

Factors Influencing Survival and Fitness Through Learning and Memory in Honeybees *Apis dorsata* and *Apis mellifera*

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Doctor of Philosophy in Animal Biology
by

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Ph.D. Thesis



(A Central University established in 1974 by an Act of Parliament)

DECLARATION

I, **Meenakshi Vijaykumar** declare that this thesis entitled “**Factors Influencing Survival and Fitness Through Learning and Memory in Honeybees *Apis dorsata* and *Apis mellifera***” submitted by me under the supervision of Prof. Aparna Dutta Gupta and co-supervision of Dr. Joby Joseph is a bonafide research work which is also free from plagiarism. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Date: 1st September 2020

Meenakshi Vijaykumar

15LAPH02

A handwritten signature in blue ink, appearing to be "Meenakshi", written in a cursive style.



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CERTIFICATE

This is to certify that this thesis entitled “**Factors Influencing Survival and Fitness Through Learning and Memory in Honeybees *Apis dorsata* and *Apis mellifera***”, submitted by **Meenakshi VK, 15LAPH02** in partial fulfillment of the requirements for award of Doctor of Philosophy in the **Department of Animal Biology, School of Life Sciences** is a bonafide work carried out by her, under our supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

Parts of this thesis:

A. Published in the following publications-

- i. **Vijaykumar, M., Mogily, S., Dutta-Gupta, A., and Joseph, J. (2019). Evidence for absence of bilateral transfer of olfactory learned information in *Apis dorsata* and *Apis mellifera*. J. Exp. Biol , 222(8), jeb196584.**
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1	AS 801	Analytical Techniques	4	Pass
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3	AS 803	Lab Work & Seminar	5	Pass



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*This thesis is dedicated to
My mentor, Late Prof.Aparna Dutta Gupta and my
family...*

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Abbreviations

Å	Angstrom
AL	Antennal lobes
ALP	Alkaline phosphatase
APN	Aminopeptidase
CaMKII	Calcium/Calmodulin dependent kinase II
CS	Conditioned stimulus
DA	Dopamine
ddH ₂ O	Double distilled water
DDT	Dichloro-Diphenyl Trichloroethane
DTT	Dithiothreitol
EAG	Electroantennogram Recording
EPSP	Excitatory post synaptic potential
ERG	Electroretinogram Recording
ERK1/2	Extracellular cell regulating kinase 1 and 2
GABA	Gamma-aminobutyric
GPCRs	G-protein coupled receptors
Hrs	Hours
Hz	Hertz
IgG	Immunoglobulin G
IPSP	Inhibitory post synaptic potential
ITI	Inter trial interval
JH	Juvenile hormone
KC	Kenyon cells
kDa	Kilo dalton
LFP	Local field potential
LH	Lateral horns
LTM	Long term memory
LTP	Long term potentiation
MΩ	Mega Ohms
M	Molar

MB	Mushroom bodies
MBONs	Mushroom body output neurons
µg	Micrograms
mg	Milligrams
ml	Milliliters
mM	milliMolar
OA	Octopamine
ORGs	Olfactory recetor genes
ORNs	Olfactory receptor neurons
PER	Proboscis extension reflex
PKC	Protein kinase C
PKG	Protein kinase G
PL	Protocerebral lobe
PNs	Projection neurons
PSP	Postsynaptic potentials
RH	Relative Humidity
S	Second
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
STM	Short-term memory
UR	Unconditioned reflex
US	Unconditioned stimulus
WM	Working memory
V	Volts
Vg	Vitellogenin
v/v	Volume/Volume
w/v	Weight/Volume

INTRODUCTION

Introduction

The foundations

One of the earliest mentions of the brain and the role it plays dates back to 17th Century BC, as documented in the Edwin Smith Medical papyrus (Middendorp et al., 2010). Of the entire gamut of complex functions performed by the brain, the capacity for it to learn and store information has fascinated both philosophers and scientists for ages. Early references of learning and memory date back to the works of Plato followed by those of his pupil Aristotle (~ 370 BC) who suggested that memory was a substance in the minds of men that was pliable like wax and could be molded depending on the person's experiences (Clark, 2018). The capacity for memory to exist as physical traces in humans was hypothesized by René Descartes in the early 1600s. It is from these ideas seeded by philosophers and mathematicians that the need to research and understand memory from the biological perspective picked up a rapid pace. The famous psychologist William James was among the first to describe the plasticity of memory formation. It was the path-breaking works of Herman Ebbinghaus, a German psychologist that laid the foundation for the understanding of the functioning of memory. In an interesting approach, Ebbinghaus compiled a series of nearly 2,300 "nonsense syllables" to understand memory in its pure form, or recollection without prior knowledge (Murre and Dros, 2015). In these experiments, he or a participating candidate would memorize these nonsense syllables and attempt recollection at varying time points. A comparison of the rate of retention or the rate of forgetting was made depending on whether the syllable to be learnt was rehearsed versus not rehearsed (Ebbinghaus 2013; Murre and Dros, 2015). In the process, he demonstrated a forgetting curve which is similar in principle to a learning curve. He also worked out the

concepts of memory savings and overlearning, which he published as his work titled “Memory: A Contribution to Experimental Psychology published in 1885”. Though most of the initial work was carried out in humans, it was soon realized that the core principles could be applied across the animal kingdom, right from the complex humans to rodents and the invertebrates such as arthropods, nematodes and cnidarians.

While studying the physiology of digestion in dogs, Ivan Pavlov and his co-workers in the late 1800s deduced important aspects of learning and memory through classical conditioning (R. E. Clark, 2018). Classical conditioning involves the use of a stimulus or a cue which when overlapped with a reward is learnt as information by the animal. After multiple learning trials or repetitions when the animal is presented with a cue, such as, the ringing of a bell as in Pavlov’s experiments, it exhibits an innate behavioral output, which in the case of Pavlov’s dogs was drooling in anticipation of the reward (food). The training stimulus or cue is termed the conditioned stimulus (CS), the reward or food is termed the unconditioned stimulus (US) and the animal’s innate response is called the innate or unconditioned reflex (UR). Karl Lashley (1915) while working with lesion experiments on the rat brain under the guidance of James. B Watson brought forth the idea of “mass action” of the brain which suggested that learning and memory is distributed across the brain rather than localized to one region. In his experiments, Lashley would introduce lesions in specific areas of the rat’s cortex either before or after the animals were trained in maze navigation and check for the animal’s ability to navigate. Pavlov’s observations along with the observations made by Karl Lashley provided the foundation on which the pillars of behavioral neuroscience were built. The idea of how memory formation takes place has evolved from the possibility of memory being stored across the brain as a whole to the present view, which suggests that different parts of the brain play different roles in the acquisition and formation of memories, which can then be transferred to regions such as the

cerebral cortex in humans and α , β lobes in insects. Retrieval of the learnt information then involves specific neuronal activity in a particular storage center.

Memory is formed and stored in phases

The process of storing memories as long term memories involves a crucial process called consolidation which makes memories resistant to disruption (Clark, 2018; Squire et al., 2015). In humans, the interplay of neural processes between the hippocampus and neocortex helps in the final consolidation of acquired information in the neocortex (Squire et al., 2015). In the relatively simple insect brain consolidation is carried out by the mushroom body, after which long term memory becomes completely dependent on the α , β lobes (Cervantes-Sandoval et al., 2013; Hourcade et al., 2010). Upon acquisition of information the initial memory also termed the short-term memory (STM) remains fragile and capable of fragmentation via interference. The primary memory formation process or the STM formation holds the acquired information for long enough to allow consolidation. The STM is limited by time and the amount of information that can be memorized, though these two aspects are topics of much debate (Cowan, 2008). STM is a sub-category under the multicomponent working memory (WM), which is a process that holds utilizable information for short amounts of time and retrieves it when provided with an appropriate stimulus (Aben et al., 2012; Atkinson and Shiffrin, 1968; Baddeley, 2010; Chai et al., 2018; Cowan, 2008) for example the memorizing of a onetime password before typing it into the code input box. The steps involved in memory formation were well described by the Atkinson-Shiffrin (1968) memory model (Fig 1A) and they were modified to include working memory as described by the Baddeley and Hitch's working memory model (1974) (Fig 1B) (Atkinson and Shiffrin, 1968; Baddeley and Hitch, 1974). While the Atkinson-Shiffrin model paints a general picture of how memory is formed, the Baddeley-Hitch working memory model

includes the short term processing and memory storage capacity of the sensory systems via the introduction of the visual, auditory system (Visuo-spatial sketch pad and phonological loop).

