisition and memory formation (reviewed by Riedel, et al. 2003).

Several genes coding for mGluR were characterized in the honey bee. Funada, et al. (2004) identified two distinct genes for mGlu receptors, *AmGluRA* and *AmGluRB*, using cDNA, and RT-PCR. However, Mitri, et al. (2004), used sequence comparison and three-dimensional modeling, reached the conclusion that the gene *AmGluRB* (named *HBmXR* in the article), although in its overall sequence similarity falls into the mGluRs, produces a protein that probably does not bind glutamate, due to an amino acid substitution in the ligand binding pocket. *AmGluRA* is a genuine metabotropic glutamate receptor (Kucharski, et al. 2007), which shares 65% amino acid identity with *Drosophila* and 43-45% amino acid identity with vertebrate mGlu receptors. Injections of both *AmGluRA* agonists and antagonists prior to training, but not post training, impaired 24 hour memory in an olfactory PER, but had not effect on 1 hour memory (Kucharski, et al. 2007). These results show that *AmGluRA* is required for long term memory formation.

iGluRs are not well studied in the honey bee and only one gene for the NMDA receptor was found. The NR1 subunit is common to all NMDA receptor variants and its splice variants influence diverse NMDA receptor properties, such as pH responsiveness, $Zn^{2+}$ inhibition, and the deactivation rates (Cull-Candy, et al. 2001). *AmNR1-1* is expressed in the brain, neurons and glial cells of the honey bee and shares 67.5% amino acid identity with *Drosophila* and 48%

identify with humans (Zannat, et al. 2006). Treatments with MK-801, a NMDA antagonist, impaired 24 hour memory when injected either before training or before testing, indicating NMDA receptors affect recall of long term memories, but not perception, acquisition or 1hour memory (Si, et al. 2004).

Octopamine: Octopamine is a neurotransmitters that is associated with several complex behaviours in insects (Roeder 2005). Ventral unpaired median (VUM) neurons receive input from sucrose receptors and the activation of the neuron $VUM_{mx1}$ instead of sucrose reward in an olfactory PER trials is sufficient for learning to occur (Borsuk-Bialynicka, et al. 1993). VUM neurons stain with an antibody against octopamine, which suggests that octopamine is the neuron's main transmitter (Kreissl, et al. 1994). Paired injections of octopamine with an odour, led the honey bees to respond to the odour as though it was paired with sucrose (Hammer and Menzel 1998), suggesting that it is important for acquisition. In 2003, Grohmann, et al. (2003) identified and characterized *Amoa1*, the octopamine receptor gene, using cDNA, *in situ* hybridization, and transiently transfected human embryonic kidney (HEK) 293 cells. Octopamine injections and oral treatments increased sucrose responsiveness (Scheiner, et al. 2002), once again implicating the neurotransmitter in acquisition.

Tyramine: Tyramine is thought to act mainly as the metabolic pre-curser for octopamine (Braun and Bicker 1992). However, Blenau, et al. (2000), using membrane homogenates of honey bee brains, found that tyramine inhibited cAMP production more potently than octopamine. The authors also identified and characterized a receptor gene, *AmTyr1*, suggesting tyramine has a role not dependent on octopamine in the honey bee. Since tyramine has similar affects to octopamine

(Braun and Bicker 1992) and increases sucrose responsiveness (Scheiner, et al. 2002) it probably also affects acquisition.

Dopamine: Dopamine is crucial for aversive olfactory learning in honey bees and other invertebrates (reviewed by Giurfa 2007). Vergoz, et al. (2007) injected dopaminergic receptor antagonists into the honey bee brain, which supressed aversive learning in a sting extension response conditioning, indicating it affects aversive acquisition. Dopamine receptors are categorized into two groups: D1-like receptors, which increase cAMP, and D2-like receptors, which either reduce cAMP or act through a different pathway (reviewed by Missale, et al. 1998). In honey bees, *Amdop1,* which shares 93% amino acid identity with *Drosophila* and 76% with humans, and *Amdop2,* which shares 76% amino acid identity with *Drosophila,* code for a D1-like receptor (Blenau, et al. 1998; Humphries, et al. 2003), and *Amdop3* codes for a D2-like receptor (Beggs, et al. 2005; Humphries, et al. 2003). Agarwal, et al. (2011) conditioned honey bees in a place avoidance paradigm, where a mild electric shock was applied to one side of the platform. Dopamine treated bees spent less time in the punishment zone during training, while pimozide, a dopamine antagonist, lead to longer time spent in the punishment zone. Dopamine treated honey bees also learned better than untreated bees. Scheiner, et al. (2002) injected dopamine into honey bees' thorax and saw a decrease in sugar responsiveness, but feeding dopamine to the honey bees had no effect. Both injections and oral treatments with a dopamine receptor agonist, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (6,7-ADTN), decreased sucrose responsiveness.

Acetylcholine: Acetylcholine, acting through either muscarinic or nicotinic acetylcholine receptors, helps encode new memories in animals (reviewed by Hasselmo 2006)). One

muscarinic acetylcholine receptors (mAChR) was identified in the honey bee (Whitfield, et al. 2002), and pharmacological manipulations suggest that mAChRs are involved with memory retrieval in honey bees. For example, scopolamine and atropine, both muscarinic acetylcholine receptor antagonists, impair shorter term memory retrieval in honey bees (Gauthier, et al. 1994; Lozano and Gauthier 1998), but pirenzepine, also a muscarinic acetylcholine receptor antagonist, has no effect on learning and memory (Lozano and Gauthier 1998). Neuronal nicotinic acetylcholine receptors (nAChRs) effect learning and memory in mammals (Hogg, et al. 2003). In mice, there are 12 different neuronal subunits, and knockdown of the different subunits produce different impairments, among them are impairments to learning and memory (reviewed by Lindstrom 2001). In honey bees, 11 genes coding for nAChR subunits were found (Table 2-3) (Jones, et al. 2006; Thany, et al. 2005; Thany, et al. 2003). Lozano, et al. (1996) injected nicotinic receptor antagonist into the honey bee brain prior to training and found that it impaired acquisition and 1 hour memory; injections post training also produced impairment at 1 hour memory, implicating it in recall of short term memory. Thany and Gauthier (2005) injected nicotine (an agonist) post training and saw improvement in short term memory (30 and 60 min), but not 5 or 10 minute memory, indicating that it is acting at the end of consolidation to facilitate recall.

Imidacloprid, a chlorinated derivative of nicotine, is an insecticide that activates nACh receptors (Buckingham, et al. 1997; Leech, et al. 1991; Sattelle, et al. 1989). Guez, et al. (2001) treated honey bees with Imidacloprid, which lead to a slower habituation in 7 day old honey bees and a quicker habituation in 8 day old bees 15min and 1h after the treatment, and a slower habituation in both age groups 4 hours after treatment. In a follow up study, Guez, et al. (2003) found that it was the metabolite of imidacloprid, olefin, that increased the number of trials

needed for habituation in both age groups and that another metabolite, 5-hydroxy-imidacloprid, decreased the number of trials in the 8 day old bees. This indicates that these metabolites are specific agonists of two nACh receptor subtypes. Decourtye, et al. (2003) treated honey bees with Imidacloprid and 5-hydroxy-imidacloprid and saw a reduction in olfactory PER performance. Later, Decourtye, et al. (2004) tested olfactory performance in free-flying honey bees, which were taught to distinguish between different feeders based on odours, and tested olfactory PER performance, as well. Imidacloprid treated honey bees performed worse in both tasks compared to untreated controls. Unfortunately due to the nature of the PER protocol used in these studies, it is impossible to determine which learning and memory process is affected by Imidacloprid.