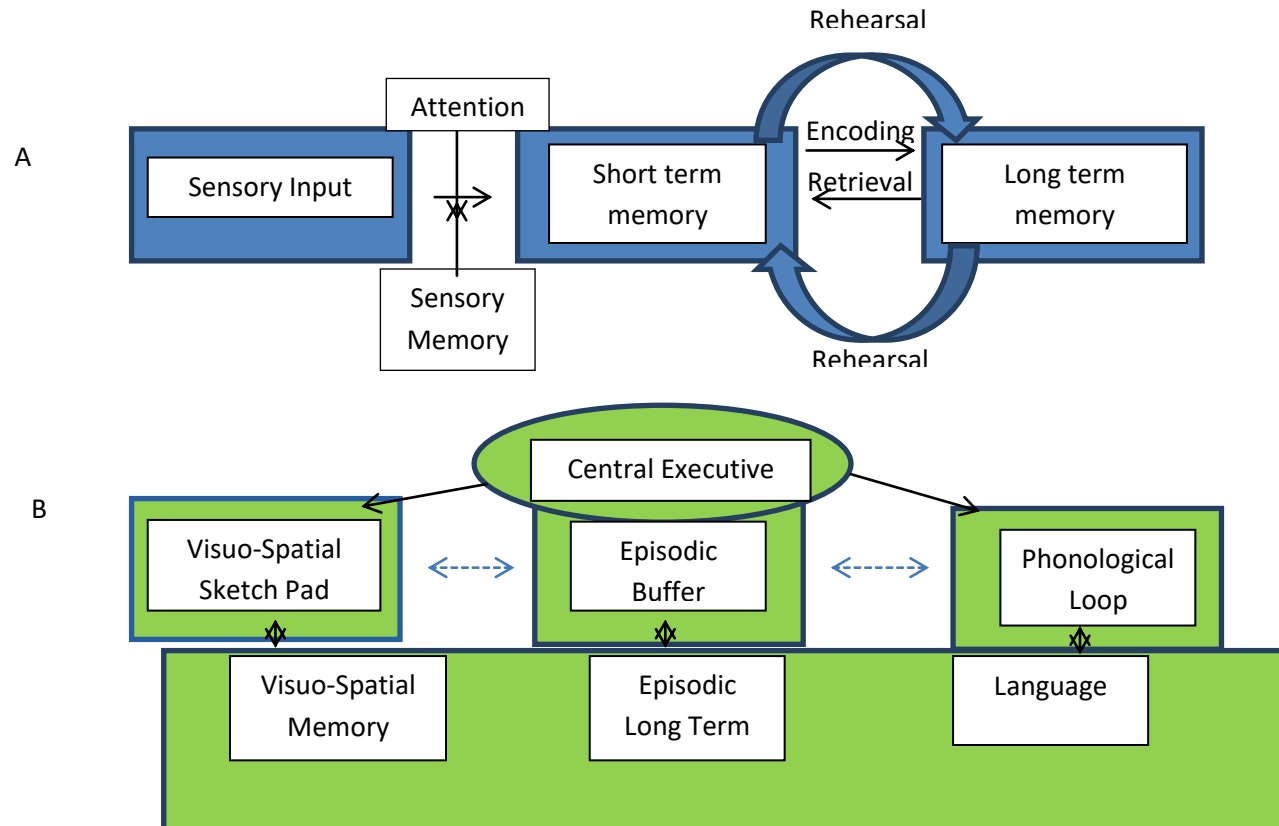


Fig 1: A. Representation of the Atkinson-Shiffrin memory model, the formation of LTM depends on the rehearsal of the acquired information and the level of attention during information acquisition
 B. The representation of the Baddeley-Hitch memory model, which highlights the importance of a central executive structure in the brain such as the Pre-frontal cortex (PFC), the stimulus such as visual or auditory and the need to have rehearsals of the memory such as the episodic buffer which assists in the formation of episodic memory.

The episodic buffer showed the importance of rehearsing and providing the stimulus for multiple trials, such that the information is processed and stored optimally. The formation of long term memory(LTM) also involves the synaptic strengthening between interacting neurons and the

formation of new connections and synapses between neurons, in a process termed long term potentiation (LTP). This strengthening of synaptic connections is encouraged by learning and rehearsing the information as highlighted by the episodic buffer in Baddeley-Hitch's model. The practical application of the memory models was seen when behavior biologists began experimenting on both vertebrate and invertebrate animal systems. A major breakthrough came with Eric Kandel's work on the sea slug, *Aplysia* which earned him the Nobel Prize in Medicine in 2000. In a behavioral experiment called the *Aplysia* gill and siphon withdrawal reflex, Kandel was able to unravel various aspects of memory, including, long term and short term memory via observing a process called habituation. In the experiment, when the siphon of the animal was touched with a probe, it would withdraw its siphon as a defensive reflex, over repeated trials the animal learnt that the probe was harmless and the withdrawal response would gradually subside over the period of trials. This process was termed habituation (Beeman, 2013; Schwiening, 2012) and this study laid the foundation for the understanding of non-associative memory.

The physiology of memory

The cellular and physiological mechanisms of memory formation are complex and a basic framework was laid down by the works of Hodgkin and Huxely (1947). They provided a series of mathematical equations using the giant squid as their animal model and by working on its axon. Their mathematical model described the excitability of neurons and how they are capable of generating an action potential, which is propagated to the connecting neuron and so forth (Beeman, 2013; Schwiening, 2012). Using a technique called voltage clamp, where the voltage of the excitable neuron is maintained at one value, they were able to observe and assess the current flow of various ions upon voltage changes across the axon, hence bringing to light the functioning of voltage-gated ion channels, participating ions (Na^+ , K^+ , Cl^- , Ca^{2+}) and

neurotransmitters [Acetylcholine, Dopamine, Serotonin, Glutamate, Gamma-aminobutyric (GABA), Histamine and Nor-epinephrine]. Today the study of the neurobiology of memory involves multiple components, such as the understanding of neurogenesis, the molecular mechanisms and genetics involved in neuroplasticity and LTP, the physiology of the interacting neurons, and finally the constructing of maps or connectomes by locating, visualizing and mapping the interacting neurons. By building a digital atlas, neuroscientists and behavioral biologists can further study and record how neuronal plasticity, changes during learning and LTP across the animal's brain manifest as behavior or an output by the organism as a whole.

The role of kinases in the formation and consolidation of memories is well established (Giese and Mizuno, 2013; Mizunami et al., 2014). From the 250 kinases expressed in mammals calcium/calmodulin dependent kinase II (CaMKII), extracellular cell regulating kinase 1 and 2 (ERK1/2), protein kinase G (PKG), protein kinase C (PKC) are a few kinases that regulate the formation and storage of memories. How the signaling cascade regulates, modulates, and promotes optimal behavioral output is a topic of much interest and research. In insects such as crickets, CaMKII, NO-cGMP, and protein kinase A, play a vital role in long term memory (LTM) formation (Matsumoto et al., 2018) suggesting that the pathways involving protein-dependent memory are conserved across the animal kingdom.

Memory Traces and Oscillations

An interesting phenomenon discovered by Hans Berger in 1929 was that of neural oscillations. The rhythmic pattern created by the synchronized excitatory and inhibitory activity of populations of neurons in a region of the brain is called oscillations. In humans, Electroencephalogram recordings (EEG) have provided evidence of oscillations, termed, Theta

oscillations ranging in the frequency of 2-12 Hz and Gamma oscillations ranging in the 25-140 Hz frequency, that play a crucial role in memory formation and other important cognitive functions (Baars and Gage, 2013; Hanslmayr et al., 2012; Kay, 2014). The gamma oscillations in rodents have been shown to be functionally similar to those observed in humans (Kay, 2014). In insects, Local field potential (LFP) recordings from sensory integration and memory centers of the brain such as the Mushroom body calyx have shown that oscillations ranging in the 8-20 Hz frequency also called the Alpha oscillations might play a role in the cognitive abilities of the animal and information binding (Hanslmayr et al., 2012; Popov and Szyszka, 2020; Stopfer et al., 1997).

Interhemispheric communication

Interhemispheric neuronal connections shunt stimulus and learnt information between the two lobes of the brain in order to assist bilateral coordination. This capacity is enabled by the corpus callosum in mammals, by the anterior, posterior, habenular commissure in lower vertebrates and the bilateral neurons in arthropods (Aboitiz and Montiel, 2003; Gazzaniga, 2000; Suarez et al., 2014). The corpus callosum is an “evolutionary innovation” seen only in eutherian mammals and much of what is known about its function has come from studies in human patients who have undergone a corpus callosotomy (Gazzaniga, 2000, Gazzaniga, 2014; Mahrshahi, 2006). In insects, bilateral neurons associated with the central complex are well established as seen in *Bombyx mori* and *Schistocerca gregaria* (Namiki and Kanzaki, 2016; Vitzthum et al., 2002). Many of the known bilateral connections are made by the visual neurons and their association with the central complex has been suggested to coordinate navigation and flight activity (Namiki and Kanzaki, 2016; Strausfeld and Hirth, 2013; Vitzthum et al., 2002). At the olfactory level, bilateral projections have been seen between the antennal lobes in *Drosophila* and the neurons of

the lateral horn in *Schistocerca americana* (Gupta and Stopfer, 2014; Horne et al., 2018; Lin et al., 2018). However other than the above mentioned examples very little is known about bilateral olfactory connections and their role in other insect species. In a behavioral study using honey bees evidence was provided for the possible transfer of olfactory learnt information from the trained side of the brain to the unexposed or untrained side (Sandoz and Menzel, 2001). In this experiment, the bee's antennae were separated using a plastic wall and one of the antennae was trained in olfactory conditioning while keeping the other isolated from the training procedure. The results showed that the information learnt on one side was transferred to the untrained side over a period of 3 hours. Evidence was also provided for the discriminating capacity of these insects trained with one antenna, as when trained using two odors, one rewarded and the other unrewarded, the insect was able to recall both the rewarded odor and discriminate it from the unrewarded odor. Using transcriptomics, Guo et al., (2016) demonstrated a significant change in the levels of learning and memory related genes such as C-Fos, CaMKII, 24 hrs post-training, on the untrained side. In this experiment, the foragers were trained with one antenna open and the other sealed completely from the training procedure using a silicon paste. The animals were however not checked for retention or transfer of olfactory information on the untrained side at 24 hrs (Guo et al., 2016). These results suggested the possible presence of a commissure of bilateral neurons in honey bees that not only played a role in transferring the learnt information but also the identity of the odor in a temporal manner. Given the lack of physical evidence of bilateral connections at the olfactory level, behavioral results of bilateral transfer of information is intriguing and make the search for bilateral olfactory neuronal processes an interesting question.

Electrophysiology as a tool to study neurons

Electrophysiology has been a very useful tool in the field of neurophysiology for the study of the neurons and their electrical activity (Fig 2). It gives the researcher the ability to measure the changes in the membrane potential of the neurons. A glass electrode filled with an electrically conductive fluid such as potassium chloride, lithium chloride, or saline and a metal wire usually silver or tungsten is guided to either the extracellular space of the neurons or gently inserted into the neuron (Fig 2). The difference between electrodes used for intracellular and extracellular recordings is the impedance produced by the electrode-solution interface circuit which mostly resides at the tip of the electrode. The impedance of a conducting element refers to the resistance to current flow and is inversely proportional to the conductance. High impedance electrodes (100-200 M Ω) are used to record intracellularly while electrodes with lower impedance (1-10 M Ω) are used for extracellular recordings. The impedance also depends on the length of the electrode shank and the conducting electrolyte fluid in the glass electrode. The electrophysiological set up consists of the glass electrode inserted into a micromanipulator, which sends the signal of the recording to an amplifier which amplifies the signal. The recording from the electrode is compared to the signal from a ground wire; this signal output is made visible in its waveform on an oscilloscope while a digitizer helps digitize the signal from the amplifier (Fig 2). Propagation of action potential requires changes both at the conductance and potential level in the postsynaptic cell. These changes are termed postsynaptic potentials (PSPs.) PSPs could either be an increase in the membrane potential above the resting potential (-70 mV) and beyond the threshold voltage called the excitatory postsynaptic potential (EPSP) or a decrease in the membrane potential generating a negative potential also termed inhibitory postsynaptic potential (IPSP). Whether a postsynaptic cell will generate an EPSP or IPSP depends on the neurotransmitter that binds to the cells receptors. For example, binding of

glutamate might cause the opening of the Na^+ gated ion channels which will increase the membrane potential beyond -55 mV which would depolarize the cell and generate an action potential. On the other hand, if a transmitter such as GABA were to bind to the cell's GABAergic receptors, the Cl^- channels would open turning the membrane potential more negative causing it to hyperpolarize and inhibit the further propagation of the action potential. Extracellular recordings provide information of the activity of a subset of neurons around the electrode which is the summation of all the EPSPs and IPSPs in the postsynaptic neuron which if above -55 mV could generate an action potential (Carter and Shieh, 2010).

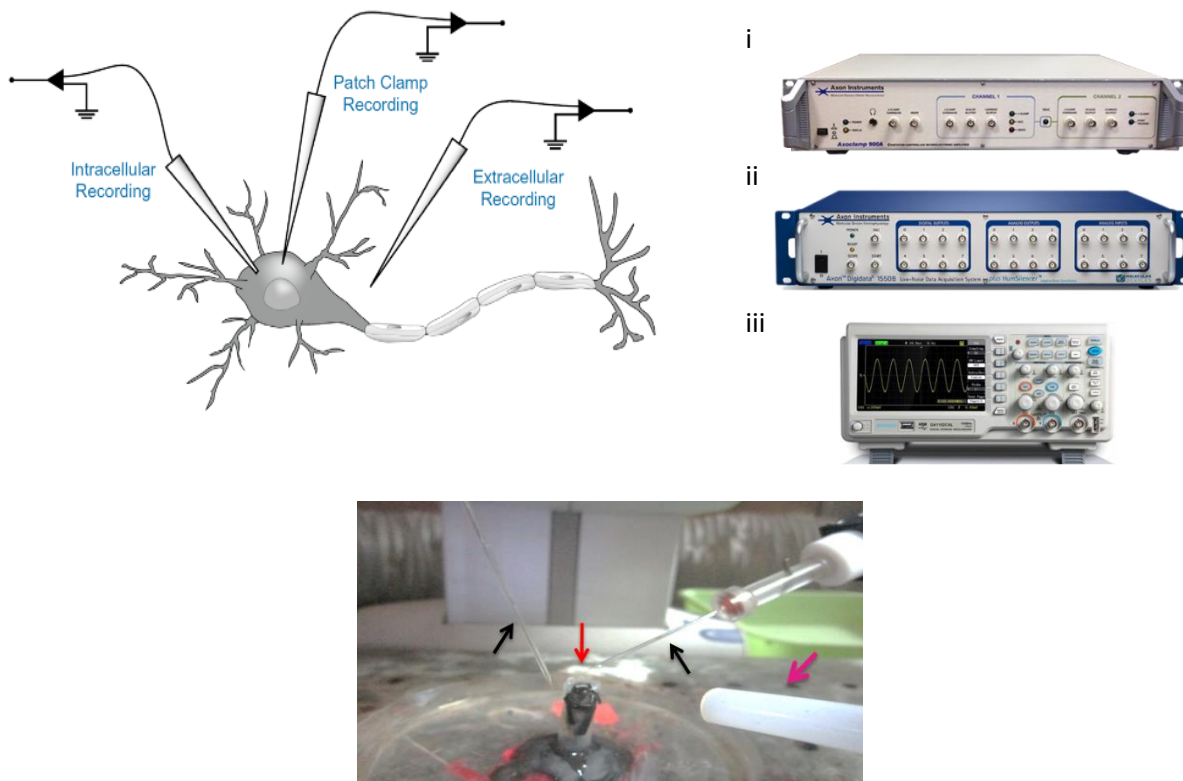


Fig 2: The top left panel represents the different types of electrophysiological recordings that can be made, patch-clamp and intracellular recordings require the electrode to make contact or be inserted into the neuron whereas extracellular recordings are made from the space surrounding the neurons (Top left panel figure adapted from Carter and Shieh, 2010) i. Amplifier ii. Digitizer iii. Oscilloscope B. The recording setup in the laboratory, the experimental insect mounted and

prepared for the recording session (red arrow), the two glass electrodes to carry out intracellular and extracellular recordings (black arrow), the odor stimulus valve (pink arrow).

Olfaction - A highly conserved sensory stimulus

Olfaction is a sensory process crucial to survival and involves sensing the presence of volatile chemicals in the air or sapid molecules in solution. The olfactory processes are highly conserved and involve the same fundamental steps in most multicellular eukaryotes. While terrestrial animals sense and discriminate air-borne odor molecules and their concentration, fish and aquatic animals gather information of the dissolved chemicals in the water via the same olfactory processes as the terrestrial animals (Ache and Young, 2005). Olfaction provides the animal with abundant chemical information of its surroundings. An evolutionarily conserved feature across the animal kingdom is the presence of G-protein coupled receptors (GPCRs) on the olfactory receptor or sensory neurons which are the first set of neurons to interact with the odor molecules. In mammals, the olfactory sensory neurons are present in the nasal epithelium while in insects such as the honey bee the olfactory receptor neurons are present inside the sensilla which are hair-like structures on the animal's antennae. Different species exhibit a different number of GPCRs that respond to either a similar set of odors or different sets depending on the genetic variation of the receptor which is controlled by the olfactory receptor genes (ORGs). Over the period of evolution, the ORGs have undergone a number of mutations under selective pressure including duplication, positive selection, or inactivation and a number of ORGs are now present as pseudogenes in higher complex primates (Ache and Young, 2005). Rodents have nearly 1400 ORGs while the ORGs characterized in humans so far are just over 400, honey bees on the other hand exhibit the presence of 170 ORGs. Each ORG allele produces one variant of the GPCRs which is then expressed on the surface of one olfactory receptor or sensory neuron in an

interesting one receptor one neuron phenomena, and these neurons will then respond to an odor or perhaps a structurally closely related odor. Olfactory neurons responding to one smell will converge into an olfactory tract that conducts the odor information to an olfactory bulb. Multiple tracts then synapse onto the olfactory bulb which is called the antennal lobe in insects. The organization of the mammalian olfactory bulb and insect antennal lobe is strikingly similar (Ache and Young, 2005). Functional units of the antennal lobe or olfactory bulb called glomeruli containing projection neurons now relay this information to higher-order information and memory processing centers of the brain such as the cerebral cortex in mammals and mushroom body calyx in insects (Ache and Young, 2005).

Insects as a model system for memory and learning

Insects have been successfully used as behavior, neurophysiology, learning, and memory models for decades. Their capacity to exhibit sophisticated foraging, defense, survival, and social behaviors despite their relatively simple brains with fewer neurons compared to complex mammals makes it feasible for neuroscientists and behavior biologists to ask and answer crucial neuroethological questions. Whether insects can be considered as cognitive animals is still a topic of much debate with evidence in support of both outcomes. While a group of scientists propagates the idea that, what insects exhibit as complex behavior might just be an output to survive or rapidly adapt to a particular situation (Webb, 2012) the other groups strongly support and provide evidence in support of the insects displaying higher cognitive abilities and behaviors that cannot be described as simple adaptation or survival (Chittka et al., 2019; Giurfa, 2012). In an experiment using *Drosophila* larvae Russell et al., (2011) demonstrated that the animals when reared on feed containing high salt levels (0.3M) exhibited a positive unconditioned response (UR) to an odor attractant (CS) when the odor was positively reinforced or rewarded.