*Early growth response protein 1 (Egr-1)*: *Egr-1* is a transcription factor that is associated with learning and novelty detection in many vertebrates (Knapska and Kaczmarek 2004). A honey bee homolog of Egr-1, *Egr,* which shares 89% amino acid identity in the DNA-binding domain with the mouse was found using BLAST (Lutz and Robinson 2013). Using both *in situ* and qRT-PCR analysis, this gene was found to be upregulated in the mushroom bodies, when honey bees were exposed to a novel environment, suggesting an association with learning in bees (Lutz and Robinson 2013).

*Malvolio*: The *malvolio (mvl)* gene was found to influence responsiveness to sucrose in *Drosophila melanogaster* (Rodrigues, et al. 1995). The gene encodes a manganese transporter (Supek, et al. 1996) that when interrupted by mutation, reduces responsiveness to sucrose, which can be rescued by oral treatment with manganese (Orgad, et al. 1998). Ben-Shahar, et al. (2004) identified and characterized the honey bee ortholog, *Amvl*, which shares 80% protein sequence

similarity to the *Drosophila* gene, using cDNA, RT-PCR, and in-situ *hybridization*. The authors also treated honey bees with manganese and zinc (an antagonist of *malvolio* (Orgad, et al. 1998)) and found that honey bees treated with manganese have a significantly increased sucrose responsiveness when compared to sugar control, zinc, and zinc+manganese treated bees.

*Foraging*: The *foraging* gene encodes cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG), and is over 70% similarity to *Drosophila dg1* and *dg2*, and mammalian *pkg1* genes (Ben-Shahar 2005; Ben-Shahar, et al. 2002). It is found in two splice variants, one which correlated with sucrose responsiveness (Thamm and Scheiner 2014). The authors also treated bees with cGMP and found that increased PKG activity increased sucrose responsiveness.

**Population genetics of learning and memory in honey bees**

Although learning and memory are believed to enhance fitness, we do not currently know the extent of natural genetic variation in learning and memory genes, and the degree to which learning and memory genes evolve through natural selection. Population genetic studies of two candidate genes (*foraging* and *Erk7*) that are involved in honey bee neurobiology documented evidence of positive selection on portions of *Erk7* (Kent, et al. 2011) and portion of *foraging* (Harpur, et al. 2014). Additional studies documented that genes associated with neurobiology, and genes associated with worker behavioural traits, are located in parts of the honey bee genome containing the highest rates of genetic diversity, and where natural selection works most efficiently because of high recombination (Kent, et al. 2012; Kent and Zayed 2013). Recently, Harpur, et al. (2014) sequenced the genomes of 39 *Apis mellifera* and its sister species *Apis cerana* and identified genes and regulatory sequences with signatures of positive selection. Genes with signatures of positive selection were enriched for many gene ontology terms

associated with learning and memory, including: Olfactory receptor, odorant binding, sensory perception of chemical stimulus, cognition, sensory transduction, and G-protein coupled receptor proteins and signalling pathways (See Dataset S4 in ref. Harpur, et al. Accepted). G-protein coupled receptor proteins act by translating sensory information into cellular response, and are important for learning and memory in animals (Poulin, et al. 2010). High genetic variation and positive selection in genes associated with learning and memory indicates that learning and memory is evolutionary important.

**Conclusion**

The honey bee is a model eusocial organism and researchers in the field have made great strides in understanding the genetics of learning and memory in the bee over the two decades. Learning and memory has a strong genetic component in the honey bee; most learning and memory related phenotypes studied in the bee had high heritability (~25-50%). Alleles which influence variation in learning and memory often have additive effects, and can sometimes have pleiotropic effects on different aspects of learning and memory. It is becoming increasingly apparent that genes involved in neurobiology and behaviour are conserved across evolutionary time (Reaume and Sokolowski 2011; Zayed and Robinson 2012). It is not surprising, then, to observe that genes and pathways that influence learning and memory in vertebrates and in *Drosophila* also influence learning and memory in honey bees. High sequence similarity (~80%) for some learning and memory genes have been observed in honey bees and mammals (Eisenhardt, et al. 2001), which indicates the presence of a core set of for learning and memory genes that existed in the ancestor of insects and mammals approximately 545 to 670 million years ago (Erwin 1999) . This indicates that some learning and memory pathways are as old as

certain immunity proteins (Krem and Cera 2002) and Hox gene cluster (de Rosa, et al. 1999; Garcia-Fernàndez 2005).

There could also be novel genes for learning and memory in the honey bee. The honey bee genome has 182 orphan genes, with no orthologs or homologues in other organisms (Johnson and Tsutsui 2011). From the 259 genes that exhibited differentially expression after olfactory learning 89 are of unknown function (Wang, et al. 2013), suggesting that novel genes are associated with learning and memory in honey bees. A population genomics study also discovered that novel genes play an important role in adaptive evolution in the honey bee (Harpur, et al. 2014). Recent studies of *Drosophila* revealed that approximately 50% of novel genes are expressed in the brain and that these genes regulate behaviour (Chen, et al. 2013). Going forward, it will be important to determine the degree to which novel genes contribute to learning and memory in the honey bee.

I recommend and encourage researchers in the field to employ other assays to learn about the genetics and molecular biology of learning and memory in honey bees. Most of the research on the genetic and molecular basis of learning and memory in honey bees was based on the PER paradigm. PER, although a useful method, has some disadvantages. For example, bees have to be restrained, which likely imposes some stresses and precludes the observation of learning in the context of a honey bee's natural behavioural repertoire. Moreover, certain types of learning and memory, such as spatial learning, cannot be comprehensively investigated using PER. Also, there is no standard way to perform the assay, which leads to some discrepancies in the literature (Frost, et al. 2011). Honey bees have been trained to navigate mazes (Zhang 2006), recognize faces (Dyer, et al. 2005) and different visual patterns (Srinivasan 1994). Developing new high

throughput learning and memory assays that utilize free flying bees would greatly facilitate studies of the genetics and molecular basis of learning and memory in bees.

## Tables and Figures

Table 2-1. Glossary. The definitions of specific terms as used in this review.

| | |
|---|---|
| **Proboscis Extension Response (PER)** | The extension of a bee's proboscis when sucrose is touched to her antenna. |
| **Acquisition** | The process of learning new information and relationships. |
| **Classical Conditioning** | A type of learning where an unconditioned stimulus is paired with an unconditioned response. |
| **Unconditioned Stimulus (US)** | A stimulus naturally capable of eliciting a specific response. |
| **Unconditioned Response (UR)** | A response that occurs naturally to a stimulus without learning. |
| **Conditioned Response (CR)** | A response that occurs to a stimulus after learning. |
| **Conditioned Stimulus (CS)** | A stimulus learned to elicit a specific response. CS+ refers to training with a positive reinforcement. CS- refers to training with positive punishment or no reinforcement. |
| **Latent Inhibition** | A familiar stimulus takes longer to associate with a meaning than a new stimulus. |
| **Positive Reinforcement** | When a targeted behaviour is followed by a pleasant stimulus and as a result the likelihood of that behaviour increases. |
| **Negative Reinforcement** | When a targeted behaviour is followed by the removal of an unpleasant stimulus and as a result the likelihood of that behaviour increases. |
| **Punishment** | When a targeted behaviour is followed by an unpleasant stimulus and as a result the likelihood of that behaviour decreases. |
| **Extinction Response** | A reduction in a conditioned response after repeated exposure to a conditioned stimulus without the associated reward. |
| **Habituation** | A reduction in response after repeated exposure. |
| **Sensitization** | An increase in response after repeated exposure. |
| **Discrimination** | Learning to distinguish two stimuli. |
| **Aversive Learning** | A type of learning where an unconditioned response is paired with an unconditioned unpleasant stimulus. |
| **Reversal Learning** | An animal is first trained to differentiate between 2 stimuli using reward and punishment, and then trained the opposite pattern, where the previously rewarded stimulus is now punished and vice versa. |
| **Haplodiploidy** | A sex determining system where males develop from unfertilized eggs and are haploid and females develop from fertilized eggs and are diploid. |
| **Narrow sense heritability ($h^2$)** | Takes into account additive genetic effects from a combination of alleles. $h^2 = \frac{V_a}{V_t}$ , a value of 1 indicates that all of the variation can be accounted for by allelic effects. A value of zero indicates no effect of alleles on the trait. |

| | |
|---|---|
| **Broad Sense heritability ($H^2$)** | Takes into account additive, dominant, epistatic, and maternal and paternal effects. $H^2 = \frac{V_g}{V_t}$, a value of 1 indicates that all variation is genetic. A value of zero indicates all the variation is due to environmental effects. |
| **Forward genetics** | Focus on discovering the genetic basis of a phenotype. Usually includes crossbreeding and genetic mapping. |
| **Reverse genetics** | Focus on discovering the phenotype that is generated by a gene(s). Usually includes mutating the gene(s) in question to generate an aberrant phenotype. |
| **Additive genetic effects** | The phenotypic trait is inherited such that the combined effects of alleles equals to the sum of their individual effects. |
| **Dominant genetic effects** | The phenotypic trait is inherited such that the combined effects of alleles equals to one of the individual alleles. |
| **Parthenogenesis** | Form of asexual reproduction where females produce identical female offspring from unfertilized eggs. |
| **Caste** | The female honey bees can be divided in two castes: the queen, the only reproductive, and the worker, which preforms all of the non-reproductive duties. Caste is determined environmentally, not genetically. |
| **Nurse (social role)** | Usually a young worker (0-2 weeks of age), performing in-hive activities |
| **Forager (social role)** | Usually old worker (2 weeks until death), performing out-of-hive activities of gathering nectar and pollen. |
| **Single Drone Insemination (SDI)** | A virgin queen is artificially inseminated using only one drone. The resulted workers are 75% genetically identical. |
| **Sucrose Responsiveness** | A test to determine at which concentration of sucrose the honey bee responds with proboscis extension. The lower the sucrose concentration the higher the responsiveness. |

Table 2-2. Quantitative heritability of learning and memory.

| Phenotype | Cross | Method | Result | Reference |
|---|---|---|---|---|
| Acquisition | Naturally mated queens. | Patriline (broad sense) | $H^2$=0.22 | (Laloi and Pham-Delegue 2010) |
| Discrimination | Tested workers laid eggs to produce workers to be tested (parthenogenesis). | Parent-offspring regression (broad sense) | $H^2$ = 0.45 | (Brandes 1988) |
| Discrimination | Tested workers laid eggs to produce workers to be tested (parthenogenesis). | Sib analysis (broad sense) | $H^2$ = 0.54 | (Brandes 1988) |
| Discrimination | Tested workers laid eggs to produce workers to be tested (parthenogenesis). | Realized heritability (broad sense) | $H^2$ = 0.39 | (Brandes 1988) |
| Discrimination | Tested workers laid eggs to produce queens (parthenogenesis), which were mated with drones, to produce workers to be tested. | Offspring – parent regression (narrow sense) | $h^2$ = 0.43 | (Brandes 1988) |
| Discrimination | Workers tested to produce G1. | One-way ANOVA (source population - narrow sense) | $h^2$= 0.235 | (Brandes 1991) |
| Discrimination | Tested workers laid eggs to produce workers to be tested (parthenogenesis). | Nested ANOVA (G1- narrow sense) | $h^2$=0.588 | (Brandes 1991) |
| Discrimination | G1 workers tested to produce G2. | One-way ANOVA (G1-broad sense) | $H^2$= 0.420 | (Brandes 1991) |
| Discrimination | G1 Tested workers laid eggs to produce G2 workers to be tested (parthenogenesis). | One-way ANOVA (G2-broad sense) | $H^2$= 0.478 | (Brandes 1991) |
| Extinction | Naturally mated queens. | Patriline (broad sense) | $H^2$=0.13 | (Laloi and Pham-Delegue 2010) |

Table 2-3. Neurotransmitter receptor/transporters which are implicated in learning and memory with their corresponding genes. *AmGLuRB** was found to not code a protein which responds to glutamate in a later study (Mitri, et al. 2004).

| Phenotypes | Receptor/Transporter | Expression | Gene | Reference |
|---|---|---|---|---|
| Acquisition | Tyramine receptor | Brain and subeseophageal ganglion | *Amtyr1* | (Blenau, et al. 2000) |
| Appetitive Learning Acquisition | Octopamine Receptor | Mushroom body intrinsic neurons | *Amoa1* | (Grohmann, et al. 2003) |
| Aversive Learning Acquisition | D1 like dopamine receptor | Brain neuropils, including the mushroom body | *Amdop1* | (Blenau, et al. 1998) |
| Aversive Learning Acquisition | D2 like dopamine receptor | Mushroom body | *Amdop2* | (Humphries, et al. 2003) |
| Aversive Learning Acquisition | D2 like dopamine Receptor | Mushroom bodies, antennal lobes and optic lobes | *Amdop3* | (Beggs, et al. 2005) |
| Long Term Memory Consolidation | Glutamate Receptor | Adult and pupal brain, optic lobes and central neuropils | *AmGluRA* | (Kucharski, et al. 2007) |
| Long Term Memory Consolidation | Glutamate Receptor | *AmGluRA* - Brain, abdomen, and thorax, *AmGLuRB* - primary in brain | *AmGluRA* *AmGLuRB** | (Funada, et al. 2004) |
| Long Term Memory Recall | Glutamate transporter | Mainly in the brain: optic lobes and inner compact Kenyon cells of the mushroom bodies | *AmEAAT* | (Kucharski, et al. 2000) |
| Long Term Memory Recall | NR1 subunit of NMDA receptor | Brain, neurons and in glial cells | *AmNR1-1 (nmdar1)* | (Zannat, et al. 2006) |
| Short Term Memory Recall | Nicotonic Acetylocholine receptor gene family | Dorsal lobes, and mushroom bodies | *Amelα2, Amelα7-1, Amelα7-2* | (Thany, et al. 2005) |
| Short Term Memory Recall | Nicotonic Acetylocholine receptor gene family | In larvae: subeophageal ganglia. In adults: optic lobes, dorsal lobes, antennal lobes, calyces of MB | *Amelα3* | (Thany, et al. 2003) |
| Short Term Memory Recall | Nicotonic Acetylocholine receptor gene family | Larvae and ouoae stages, adult brains, optic lobes | *Amelα1, Amelα2, Amelα3, Amelα4, Amelα5, Amelα6, Amelα7, Amelα8, Amelα9, Amelβ1, Amelβ2* | (Jones, et al. 2006) |