This result was interesting since the salt concentrations in the unconditioned stimulus (US) or feed reward were high enough to induce aversion in the control group of insects (Russell et al., 2011). The argument made was that the behavior of the insect displaying a positive UR in response to an aversive US by virtue of having an attractive CS would have to do with the basic odor neural circuitry, perhaps involving a rewarding molecule such as dopamine being produced upon odor sensing which might override the animal's innate aversive behavior. This was debated as an example of how insects might not be as cognitively astute as the mammals and higher-order primates (Russell et al., 2011; Webb, 2012). In an equally robust argument, it has also been shown that insects can display a repertoire of sophisticated cognitive behavior, such as imitation of con-specifics, communication, navigation, and eusocial behavior (Chittka et al., 2019; Giurfa, 2012). It is however clear that much needs to be learnt about the neural mechanisms behind insect behavior and that study of insect behavior does provide important clues about how cognition evolved.

Social behavior is the hallmark of some insect species such as the eusocial insects belonging to the order Hymenoptera. Within the realm of social behavior, very intriguing and complex behavioral observations have been made (Chittka et al., 2019; Giurfa, 2012; Giurfa and Sandoz, 2012). The bumblebees for example have been shown to replicate con-specific foraging bumblebees as when the demonstrator lands on a flower, the replicator with no prior knowledge of the flower or the quality of the nectar will blindly follow the leading bumblebee (Giurfa, 2012; Leadbeater and Chittka, 2009). Wood crickets, *Nemobius sylvestris*, have also been shown to display a unique imitation behavior where they imitate wood crickets that have been trained and have learnt to hide from predators such as spiders. This behavior is especially interesting since the wood crickets imitating hiding have not been exposed to the threat but are simply

imitating the experienced insects. Navigation studies in insects such as the desert ants, honey bees, and bumblebees have paved the way for understanding insect behavior. A structure in the insect brain termed the central complex has been shown to play a pivotal role in combining several cues including the polarized sunlight, visual cues of the animal's present location, etc (Evangelista et al., 2014; Strausfeld and Hirth, 2013; Strauss, 2002).

In addition to optimized visual cues, the olfactory system plays a vital role in guiding an insect's foraging behavior and also in the formation of associative and non-associative memories. The heightened olfactory capabilities of insects such as the honey bee have been utilized by behavior and neurobiologists to understand the neural mechanisms behind memory formation and how the stored memory translates into elegant behavior outputs (Chittka et al., 2019; Giurfa, 2012; Giurfa and Sandoz, 2012; Menzel, 2001; Menzel, 2012; Webb, 2012). Insects can be trained in variations of the Pavlovian form of conditioning, wherein the animal learns to associate a stimulus such as smell (CS) with a reward (US) and displays an innate behavioral response (UR) which in case of insects could be the extension of the proboscis (Fig 3) or the palp movement as exhibited by grasshoppers in anticipation of the reward (Bitterman et al., 1983; Giurfa and Sandoz, 2012; Menzel, 2001). So far olfactory Pavlovian conditioning has proven to be a highly reproducible and robust method to study insect behavior and the underlying neural mechanisms (Giurfa and Sandoz, 2012).

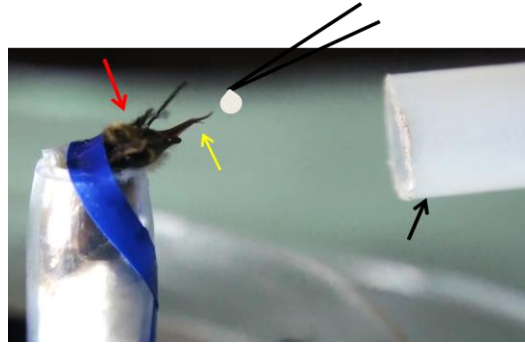


Fig 3: A honey bee undergoing olfactory conditioning, the odor valve (black arrow) provides the odor stimulus to the insect (red arrow), during which period a sugar reward (white teardrop) is presented to the insect. The animal over a period of five such trials learns to associate the odor with the reward and extends its proboscis in anticipation of the reward (yellow arrow) when provided with the odor alone.

The honey bee, a behavioral biologist's muse

The honey bee is an eusocial insect belonging to the order Hymenoptera. Of the nearly 20,000 species of bees, 7 species of honey bees have been classified and 4 of the 7 species are present in the Indian subcontinent. In a hive, the adult bees consist of a single queen, sterile female workers, and a few male drones. These insects exhibit a highly organized form of social behavior, where a single queen is responsible for the egg-laying and propagation of the hive while the remaining sterile worker bees are divided into working classes dependent on their age. This age-dependent job execution is termed age-dependent polyethism. Starting with the newly eclosed honey bee, the workers are categorized into cleaners, nursers, scouts, defenders, and foragers. The cleaners are responsible for the basic hive hygiene and the insect takes on the job of a cleaner immediately post eclosion, 3-7 days later the cleaners progress to become nursers, the nurser gradually transforms through the jobs of a comb maker, hive fanner, guard and approximately 21 days later begins foraging (Vance et al., 2009). The graduation through job classes also involve

changes at the genetic, physiological and anatomic level. For example the nursers have copious amounts of glands termed the hypopharyngeal glands that produce the enzymes required for the making of honey and royal jelly (Fahrbach and Robinson, 1996; Ueno et al., 2015; Vance et al., 2009). These glands are present below the head cuticle in nursers and as the nurser transforms into a forager the glands begin to degenerate (Deseyn and Billen, 2005). The ratio of two hormones, Juvenile hormone (JH) and Vitellogenin (Vg) also plays a crucial role in the physiology and behavioral programming of the worker bees (Fahrbach and Robinson, 1996). In addition, genetic changes such as the upregulation of the genes, *Apis mellifera foraging* (*Amfor*), *Apis mellifera buffy* (*Ambuffy*), *Apis mellifera matrix metalloproteinase1* (*AmMMP1*), have been shown to regulate the behavior and neurocircuitry of the worker bees (Heylen et al., 2008; Ueno et al., 2015). Further anatomical changes such as reduced fat bodies, increase in the size of the mushroom bodies in the bee brain are also well documented (Farris et al., 2001; Nilsen et al., 2011)

The foragers are crucial to the survival of a honey bee hive. They are saddled with the task of collecting pollen, nectar, water, and propolis from relevant foraging sources and providing the hive with the collected resource. The Western honey bee *Apis mellifera* can travel upto distances of 15 Km while the Asian giant honey bee *Apis dorsata* can sometimes travel upto 30 Km one way in search of viable food sources (Giurfa and Sandoz, 2012). Karl Von Frisch who was awarded the Nobel prize in medicine and physiology in 1973 was best known for his work on honey bee perception (Giurfa and Sandoz, 2012). He was able to demonstrate the capacity of the honey bee to be trained in Pavlovian forms of conditioning by setting up artificial feeders and training the foragers to visit it. Under controlled laboratory conditions, using light as a conditioned stimulus, it was Japanese researcher Matsutaro Kubwarawho, in 1957, demonstrated

that foragers can be taught to associate the CS with a reward. It wasn't till a few years later that another Japanese researcher Kimihisa Takeda worked out a more robust protocol using olfactory stimulus as a CS (Giurfa and Sandoz, 2012). Finally in a detailed paper published by Bitterman et al, (1983) a protocol using harnessed foragers, an olfactory stimulus, and 30% sucrose as a reward was set (Bitterman et al., 1983). Using this protocol and variations of the same, bee researchers have been able to unravel many aspects of the animal's olfactory neurocircuitry, memory, learning capacities, neurophysiology, foraging and feeding behavior, etc. (Giurfa and Sandoz, 2012; Menzel, 2001; Menzel, 2012; Wright et al., 2010).

The tasks of the honey bee foragers require them to be exceptional navigators, which involves a multitude of neuronal processes to be heightened and optimized to their needs. While foraging the animal needs to use visual cues such as the polarizing light of the sun to find the direction from the hive to the foraging patch and the color of the floral patch. Studies have shown that the foragers memorize landmarks which help them travel to and fro while in novel routes they activate a property called path integration where the animal cumulates the distance from the starting point namely the hive and hence can estimate the path back from the foraging site (Chittka et al., 1995; Collett, 2019; Pahl et al., 2011). At the foraging site, the animal now has the immense task of collecting the best quality pollen or nectar for the hive, for which it needs a well equipped olfactory system that will guide it to the perfect source (Wright et al., 2018). The honey bee forager discriminates between a wide array of odors and this discrimination is based on the chemical properties of the odor (Devaud et al., 2015; Laska et al., 1999). Once the collected food is taken to the hive the returning forager communicates the position of the source to its hive counterparts by performing a waggle dance (Barron and Plath, 2017). Through the course of foraging the forager needs to maintain a memory of the completed task and each step

involved in that day's task. To this end it has been shown that honey bee foragers exhibit short term (0-3 hrs), mid-term (6-12 hrs) and long term memory (24hrs-weeks) (Menzel, 2001; Menzel, 2012) that can be studied and observed under controlled laboratory conditions as described in the section above. For these reasons the honey bee forager has proven to be a very useful learning and memory model, given its capacity to rapidly assimilate new information and retain the learnt information for days, it is also utilized by neuroscientists to help understand the neurocircuitry of learning and memory and the changes that take place at the neuronal level during learning and memory formation (Menzel, 2001).