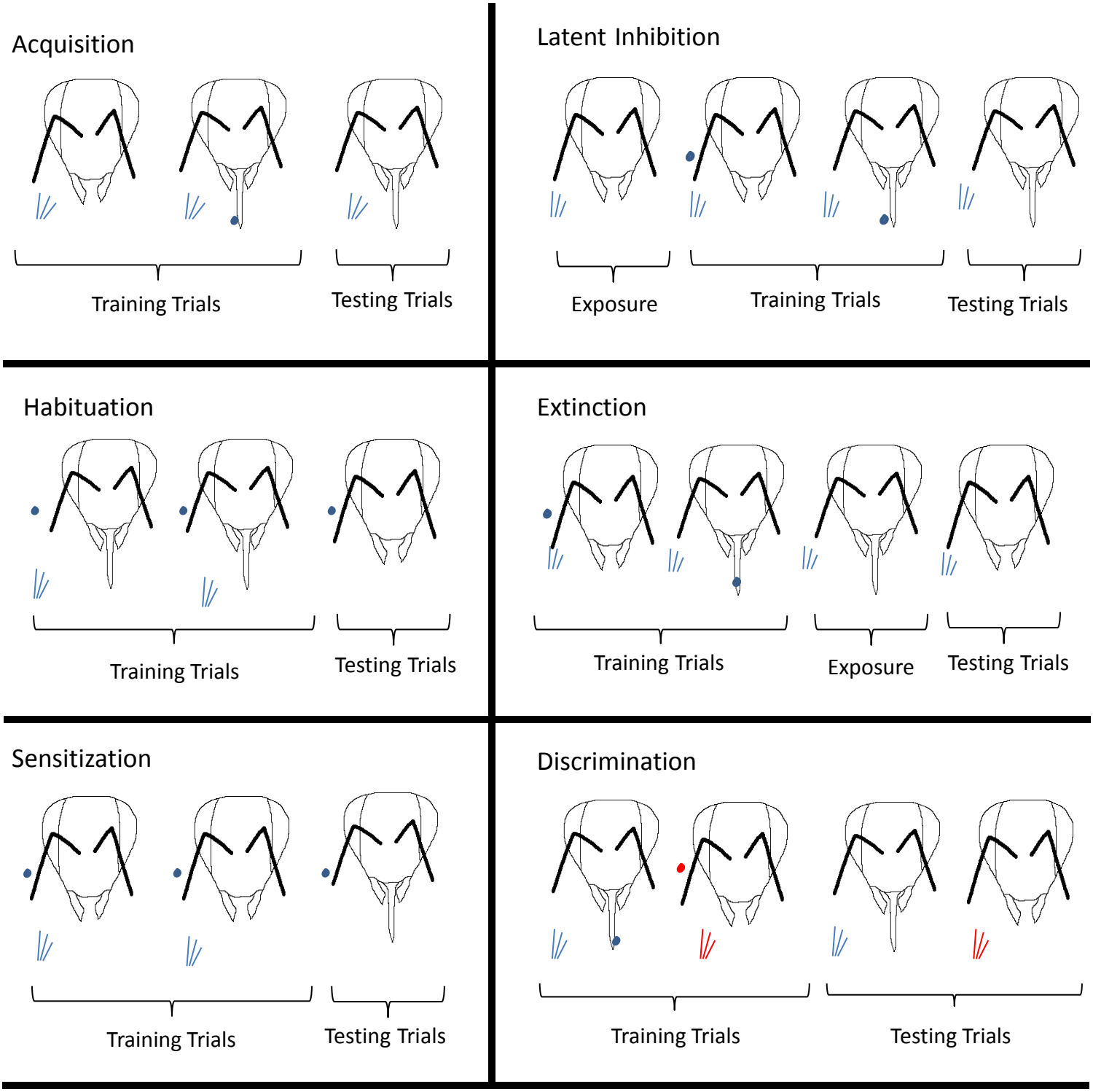


Figure 2-1. Different learning and memory measures using the proboscis extension response (PER). **Acquisition** – pairing a scent with a sugar reward and then testing the learned response just by presenting the sent. **Latent Inhibition** - presenting the scent prior to training, which will create a delay in the acquisition. **Habituation** – presenting a sugar drop on antenna several times such until the automatic proboscis extension stops. **Extinction** – acquisition training, which is followed by exposure to the learned stimulus without reward, until response stops. **Sensitization** – presenting a low concentration sugar drop on antenna several times, until response it seen. **Discrimination** – Paining one scent with sugar reward and a different scent with salt punishment.

# Chapter Three

# Effects of Social Interactions on Learning and Memory in the Honey Bee, *Apis mellifera*

## Introduction

Maternal care is a characteristic of mammals and social interactions with conspecifics is often typical. Social isolation in mammals often leads to abnormal brain development and behaviour (Fone and Porkess 2008; Harlow, et al. 1965; Koike, et al. 2009; Pan, et al. 2009). In rats, for example, social isolation often leads to hyperactivity (Einon and Morgan 1978; Gentsch, et al. 1981; Morgan 1973; Syme 1973), increased locomotor and startle response in novel environments (Gentsch, et al. 1982a; Gentsch, et al. 1988; Gentsch, et al. 1982b; Varty, et al. 2000), increased responsiveness to amphetamines (Jones, et al. 1992; Jones, et al. 1990; Lapiz, et al. 2003), reduced accuracy in spatial memory (Lu, et al. 2003; Quan, et al. 2010; Quan, et al. 2011), and abnormal function in the hippocampus and amygdala (reviewed by Lapiz, et al. 2003). In some cases, the behavioural deficits after social isolation can be reversed by introduction to a social group (Novak and Harlow 1975; Suomi and Harlow 1972), however, in other cases, the deficits are so great that normal social interactions do not occur even after introduction to conspecifics (Baarendse, et al. 2013; Harlow, et al. 1965; Hol, et al. 1999). These studies differ in the duration of social isolation and in the stage of life during which the isolation occurs, which suggests that there may be a sensitive period during which social interactions are crucial for typical development.

There is some evidence that, like species in other taxa, insects also suffer developmental abnormalities when raised in isolation. Fruit flies, which are surrounded by conspecifics during their larval stage, exhibit similar aggressive behaviour to rats when raised in isolation (Valzelli and Garattini 1972; Wang, et al. 2008) and have reduced Mushroom Body fibers (Technau and Technau 2007), an area associated with learning and memory in insects. Lihoreau, et al. (2009)

showed that socially isolated cockroaches exhibit exploration avoidance, reduced willingness to interact socially and reduced ability to assess mating partners.

Honey bees are highly eusocial insects, where an individual in surrounded by thousands of conspecifics from birth and is provided for by its siblings until it is mature (Winston 1991). Female workers interact with thousands of sisters and work cooperatively to feed the brood, maintain the hive, and forage for pollen and nectar. Honey bees have a high capacity for learning as well, gaining information from their conspecifics about the location and profitability of food locations, through the well-described "waggle dance' (Von Frisch 1967). These robust social interactions a honey bee experiences and their cognitive abilities make it an ideal organism to study the effects of social interactions on learning and memory.

In a recent paper, Maleszka, et al. (2009) compared learning in honey bees raised in isolation and those raised in groups of 50 and found that those raised in isolation had lower accuracy in a one-trial association task. This result indicates either a deficit in reward perception, learning, memory or a combination. Sucrose responsiveness is known to affect learning in honey bees (Scheiner 2004; Scheiner, et al. 2003; Scheiner, et al. 2001), so the results could be due to the bees raised in isolation having a lower sucrose responsiveness and not due to any cognitive deficits. Therefore, I decided to examine how social interactions affect learning and memory in honey bees, while assessing sucrose responsiveness and using a more complex measure for learning and memory.

The mammalian studies indicate that a sensitive period exists in the beginning of an individual's life during which social isolation leads to atypical behaviour that is irreversible (see above). In honey bees, most extensive brain gene expression differences related to age is

between newly emerged and 4 day old bees (Whitfield, et al. 2006) and reliable acquisition and retention cannot be achieved before honey bees are 6 days of age (Ray and Ferneyhough 1997). Therefore, age 0 to 6 days makes a good potential sensitive period in honey bees. In order to test how social interactions affect learning and memory in honey bees, I placed honey bees from emergence into three different interaction conditions and tested the bees at 6 days. First, I tested them for sucrose responsiveness then the bees were tested on discrimination learning and memory using the Proboscis Extension Paradigm (PER).

**Methods**

*Bees*

The bees were taken from an apiary located at the York University Research Apiary (Toronto, Ontario, Canada). I collected brood frames from two honey bee colonies (c3 and c6) maintained at the apiary in the summer of 2013. The bees had a mixed genetic ancestry with major contributions from the East European population group (C group: *A. m. ligustica* and *A. m. carnica*) and minor contributions from the West European population group (M group: *A. m. mellifera*) (Harpur, et al. 2013, 2012). Brood frames were kept at 33$^{o}$C and were checked for emerging brood daily. Every 24 hours, I assigned the newly emerged honey bees into a group of 32 bees, 8 bees, or 1 bee. They were housed in a 908mL container (12.5 x 12.5 x 6.5 cm) with air holes in a 33$^{o}$C incubator (separate from the brood). 30% sucrose (Sigma) and a pollen patty (Bee-Pro Patties, Mann Lake LTD) were provided in excess. Dead honey bees were removed daily and if more than 20% mortality was observed in groups of 8 or 32 bees, the particular group was not used in testing. This occurred in 7/139 boxes which housed 8 or 32 bees.

*Sucrose Responsiveness*

I used a standard protocol for measuring sucrose responsiveness in honey bees (Scheiner 2004). Briefly, when the honey bees were 6 days old, they were chilled at -20$^{o}$C for about 2 minutes, until they became immobile. They were then harnessed using a modified 1000µL pipette tip with the tapered end was removed, and plasticine in order to secure their thorax and legs (Fig. 3-1). The bees were fed 1µL of 30% sucrose and were left for 1.5hours on the bench top to recover, which was previously shown to be sufficient recovery time (Frost, et al. 2011). Both antennae were touched with a droplet of solution and the absence or presence of the proboscis extension was recorded. The solution concentrations were: 0%, 0.1%, 0.3%, 1.0%, 3.0%, 10%, and 30% sucrose and they were applied in an ascending order. The inter trial interval (ITI) was 3 minutes. The gustatory response score (GRS), which is a measure of sucrose responsiveness (Scheiner, et al. 2004), was calculated by summing the total proboscis extensions on an individual honey bee. Thus the possible GRS score range from 1 to 7, where 7 represents the most responsive bees. Honey bees that failed to respond to 30% sucrose were excluded from further testing since the reward in the discrimination learning was 30% sucrose (no significant difference found between group condition and failure to respond to 30% sucrose (chi-square test $x^2$=1.73 , df=2, p=0.421))

*Discrimination Learning*

I measured discrimination learning using a well-established (Bitterman, et al. 1983) olfactory conditioning procedure by measuring the proboscis extension response (PER) with the method adapted from (Ben-Shahar, et al. 2000). I tested the honey bees in a well-ventilated area. I used odour, either geranoil (Sigma) or 1-hexanol (Sigma), as the unconditioned stimulus (CS)

and either salt (3M NaCl (Sigma)) or 30% sucrose as the conditioned stimuli (US). I delivered each odour for 6 seconds using a syringe with a filter paper containing 1µL of undiluted solution. While the odour was being delivered, I placed a droplet of the US on the antennae and if the solution was sucrose, the honey bee extended her proboscis and was allowed to feed for 1 second. The learning phase consisted of 12 trials, 6 with one odour paired with reward (CS+) and 6 with the other odour paired with punishment (CS-) in a pseudorandom order. The ITI was 3 minutes and proboscis extension was recoded in each trial. If the honey bee responded spontaneously to the initial odour presentation, I removed her from testing. Both odours were used as the rewarded (CS+) and punished (CS-) stimuli in different blocks, where all 3 conditions were tested in a block. I tested 1 hour memory by exposing the bee to CS+ and then to CS- without reward or punishment. I measured 24 hour memory by exposing the bees to CS- and then to CS+. After testing, each honey bee was frozen in -80$^{o}$C for possible future analysis.

I classified bees as having a "good" discriminatory learning performance when the bee met the set criterion. The criterion was that a bee would make no more than one mistake after receiving the CS+ and no more than one mistake after receiving the CS- (either extension to a CS- or no extension to CS+ before US delivery) after the 2$^{nd}$ conditioning trial (when she underwent one exposure to CS+ and one CS-). The bees were then tested at one hour and 24 hour delays to see if the discrimination was retained.

*Statistics*

Statistical analyses were performed using R (version 3.1.1) (Team 2005) with the package Analysis of Overdispersed Data (aod). Plotting of data was performed with the help of the package Scientific Graphing Functions for Factorial Designs (sciplot). The discrimination

data was analysed by building a logistic regression model since the response variable is binary. When a coefficient is different from zero (thus having a p value of less than 0.05), than the variable has a significant influence on the dependent variable (response). The models with the lowest AIC value and the highest Akaike weight (Burnham and Anderson 2002) were chosen to evaluate the data (Table 3-4, 3-5, 3-6). For analysis of 1 hour and 24 hour memory, only those observations where the bee met the initial discrimination criterion were analyzed

**Results**

Increased social interactions were associated with decreased sucrose responsiveness in our experiment. The GRS score was lowest in bees raised in groups of 32, intermediate in bees raised in groups of 8, and was the highest in bees raised in isolation; all significantly different from the other two conditions (Pairwise comparisons using Wilcoxon rank sum test, $p<0.01$, $N=276$) (Fig. 3-2).

As expected from previous studies, the bees with the highest GRS scores had the best discrimination performance (Logit Regression Analysis, Table 3-1, $p<0.0001$, $N=276$). For memory analysis, only "good" discriminatory learners were included. GRS did not have a significant effect on 1 hour memory (Logit Regression Analysis, Table 3-2, $p=0.58$, $N=123$) or on 24 hour memory (Logit Regression Analysis, Table 3-3, $p=0.32$, $N=123$) (Fig. 3-3).