The honey bee olfactory circuit an overview

Honey bee olfaction begins with the antenna sensing the odor molecules (Fig 4). Hair-like structures on the surface of the antenna called sensilla are the first to interact with the odor molecules. This interaction results in the activation of the olfactory receptor neurons (ORNs) by the binding of odor molecules to the sensilla. The ORNs relay this information to neuropiles called the glomeruli, in each of the two antennal lobes which have projection neurons (PNs) and local interneurons arborizing onto them. From the antennal lobes, approximately 600 projection neurons carry forward the odor information received from the ORNs to the lateral horns (LH) and Mushroom bodies (MB) where they synapse onto ~183,000 intrinsic neurons of the MBs called Kenyon cells (KC) (Kropf and Rössler, 2018). The KCs have been shown to have sparse coding and also exhibit a temporal pattern of coding the olfactory information (Gupta and Stopfer, 2014; Kloppenburg and Nawrot, 2014). The KCs carry this decoded and packaged olfactory information to a class of neurons called the mushroom body output neurons (MBONs). The MBONs transfer the information to higher-order memory centers such as the α and β lobes in the pedunculus of the brain and are an interesting class of neurons that have been suggested to play a

role in designating a valence to the olfactory information rather than an identity (Aso et al., 2014; Traniello et al., 2019). Information processing begins with the ORNs at the first level followed by the PNs at the second level, and the MBs and α and β lobes in the pedunculus at the third and fourth levels also called higher levels.

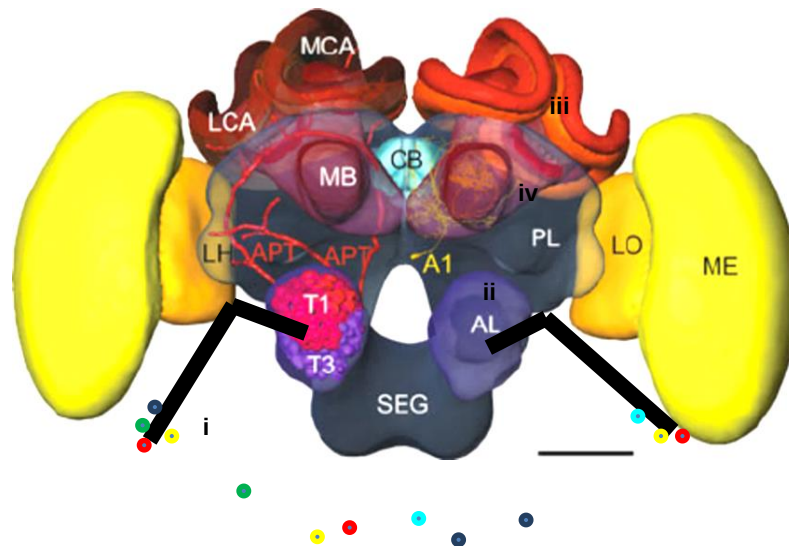


Fig 4: A representation of the honey bee olfactory circuit, i. The odor molecules (colored dots) are sensed by the sensilla on the antennae ii. The ORNs carry the odor information to the antennal lobes (AL) iii. From the AL the information is carried by the projection neurons via the projection neuron tracts APT to the iii. Mushroom body calyces (MCA, LCA) iv. The Kenyon cells (KCs) relay this information to the higher-order memory and learning centers namely the α and β lobes in the mushroom body pedunculus (MB). Figure adapted from (Rybak, 2012).

i) First level of olfactory processing- Transmission of odor stimulus from ORNs to Antennal lobes

In the first level of odor processing, ORNs are activated by the heterogeneous mixture of odor molecules present in the insect's environment (Fig 5). The odor molecules enter the pores of the sensilla where odor binding proteins bind to and transport them to the receptors

present on the ciliary dendritic processes on the surface of the ORNs (Jenkins et al., 2009). The olfactory information coding at the level of the ORN has a combinatorial pattern which depends on the molecular structure of the binding odor molecule (Ache and Young, 2005; Gutiérrez-Gálvez and Marco, 2013; Q. Li et al., 2013). The receptors on the ORN surface are predominantly G-protein coupled receptors which are a seven transmembrane domain protein that are able to generate an action potential in the ORN via the cyclic nucleotide or phosphoinositide secondary messenger system (Ache and Young, 2005). The secondary messengers in an activation cascade activate the ion-gated channels allowing for Ca^{2+} influx into the cell which changes the membrane potential of the cell causing a graded voltage-dependent neuronal response that can result in the generation of an action potential. In honey bees the activated ORNs relay the odor information to the functional units of the antennal lobes called glomeruli via four neuron tracts, T1-T4. ORNs responding to one odor are clustered spatially and project into the same glomerular compartment where they synapse onto the PNs and local interneurons (Ache and Young, 2005; Kirschner et al., 2006; Kropf et al., 2014; Rybak, 2012). The local inhibitory interneurons influence the output of the PNs and are hypothesized to play a role in gain control when the concentration of the odor stimulus is high (Chou et al., 2010; Christensen et al., 1993; Dacks et al., 2010; Das et al., 2008). The local interneurons have been found to be Histamine and GABA positive (Dacks et al., 2010; Sachse and Galizia, 2002; Schäfer and Bicker, 1986). The PNs receive the odor stimulus input and via two tracts transmit this information to the KCs (Dacks et al., 2010; Kirschner et al., 2006; Rybak, 2012; Zwaka et al., 2016).

ii) Second level of odor processing, PN to KC

Approximately 900 PNs exit the antennal lobe glomeruli in five tracts, the median (m-ALT), lateral (l-ALT) (Fig 6), and three medio-lateral (ml-ALT) (Zwaka et al., 2016). The axons of the m-ALT first project to the lip and basal calyx of the MB from where they branch towards the lateral horns (LH) while the l-ALT project initially to the LH followed by sending their axonal processes to the MB calyx (Kirschner et al., 2006; Laska et al., 1999; Menzel et al., 2005; Zwaka et al., 2016)

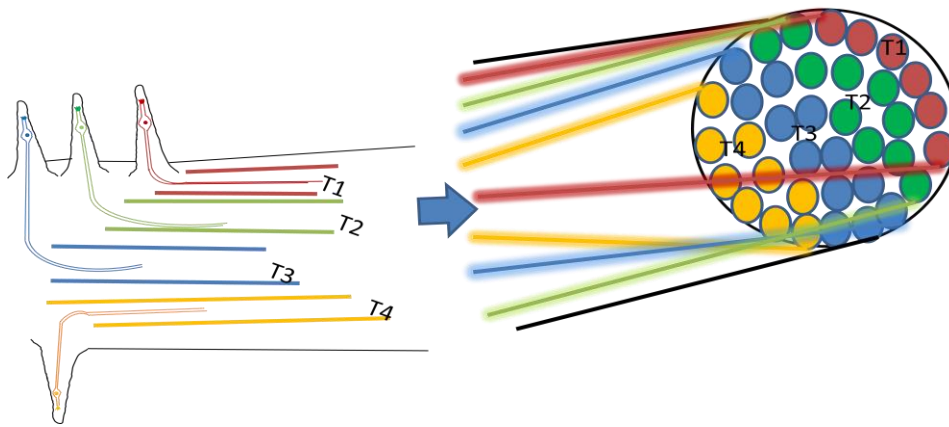


Fig 5: Different ORN's represented as the four different colors (Red, Green, Blue, Yellow) travel through four neuronal tracts, (T1-T4) each with similarly responding ORNs to the glomeruli of the antennal lobe. Here the tracts synapse onto the PNs in the glomeruli with the neurons responding to one odor synapsing onto the same PN. The glomeruli receiving input from the different tracts and are spatially segregated as represented by the four colors (T1-Red, T2- Green, T3- Blue, T4-Yellow). The image is not to scale and solely for representation purposes.

The ml-ALT on the other hand project into the protocerebrallobe (PL) without passing through the MBs. The m-ALT and l-ALT receive input from single glomeruli and exit as uniglomerular projections (uPNs) while the ml-ALT receive multiglomerular input exit the antennal lobes as multiglomerular projections (mPNs). The glomeruli onto which ORN tracts T2-T4 synapse provide input to the m-ALT tracts while l-ALT receives

primary input from T1 and some from T2 and T3 glomeruli clusters (Zwaka et al., 2016). There appear to be functional differences between the m-ALT and l-ALT PNs based on how the synapse onto the MBs, and the question is currently still being researched (Grünewald, 2003; Kropf and Rössler, 2018)

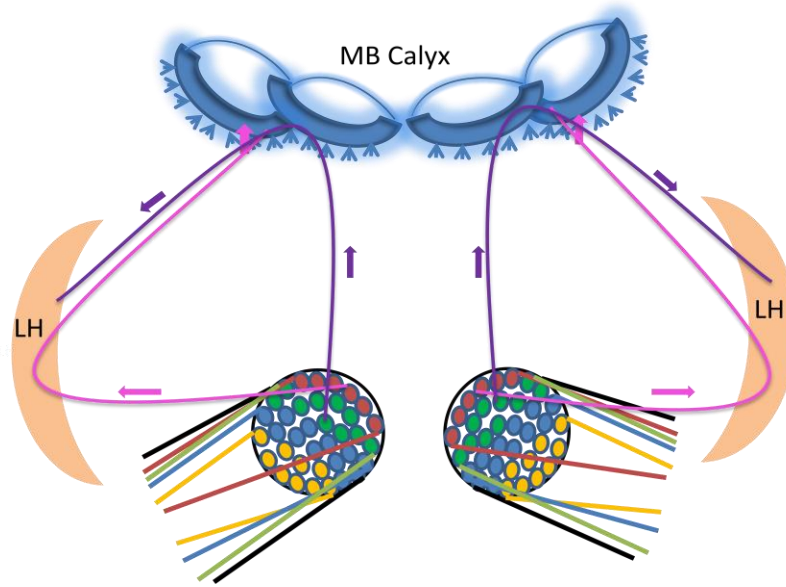


Fig 6: Uniglomerular olfactory information is conducted via two PN tracts, the m-ALT (Purple tract) and the l-ALT (Pink tract) to the MBs. The m-ALT projects into the MB first followed by sending its axonal processes to the lateral horn (LH) while the l-ALT first project into the LH followed by the MBs. The PNs synapse onto the KCs in the MBs as represented by the blue arrows across the MB calyx. The image is solely for representation and not to scale.