After controlling for the impact of sucrose responsiveness on learning and memory, I found that social isolation was associated with reduced discrimination learning performance in our experiment (Logit Regression Analysis, Table 3-1, $p=0.016$). No association on 1 hour (Logit Regression Analysis, Table 3-2, $p=0.51$) or 24 hours (Logit Regression Analysis, Table 3-3, $p=0.32$) memory was found.

Figure 3-3 depicts the honey bees' performances in the discrimination task and the 1 hour and 24 hour memory tasks. The coloured lines represent regression lines for each group condition, which shows that GRS matched bees that were raised in groups of 32 had better discriminatory performance than bees raised in groups of 8 or 1. (Fig.3-4A). Group conditions were not associated with 1 hour or 24 hour memory (Fig. 3-4B, 3-4C)

**Discussion**

In order to investigate the effect of social interactions have on learning and memory in honey bees, I randomly assigned emerging honey bees to one of three different conditions: isolation (1 bee), 8 bees, and 32 bees. In previous studies, sucrose responsiveness affected certain types of PER learning and memory (Scheiner 2004; Scheiner, et al. 2003; Scheiner, et al. 2001), therefore, I measured sucrose responsiveness before the training trials. I found that social interactions affected sucrose responsiveness, where the fewer social interactions the bee experienced the more responsive she was to sucrose. These results are in line with literature on mammals, where rats raised in isolation are more sensitive to low saccharine solutions (Hall, et al. 1998b), consumed more sucrose solution (Hall, et al. 1998b; Hall, et al. 1997) and are more responsive to food rewards (Harmer and Phillips 1998; Jones, et al. 1990).

As before (Scheiner 2004; Scheiner, et al. 2003; Scheiner, et al. 2001), sucrose responsiveness affected learning, with those bees that were the most responsive to sucrose showing the most accurate discrimination. It is possible that more responsive bees perceive the same concentration of sucrose as sweeter and thus as a better reward. Scheiner, et al. (1999) showed that reward concentration is positively correlated with acquisition scores. So, even though all of the honey bees received 30% sucrose as their reward, those with higher sucrose

responsiveness might have perceived it as sweeter and a more lucrative reward, which led to better performance.

When sucrose responsiveness is taken into account, better discrimination performance was seen in bees housed in groups of 32 than those housed in groups of 8 or 1, but no effects on memory were detected. These results are consistent with Maleszka, et al. (2009) who showed that honey bees raised in groups of 50 have increased one-trial acquisition when compared to honey bees raised in isolation. The fact that I saw a difference with the bees housed in a group of 32 and no differences between groups of 8 and 1, suggests that it may not be only total social isolation, but the reduction in social interactions in general that impairs discrimination learning in honey bees. The differences, if any, created by the slight increase in social interactions between 1 individual and 8 individuals, when compared to natural hive conditions, were not detected in this study. No effect of different social interactions conditions were detected on 1hour or 24 hour memory. It is impossible to compare to previous studies, since this study is the first to investigate the effects of social interactions on memory in insects. Acquisition and retention are mediated by different molecular processes (Menzel and Muller 1996), so it is possible that social interactions in honey bees affect the process implicated in acquisition, but do not affect the processes associated with memory.

I saw no effect of sucrose responsiveness on 1 hour or 24 hour memory. Scheiner, et al. (2005) showed that GRS score had an effect on 24 hour memory in tactile PER. The discrepancies between our study and theirs are likely due to the different types of learning tested (tactile or olfactory learning) and the different learning procedures used (acquisition or

discrimination learning). Also, I used a different criterion for learning since I used a different learning procedure.

It is possible that the dopaminergic system is disrupted by the reduced social interactions in honey bees. In rats, it is the dopaminergic system that is implicated in food responsiveness and consumption (Avena, et al. 2008; Hajnal, et al. 2004; Sills and Crawley 1996) and it is also disrupted during isolation (Blanc, et al. 1980; Fabricius, et al. 2010; Hall, et al. 1998a; Jones, et al. 1992; Jones, et al. 1990; Yorgason, et al. 2013). In honey bees, injections of dopamine and dopamine receptor agonist 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (6,7-ADTN) reduce sucrose responsiveness (Scheiner, et al. 2002) and dopamine injections before training do not alter memory retrieval or storage (Menzel, et al. 1988; Menzel, et al. 1990; Mercer and Menzel 1982). The dopaminergic system is involved in aversive conditioning in honey bees and other insects (reviewed by Giurfa 2007), where dopamine antagonists would impair aversive acquisition (Agarwal, et al. 2011; Unoki, et al. 2005). Since discrimination learning involves aversive acquisition (odour with salt punishment), it is likely affected by dopamine. Thus, low levels of dopamine or reduced responsiveness to dopamine in the lower social interaction groups would cause higher sucrose responsiveness and impaired learning performance, but would not affect memory, which is consistent with the results of this study. Measuring both dopamine titers and dopamine receptor expression after social isolation would help illuminate whether the dopaminergic system is indeed affected by reduced social interactions.

It is possible that the reduced social interactions altered the behavioural role of the honey bees. Normally, younger bees perform hive duties, while those age 14 days or older tend to forage outside of the hive. This division of roles is flexible, however, dependent on hive

conditions. In some cases, bees as young as 4 days can forage (Winston 1991). It is feasible that more of the bees raised in isolation were "foragers", while more of the bees raised in groups were "nurses". Foragers have a higher sucrose responsiveness than nurses (Behrends, et al. 2007) and our isolated bees had the highest sucrose responsiveness. However, nurses and foragers exhibit no significant differences in PER acquisition (Ben-Shahar, et al. 2000; Bhagavan, et al. 1994), which would not agree with our result, where bees housed in groups of 32 had higher acquisition than those housed in groups of 8 and 1. In addition, all of the bees were raised without the presence of queen pheromone. Naeger, et al. (2013) showed that an absence of a queen results in more blurred lines between the behavioural roles: bees who were previously foragers start performing typical nurse duties and vice versa. Therefore, it is not expected that the bees in the current experiment would have different behavioural roles.

Interestingly, rats raised in isolation often do not show impairment in different types of acquisition (reviewed by Fone and Porkess 2008; Green and McCormick 2013), nor do they show impairment in the acquisition of an operant discrimination task (Abdul-Monim, et al. 2003). However, impairments have been found in some associative learning tasks (Weiss, et al. 2004). Despite the robust literature on social isolation in rats, there is still some debate regarding the effect of social isolation on learning (Fone and Porkess 2008; Green and McCormick 2013). Some of the discrepancy in the rat literature may come from the different procedures used. Weiss, et al. (2001) demonstrated that using either post-weaning or pre-weaning isolation had dramatic consequences on the impairments found in prepulse inhibition and latent inhibition. Sex was also found to have an effect. Worker honey bees, on the other hand, are all female and survive without sibling care after emergence, which allows for an easier standardization of the social isolation procedure.

The variety of social conditions seen in insects provides a good opportunity to study social interaction effects on behaviour. Studying insects that are solitary, communal, or social and manipulating their social conditions at particular life stages would illuminate how crucial social interactions are for typical behaviour. My study and the Maleszka, et al. (2009) study focused on a eusocial insect and demonstrated that social interactions during a certain developmental stage are important for typical behaviour. Other studies focused on insects that are less social (Lihoreau, et al. 2009; Technau and Technau 2007; Wang, et al. 2008), but have also found that social interactions are important for typical behaviour. This demonstrates that if normally an insect experiences social interactions during a certain developmental stage, then those social interactions seem to be necessary for typical behaviour. Using insects with different social aggregation, would allow us to make more direct comparisons on the effects of social interactions in general. The diversity of social life styles within insects makes them a good model for studying the effects of social interactions on behaviour.