iii) Higher-level olfactory processing KC-MBON

The 900 PNs via the m-ALT and l-ALT synapse onto approximately 175,000 intrinsic cells of the MBs namely the KCs (Fig 7). Each PN synapses onto approximately 40 KCs and each KC receives input from 5-15 PNs. The KCs exhibit a unique information coding phenomenon called sparse coding, wherein the number of spikes generated by the KCs with the initial PN input is sparse (Demmer and Kloppenburg, 2009; Gupta and Stopfer, 2014; Kloppenburg and Nawrot, 2014; A. C. Lin et al., 2014; Szyszka et al., 2005). This

phenomenon is hypothesized to be controlled by the oscillations generated in the internal circuit of the PNs in the antennal lobe. The frequency and synchronization of the oscillations begin to increase with an increase in odor concentration in turn increasing the activity of the KCs (Gupta and Stopfer, 2014). The sparse coding nature of the KCs has been suggested to play a role in the memory and storage processes of the MB (Lin et al., 2014). The MBONs, a class of neurons that lie downstream of the KCs have been shown to play multiple roles including the encoding of learnt information and memory formation. The KCs synapse onto ~ 400 MB (A1-A7) extrinsic neurons which are located around the α and β lobes of the pedunculus (Rybak and Menzel, 1993). At the physiological level, the MBONs have been shown to be multi-modal in nature and display the capacity to alter their activity upon appetitive olfactory conditioning (Okada et al., 2007; Strube-Bloss et al., 2011; Strube-Bloss and Rössler, 2018). The A3 cluster of MBONs and a unique extrinsic neuron called the extrinsic neuron of the pedunculus (PE1) have been studied in much detail. The neurons in the A3 cluster have been shown to be GABAergic suggesting their role in olfactory learning and memory while the activity of PE1 is shown to reduce upon olfactory conditioning (Haehnel and Menzel, 2012; Okada et al., 2007). Interestingly not much is known about the activity of each subset of MBONs in honey bees and much still remains to be explored with respect to their activity.

Stressors, chemicals, and neurotransmitter blockers can hinder the process of memory formation

The MBs are crucial multi-sensory integration units, and have been shown to play a pivotal role in long term memory formation while the α and β lobes are required for the long term storage of memory (Boitard et al., 2015; Komischke et al., 2005; Malun et al., 2002; Traniello et al., 2019).

The process of memory formation was shown to be vulnerable to interference using cooling studies and chemical ablation using Hydroxyurea.

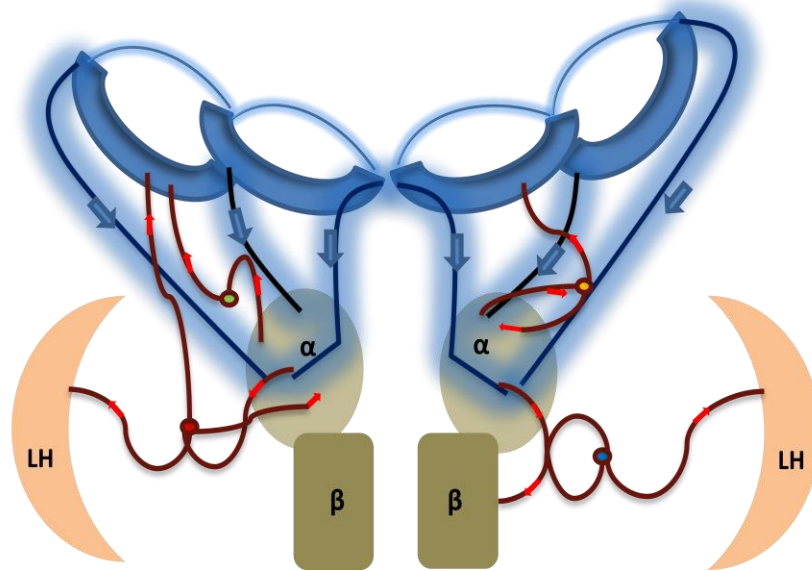


Fig 7: Higher-order olfactory memory processing takes place in the α and β lobes (green circle and green rectangle) which receive input from the KCs (blue tracts) that synapse onto the dendritic processes of the MBONs (green, yellow, red and blue dots). The soma of the MBONs are usually found adjacent to the α and β lobes as represented by the four colored dots, these subset of neurons send their processes and synapse into multiple areas of the brain including the MBs, the LH and also form feedback loops in the α and β lobes. Arrows on each of the tracts represent the direction of information flow. The image is representative and not drawn to scale.

These experiments involve the use of a cooling probe to cool neurons in a relevant region rendering them temporarily non-functional or exposing the larvae to Hydroxyurea in order to induce specific anatomic ablation during development. When the bees were checked for how cooling affected their learning and retention capacities using olfactory conditioning, it was seen that the α and β lobes play a crucial role in memory storage while the MBs are required for the process of memory formation (Erber et al., 1980). The experiments using Hydroxyurea also

highlighted the requirement of the MBs for the process of learning and solving elementary learning tasks (Komischke et al., 2005). At the synaptic level neurotransmitters are a vital part of memory formation, storage, and consolidation processes. Using neurotransmitter receptor blockers, and antagonists, researchers have shown that in addition to common neurotransmitters such as acetylcholine (ACh) the biogenic amines octopamine, dopamine, serotonin, GABA, histamine aid appetitive and aversive learning (Gauthier and Grünewald, 2012). Initial work on the role of neurotransmitters suggested that octopamine aids associative and reward associated learning (Farooqui et al., 2003; Hammer and Menzel, 1998; Scheiner et al., 2002), however, the present view on the role of neurotransmitters especially the biogenic amines suggests that each neurotransmitter plays a role in both aversive and appetitive learning. Dopamine has been extensively studied in regards to aversive learning and is seen to be required for the process of memory formation (Agarwal et al., 2011; Jarriault et al., 2018; Waddell, 2013). Recent evidence has shown that in addition to aversive learning both octopamine and dopamine have been established to play a role in reward-associated appetitive, reward-seeking and reinforcement learning and memory formation highlighting the complexity of the actual process (Barron et al., 2010; Liu et al., 2012; Waddell, 2013). Memory and learning in honey bees also depend on parameters such as motivation which can be driven by hunger and physical health of the animal. The animal's level of nutrition and the presence of parasites that impairs the insect's immune system also influence the honey bees learning and memory (Arien et al., 2015; Gage et al., 2018; Gómez-Moracho et al., 2017; Mattila and Smith, 2008).

Human interference is causing a rapid decline in the honey bee population via interference of their learning and memory

The need to understand the process of memory formation and the insect's behavior at the field level when it is learning new information has become more important in recent times given the dramatic decline in the numbers of honey bees. One of the main reasons for this decline is the inability of foragers to look for food efficiently or fly back to the hive from a floral patch as a result of perturbation to their cognition (Goulson et al., 2015; Neumann and Carreck, 2010; vanEngelsdorp et al., 2009; Wright et al., 2018). The foragers unable to navigate back to the hive die in the field out of exhaustion, and a multitude of causes have been shown to be responsible for this such as, the use of field-level pesticides, climate change, human destruction of hives, etc (Belsky and Joshi, 2019; Doublet et al., 2015; Goulson et al., 2015; Kessler et al., 2015; Reitmayer et al., 2019). Ample proof has also been provided to show how field-level pesticides such as neonicotinoids act by blocking the cholinergic receptors of the brain thereby preventing the binding of ACh to its receptor. Behavioral studies have also shown that honey bees exposed to neonicotinoids even exhibit a preference for the sugar solution with the pesticide, implying the possible addictive property of the solution (Kessler et al., 2015). This becomes especially problematic at the field level when the bees are unable to avoid the flowers containing the pesticide and will show a preference for it. Biopesticides are a more eco-friendly alternative to the neonicotinoids and so far have been shown to be harmless to non-target insects such as the pollinators (Dai et al., 2012; Jia et al., 2016). It is still to be assessed whether the extended exposure to biopesticides would affect the cognitive abilities of the honey bees and if these effects can be seen in acute doses depending on the method of field level application.

To study the learning and memory changes in response to various stressors, it is essential to first standardize the behavior of the insect under the same conditions. Very little behavioral work has been done to show the effect of acute exposure to abiotic stresses on foraging honey bees. How do simple perturbations such as isolation of foragers while they are foraging affect them? In addition, does this behavior vary between species of foraging bees, and when stressed, do foragers in the hive behave differently from the foragers on duty? A study answering these questions would give us a better insight into the cognitive capacities of the honey bees and lay the foundation to further understand the complex memory processes taking place in the honey bee's brain.