## Tables

Table 3-1. Logistic regression analysis of discrimination performance. Formula: Discrimination Score ~ Group Condition + GRS + Odour. $x^2$ =35.41 df=3 p<0.0001. N=276

| | Estimate | Std. Error | Odds Ratio | p-value |
|---|---|---|---|---|
| (intercept) | -1.40980 | 0.37798 | 0.2441919 | 0.000192*** |
| Group Condition | 0.02548 | 0.01049 | 1.0258074 | 0.015186* |
| GRS | 0.33669 | 0.06932 | 1.4003098 | 1.19e-06*** |
| Odour used as CS+ | -0.81641 | 0.26137 | 0.4420147 | 0.001786** |

Table 3-2. Logistic regression analysis of 1 hour memory. Formula = 1H memory ~ Group Condition + GRS + Sugar + Colony, $\mathbf{x^2}$=15.77, df=4 p <0.0001. N=123

| | Estimate | Std. Error | Odds Ratio | p-value |
|---|---|---|---|---|
| (intercept) | 2.74482 | 0.89693 | 15.5617445 | 0.00221** |
| Group Condition | -0.01313 | 0.01989 | 0.9869515 | 0.50906 |
| GRS | 0.07356 | 0.13338 | 1.0763282 | 0.58132 |
| Odour used as CS+ | -1.71315 | 0.52243 | 0.1802967 | 0.00104*** |
| Colony | -1.20196 | 0.52596 | 0.3006052 | 0.02230* |

Table 3-3. Logistic regression analysis of 24 hour memory. Formula =24 H memory ~ Group Condition + GRS + Sugar + Colony, $\mathbf{x^2}$ = 16.85 df=4, p<0.0001. N=123

| | Estimate | Std. Error | Odds Ratio | p-value |
|---|---|---|---|---|
| (intercept) | 0.939671 | 0.653140 | 2.5591394 | 0.150236 |
| Group Condition | -0.003002 | 0.015741 | 0.9970023 | 0.848741 |
| GRS | 0.105070 | 0.105701 | 1.1107886 | 0.320206 |
| Odour used as CS+ | -1.071226 | 0.410897 | 0.2559382 | 0.009133** |
| Colony | -1.362819 | 0.412149 | 0.3425881 | 0.000944*** |

Table 3-4. Akaike's information criterion (AIC) of the regression models of discrimination performance. A total of 276 observations were analyzed.

| Model | ID | Log-Likelihood | Number of Parameters (K) | AIC | AIC delta | Akaike Wight ($w_i$) |
|---|---|---|---|---|---|---|
| Box | 1 | -189.55 | 1 | 383.1 | 31.16 | 0.00 |
| Box + GRS | 2 | -176.97 | 2 | 359.95 | 8.01 | 0.01 |
| Box + GRS + Colony | 3 | -176.97 | 3 | 361.3 | 9.36 | 0.01 |
| GRS + Colony | 4 | -179.10 | 2 | 364.21 | 12.27 | 0.00 |
| Box + GRS + Sugar | 5 | -171.97 | 3 | 351.94 | 0.00 | 0.59 |
| GRS + Sugar | 6 | -175.02 | 2 | 356.03 | 4.09 | 0.07 |
| Box + GRS + Colony + Sugar | 7 | -171.58 | 4 | 353.16 | 1.22 | 0.32 |

Table 3-5. Akaike's information criterion (AIC) of the regression models of 1 hour memory performance. A total of 123 observations were analyzed.

| Model | ID | Log-Likelihood | Number of Parameters (K) | AIC | AIC delta | Akaike Wight ($w_i$) |
|---|---|---|---|---|---|---|
| Box | 1 | -63.25 | 1 | 130.51 | 9.40 | 0.01 |
| Box + GRS | 2 | -63.25 | 2 | 132.50 | 11.39 | 0.00 |
| Box + GRS + Colony | 3 | -61.72 | 3 | 131.43 | 10.32 | 0.00 |
| GRS + Colony | 4 | -62.00 | 2 | 130.00 | 8.89 | 0.01 |
| Box + GRS + Sugar | 5 | -58.40 | 3 | 124.79 | 3.68 | 0.10 |
| GRS + Sugar | 6 | -58.45 | 2 | 122.90 | 1.79 | 0.26 |
| Box + GRS + Colony + Sugar | 7 | -55.56 | 4 | 121.11 | 0.00 | 0.63 |

Table 3-6. Akaike's information criterion (AIC) of the regression models of 24 hour memory performance. A total of 123 observations were analyzed.

| Model | ID | Log-Likelihood | Number of Parameters (K) | AIC | AIC delta | Akaike Wight ($w_i$) |
|---|---|---|---|---|---|---|
| Box | 1 | -84.51 | 1 | 173.02 | 10.73 | 0.00 |
| Box + GRS | 2 | -84.42 | 2 | 174.84 | 12.56 | 0.00 |
| Box + GRS + Colony | 3 | -79.74 | 3 | 167.48 | 5.19 | 0.06 |
| GRS + Colony | 4 | -79.82 | 2 | 165.64 | 3.35 | 0.14 |
| Box + GRS + Sugar | 5 | -82.09 | 3 | 172.18 | 9.89 | 0.01 |
| GRS + Sugar | 6 | -82.09 | 2 | 170.18 | 7.89 | 0.01 |
| Box + GRS + Colony + Sugar | 7 | -76.14 | 4 | 162.29 | 0.00 | 0.77 |

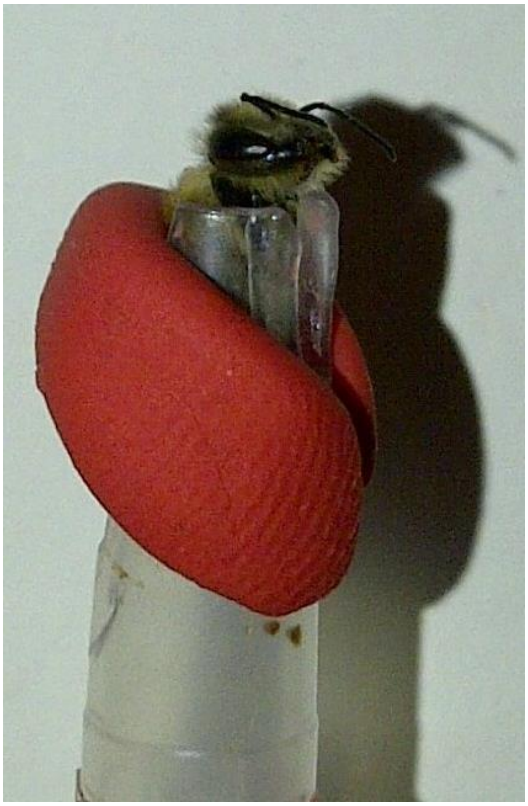

Figure 3-1. A harnessed honey bee. Honey bees were chilled until immobile and then placed into a modified 1000mL pipette secured by plasticine. Extra care was taken to ensure legs were secured and the proboscis was not blocked.

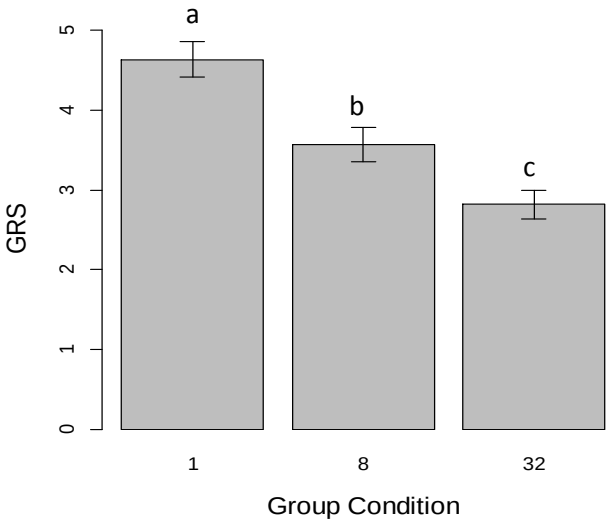


Figure 3-2. Group condition affects sucrose responsiveness (GRS). Each Group condition is significantly different from the others (Pairwise comparisons using Wilcoxon rank sum test, $p<0.01$).

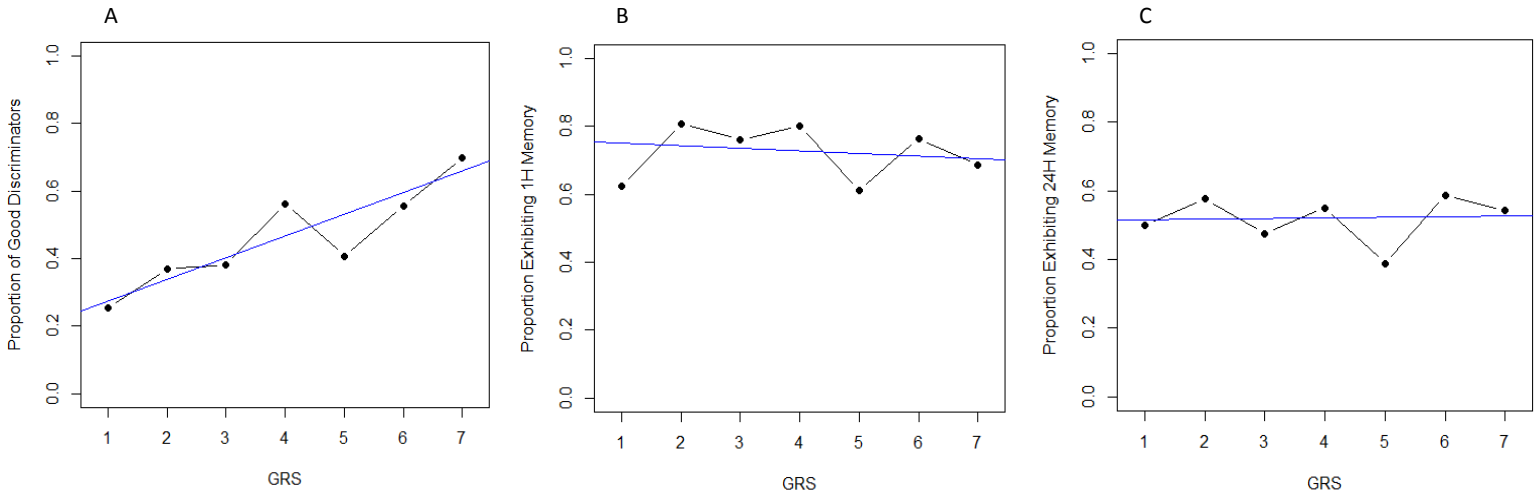


Figure 3-3. Sucrose responsiveness (GRS) affects discrimination performance, but not memory. Y axis: proportion of bees exhibiting (A) good discrimination, (B) 1 hour memory, or (C) 24 hour memory. Regression lines for illustrative purposes only. A. Proportion of bees learned significantly affected by GRS score (Table 3-1, $p<0.001$). B. proportion of bees exhibiting 1 hour memory is not significantly affected by GRS score (Table 3-2, $p=0.58$). C. proportion of bees exhibiting 24 hour memory is not significantly affected by GRS score (Table 3-3, $p=0.32$).

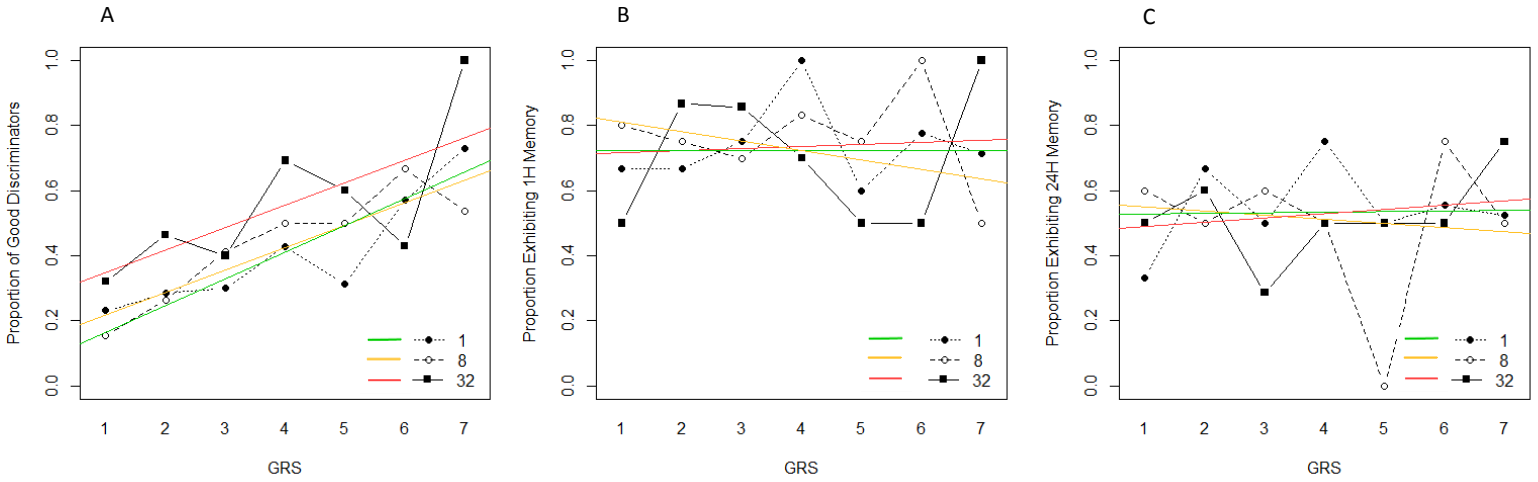


Figure 3-4. The effect of group condition on learning and memory by sucrose responsiveness (GRS).
Y axis: proportion of bees exhibiting (A) good discrimination, (B) 1 hour memory, or (C) 24 hour memory. Solid squares = group condition 32, open circles = group condition 8, solid circles = group condition 1. Regression lines for illustrative purposes only. Red line = regression of group condition 32, yellow line = regression of group condition 8, green line = regression of group condition 1. A. Proportion of bees learned significantly differ between group conditions (Table 3-1, $p=0.016$). B. proportion of bees exhibiting 1 hour memory does not significantly differ between group conditions (Table 3- 2, $p=0.51$). C. proportion of bees exhibiting 24 hour memory does not significantly differ between group conditions (Table3- 3 $p=0.84$).

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