Our hypothesis

Apis mellifera linguistica is a well-established learning and memory model and is extensively used in neurophysiology studies to provide information about the neurocircuitry of learning and memory. This species of honey bees is a semi domesticable animal that can be maintained in boxes. *Apis dorsata dorsata* is a species endemic to South East Asia and widespread throughout the Indian subcontinent. This animal is an open nesting species and efforts to domesticate it have so far proven futile. Unlike its Western sister species, very little is known about the behavior or neurophysiology of *A. dorsata*. Preliminary results from the laboratory have shown that *A. dorsata* learns as well as *A. mellifera* and makes for a stable electrophysiology insect model. With the above brief description as the background the present study was designed with three objectives in *A. dorsata* and *A. mellifera*:

1. Given the behavioral evidence in *A. mellifera*, is there a bilateral transfer of olfactory learnt information from one brain lobe to the other in honey bees *A. dorsata*?
2. Are biopesticides in the form of a spray or formulation harmless to foraging *A. mellifera* honey bees or do they affect the learning and memory capacities of the honey bees when ingested in acute doses?
3. How do honey bees *A. mellifera* and *A. dorsata* respond to isolated capture, is the behavior quantifiable and specific to foragers?

GENERAL METHODS

General Materials and Methods

1. **Honey bee collection and maintenance**

a) *Apis mellifera* collection and maintenance

Apis mellifera linguistica bee boxes were provided by The National Institute of Rural Development and Panchayati Raj (NIRD) Hyderabad, Telangana. The bee box was replete with a queen, four to five combs of brood, workers, and drones. The box was transported to the laboratory late evening once foraging was complete to avoid stress to the foraging bees. It was placed in an open space to allow free movement of the honey bee workers the next morning. Bowls of water were placed under the legs of the bee box stand to prevent ant and other insect infestation. 1.5- 2 liters of 30-50% sucrose solution was prepared once in 10 days and supplied to the hive as feed. In addition commercial bee pollen, by Theo organics, India was mixed in water to make a paste and provided to the hive once in 15 days. Box cleaning was carried out once a month.

For the behavior experiments to check for the bilateral transfer of olfactory information, foragers were collected at the entrance of the hive at 9 AM. To quantify the behavior of honey bees in isolated capture, a feeder containing 30% sucrose was set up 2 mts from the hive. The foragers were initially trained to visit the feeder by physically collecting a few foragers at the hive entrance and placing them on the feeder. One trial of this process was adequate to train the foragers to visit the feeder.

Few of the foragers visiting the feeder were tagged with testor enamel paints on the thorax. These tagged foragers were collected from inside the hive as one of the experimental groups for the experiment. Younger hive bees were gauged by their behavior of entering the brood cells and were collected.

b) *Apis dorsata* collection and maintenance

Apis dorsata dorsata is an open nesting migratory species of honey bees that cannot be domesticated. *A. dorsata* bees were either directly collected from the hive or from floral patches that the foragers were visiting, namely flowers of, *Eucalyptus globus*, *Turnera subtulata*, *Hemilia patens*.

2) Preparation of Bee Ringer's

Honey bee saline solution was prepared in accordance with the protocol published by Krischner et al., (2006). Bee's Ringer was made by dissolving equivalent amounts of the following salts, 37 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.4 mM KH₂PO₄, in 1 lt of autoclaved double distilled water (ddH₂O) and pH was maintained at 7.2.

3) Bee Dissection

Collected honey bees were anesthetized at 4°C for ten minutes. The cooled bees were harnessed into plastic holders and harnessed using insulation tape, such that their antennae, mandibles, and proboscis could move freely. The dissection was carried out under a stereomicroscope. Low melting point wax was used to tether the head, after which the head cuticle was gently removed along with the tracheal glands the hypopharyngeal glands, and membrane sheath. Bee's Ringer was added to the exposed brain to keep the animal alive (Fig 1).

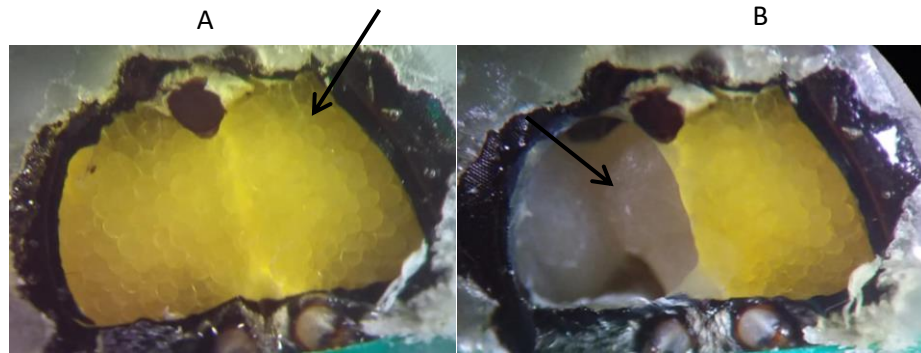


Fig 1: An exposed bee brain, A. The hypopharyngeal glands are visible as a yellow bead-like sheet (black arrow) after cutting open the head cuticle B. The brain is under the thick sheet of hypopharyngeal glands (black arrow).

4) Electrophysiology

Bees were anesthetized at 4°C for ten minutes and dissected to expose the brain. A Silver wire was chlorinated in Clorox solution for twenty minutes. The silver chloride ground wire was washed with ddH₂O and the pin of the wire was connected to the headstage in the recording rig. Borosilicate glass electrodes (1mm outer circumference and 0.5 mm inner circumference) were pulled in an electrode puller (P97; Sutter instrument, Novata, CA). The pulled electrode was filled with Bee's ringer and placed gently into the holder of a micromanipulator. The dissected bee was placed under a stereomicroscope in the recording rig, the ground wire was dipped into the Bee's Ringer solution in the wax cup that tethered the bee's head and the glass electrode was guided into the preparation to the brain region of interest using a micromanipulator. An Amplifier (Axoclamp 900A Molecular Devices U.S.A) received the signals from the glass electrode and an output signal was provided by comparing the electrode signal to the signal from the ground wire. The Axoclamp software maintained the impedance of the electrode between 1-10 MΩ. The signal was amplified to 1000X, high- pass and low-pass Bessel filtered at 1Hz and

80Hz respectively. The amplified signal was digitized using a digitizer (Digidata 1440) and the Clampex software acquired the data at a sampling rate of 10 kHz. An oscilloscope was used to visualize the waveform of the recording and a speaker provided an audible output of the recording.

In order to provide the olfactory stimulus, glass bottles with silicon stoppers containing the following odorants hexanol, nonanol, octanoic acid, and geraniol provided by Sigma-Aldrich were used. The odorants were delivered at 2 seconds (s) from the start of the 10 s trial for a period of 1 s. The release of the odor was controlled by a solenoid switch which was software controlled and the delivery rate of the odor was 0.1l/min. 10 trials of the 10 s trial were conducted for each recording and the data was analyzed using MATLAB (MathWorks Software inc).

i) Electroantennogram Recording (EAG)

The antenna of the tethered bees was placed on a small glass plate that was raised to the level of the bees head. The antenna was gently cello taped onto the glass using thin strips of insulation tape. The glass electrode was guided and placed gently on the tip of the antenna. The remaining recording and odor delivery parameters were maintained as described in the above section.

ii) Local field potential Recording (LFP)

The glass electrode was guided to gently pierce the mushroom body (MB) or the α -lobes, depending on the region to be recorded from. Bee's Ringers was used to keep the animal

alive during the recording procedure. The parameters for the recording and odor delivery were maintained as described above.

iii) Electrophoretogram Recording (ERG)

A small incision was made in the bee's head capsule and the ground wire was gently placed in the opening, care was taken not to pierce the brain. The glass electrode was placed on the eye facet and an LED light was placed close to the recording eye. The signal was high pass and low-pass Bessel filtered at DC and 80Hz respectively. The signal gain was maintained at 200, and the signal was acquired at a sampling rate of 10kHz using the digitizer and Clampex software. The light stimulus trial lasted for 10 s, the light was switched on at the 3rd s for a period of 1 s. Ten trials were carried out using the light stimulus which were software controlled.

5) Neuronal Tract Tracing and Bee brain Whole-mount Immunohistochemistry

Honey bees were collected, harnessed, and dissected. A microscopic amount of Protease (P5147, Sigma-Aldrich, India) was added to the brain using the tip of a sharp forceps. The protease was thoroughly washed off using Ringer's solution and the brain was desheathed. Fluorescent dyes Tetramethyl Rhodamine (D3308, Invitrogen, extinction coefficient 545 Å) and Dextran biotin (D7135, Invitrogen) were injected into specific regions of the exposed brain using a sharp forceps. Post-injecting the dye, Bee Ringer's was added to the brain, the preparation was covered with Aluminum foil and kept in the dark for six hours. The insect was placed under a microscope and using fine scissors and forceps the brain was gently cut out of the insect's head. 4% PFA was prepared by dissolving 4 gms of Paraformaldehyde (P5147, Sigma-Aldrich, India) in 100 ml of a 1X Phosphate buffer saline (PBS) solution. The dissected brain was placed in a 1.5ml Eppendorf

tube containing 500 μ L of 4% Paraformaldehyde (PFA) and kept overnight at 4°C. The following day the incubated brains were washed thrice in 1X PBS solution. 500 μ L of 3% Triton-X in PBS (3 % PBST) was added to the dissected brains and the brains were allowed to permeabilize for 1 hr. To the 3% PBST solution Streptavidin with secondary label Alexa fluor (S21375, Invitrogen, extinction coefficient 633Å) was added in a 1:1000 dilution. The incubated tissues were kept at 4°C for 5 days with regular shaking. Post incubation the tissues were thoroughly washed 3X in 3% PBST with a 20 min incubation period during each wash. 1 X PBS was added to the washed tissues for half an hour, 3X repetitions were done. The washed tissue was dehydrated by processing it in ascending alcohol series (30%, 50%, 70%, 80%, 90%, 100%) for 20 min each. The processed brains were then placed on a clean concave glass slide to which a drop of methyl salicylate was added. A cover glass was placed on the tissue and transparent enamel paint was used to seal the slide.

6) **Confocal Microscopy and Z-stacking**

Slides were visualized using a laser scanning confocal microscope (Leica TCS SP2, Leica Microsystems, Karl Zeiss LSCM NLO 710, Germany, Karl Zeiss LSM 780). Z-stacks were analyzed using the Image J software.

7) **Olfactory conditioning and behavior**

Honey bee foragers were collected, anesthetized and harnessed. The insects were allowed to normalize for 2 h following which olfactory conditioning was carried out. The conditioning protocol used was in accordance with the protocol published by Bitterman et al., 1983. The onset of the odor stimulus was software controlled and the odor release was controlled by solenoid valves. The insect was placed 1 cm from the odor valve and was given 14 s to normalize on the

pedestal. The odor onset lasted for 4 s, with a 1 s overlap with a 30% sucrose reward and the reward was continued to be presented for 2 s post odor switching off (Fig 2). The trials for each bee lasted 1 min including the normalizing before and after the odor conditioning. 5 repetitions of the trials with each bee was made with a 10 min inter-trial interval (ITI) between the trials. An LED light signaled the onset and offset of the odor and reward presentation. hexanol, geraniol, and citral were used as the odors for the behavior experiments.

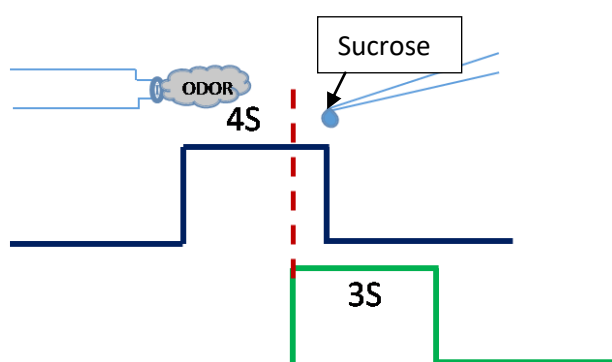


Fig 2: A diagrammatic representation of the olfactory conditioning protocol. The onset of odor is represented as the blue rectangular box (4 S) the reward presentation is shown as the green rectangular box (3S), the overlap between the odor presentation and the reward is shown by the red dotted line for, the overlap time was for 1S.

8) **Bacterial inoculation**

DOR BT-1 bacterial isolates were incubated in 100 ml of nutrient media (Himedia) for 72hrs with continuous agitation at 28°C and 180 rpm/min to allow for uniform sporulation. Post incubation the cells were centrifuged at 1100x, 4°C, the pellet was washed 3X in PBS and stored at 4°C.

9) Protein estimation by Bradford's

Bradford's assay was used to estimate the protein concentration calorimetrically in the required solutions (Bradford, 1976). 10mg /ml bovine serum albumin (BSA) was used to prepare the standard solutions of varying concentration. Into a 1.5 ml Eppendorf tube an aliquot of the requisite concentration of BSA was taken and volume adjusted to 100 μ L using 100 mM Tris-HCL (pH 7.4). 900 μ L of 1X Bradford's reagent (Bio-Rad USA) was added to the solution and allowed to incubate in the dark for 10 min. Absorbance was measured Spectrophotometrically (Shimadzu, Japan) at 595 nm using the appropriate blank solutions before measuring the incubated solutions. A standard curve of the measured absorbances was plotted and the concentrations were estimated.

10) Protein Profiling**i) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

The protocol followed was in accordance with Laemmli, 1970. A 5% stacking gel consisting of 2 ml 30% acrylamide mix, 3 ml 0.5 M Tris-HCl pH 6.8, 6.76 ml distilled water, 120 μ L 10% SDS, 50 μ L 10% of APS and 6 μ L of TEMED and a 10 % resolving gel containing 3.3ml 30% acrylamide mix, 2.5 ml 1.5 M Tris-HCl pH 8.8, 4.1 ml distilled water, 120 μ L 10% of SDS, 150 μ L 10% APS and 10 μ L TEMED were prepared. The ratio of the stacking gel to resolving gel was kept as 1/4 and 3/4 respectively. The resolving gel was first poured into the SDS-PAGE glass slabs and allowed to polymerize followed by pouring the stacking gel. A comb with the required number of wells was gently inserted into the stacking gel and allowed to polymerize. Known concentrations of the protein samples were mixed with the loading buffer which contained 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol,

and 0.002% bromophenol blue. The prepared protein samples and a protein ladder containing markers of known molecular weights (Bio-Rad, USA) were loaded carefully into the gel wells. Electrophoresis was carried out at 60 V for ten minutes till the proteins were stacked, followed by 100 V in the stacking gel. The running tank buffer consisted of 0.25 M TrisHCl, 1.92 M Glycine and 1% (w/v) SDS pH 8.3.

ii) Visualization of the Proteins

Proteins were visualized using Coomassie gel staining, the protocol followed was in accordance with Wilson et al., 1979. A staining solution containing 0.025% Brilliantblue-R 250 in 40% methanol and 7% acetic acid was poured into plastic troughs, into which the electrophoresed gels were placed. They were incubated in the staining solution overnight on a rocker. This step was followed by destaining in a destaining solution containing 40% methanol and 10% glacial acetic acid.

iii) Western blot and immunodetection

In order to confirm the presence of the protein of interest, we carried out the Western blot protocol in accordance with Towbin et al., 1979. The gel was transblotted onto a nitrocellulose membrane (Amersham, Germany). The membrane and gel were first immersed in Towbin's buffer (25 mM Tris, 192 mM glycine, and 20% methanol) for 10 min. The gel, filter paper, membrane were stacked into a cassette, and care was taken to not allow any air bubbles to enter. The cassette was placed in a transfer unit containing Towbin's buffer. The protein to membrane transfer was carried out at 25 V with 250 mA current limit at least for 14 h. Transfer of the protein was confirmed using Ponceau solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) which was followed with

extensive washing in Tris-HCl (Tris buffered saline (TBS), 10 mM Tris-HCl (pH 7.4), 150 Mm NaCl). Immunoblotting was carried out by soaking the membrane in 5% blocking solution (5% skim milk powder (w/v) in TBS) for 3hr to block all the nonspecific sites. The blot was washed (3x 15 min) with 0.1 % Tween-20 in 1X TBS (TBST). Primary antibody was diluted in a 1:1000 ratio and incubated with the blot overnight at 4°C. This was followed by incubating with the secondary antibody (1:4000). Alkaline phosphatase (ALP) or Horseradish Peroxidase (HRP) conjugated anti-mouse or anti-rabbit IgG was diluted in 5% (w/v) skimmed milk powder in TBS and soaked with the blot for 2 h. The blot was washed thoroughly to remove unbound antibodies and blot development was carried out using BCIP/NBT (G-Biosciences, USA) for ALP or chemiluminescence (Takara Bio Inc, Japan) for HRP. The blot was photographed using Kodak photoimager.

CHAPTER 1

EVIDENCE FOR ABSENCE OF BILATERAL TRANSFER OF OLFACTORY LEARNED INFORMATION IN *APIS DORSATA* AND *APIS MELLIFERA*.

Chapter 1

Introduction

The first neuron has been hypothesized to have appeared in the Ctenophores, a group of marine carnivorous invertebrates that show the presence of a neural net and the first nervous system is shown to have appeared in Cnidarians (Bucher and Anderson, 2015; Kristan, 2016; Lichtneckert and Reichert, 2007; Moroz, 2015). The Cambrian explosion resulted in the generation of the majority of the animal kingdom lineages giving weightage to the hypothesis of parallel neuron evolution (Kristan, 2016). This might also explain how despite being a basal group in the metazoan chart the Ctenophores show the presence of a neural net while the groups Porifera and Placozoa have not been established to have neurons till date (Bucher and Anderson, 2015; Moroz, 2015). The brain as a tissue with its functional units, the neurons along the course of evolution began acquiring the ability to amass external sensory cues, compute the acquired information at a cognitive level and exhibit an output such as locomotion. This behavioral output was needed both to escape a predator and to find food (Kristan, 2016). It has been theorized that the nervous system evolved from the ectodermal layer. The epithelial cells that remained in contact with the external environment triggered the need for secretory cells that were polar in nature and capable of responding to sensory cues by relaying the acquired information in the form of electrical signals (action potentials) to the next conducting cell (Bucher and Anderson, 2015). These secretory cells which were capable of responding to stimulus evolved into the neuron.

The first bilateral animals to have evolved have been suggested to be the Acoelomorphs, a group of flatworms belonging to the sub-phylum Acoelomorpha which lies in the Xenacoelomorpha phylum (Hejnol et al., 2009; Ruiz-Trillo and Paps, 2016). Whether the earliest ancestors to the

clade bilaterals are the Deuterostomes or the Acoelomorphs has been extensively debated, and recent molecular studies have shown a deep split in the evolution of the bilaterals resulting in placing Acoelomorphs as a sister group to the bilateral clade, with the Deuterostomes lying just above them (Hejnol et al., 2009). The evolutionary advantage of bilateral symmetry in animals has been posited to be in favor of the advanced mobility and locomotor activity as suggested by the “manoeuvrability hypothesis”. By allowing a polar anatomic development with the superior end consisting of the brain and the medial to inferior end consisting of the tail and locomotory appendages the animal is given the advantage of quick approach or retreat (Hollóand Novák, 2012). With the evolution of a bilateral anatomy, came the development of a bilateral brain, as seen in a majority of the eumetazoan animals (Fig 1).

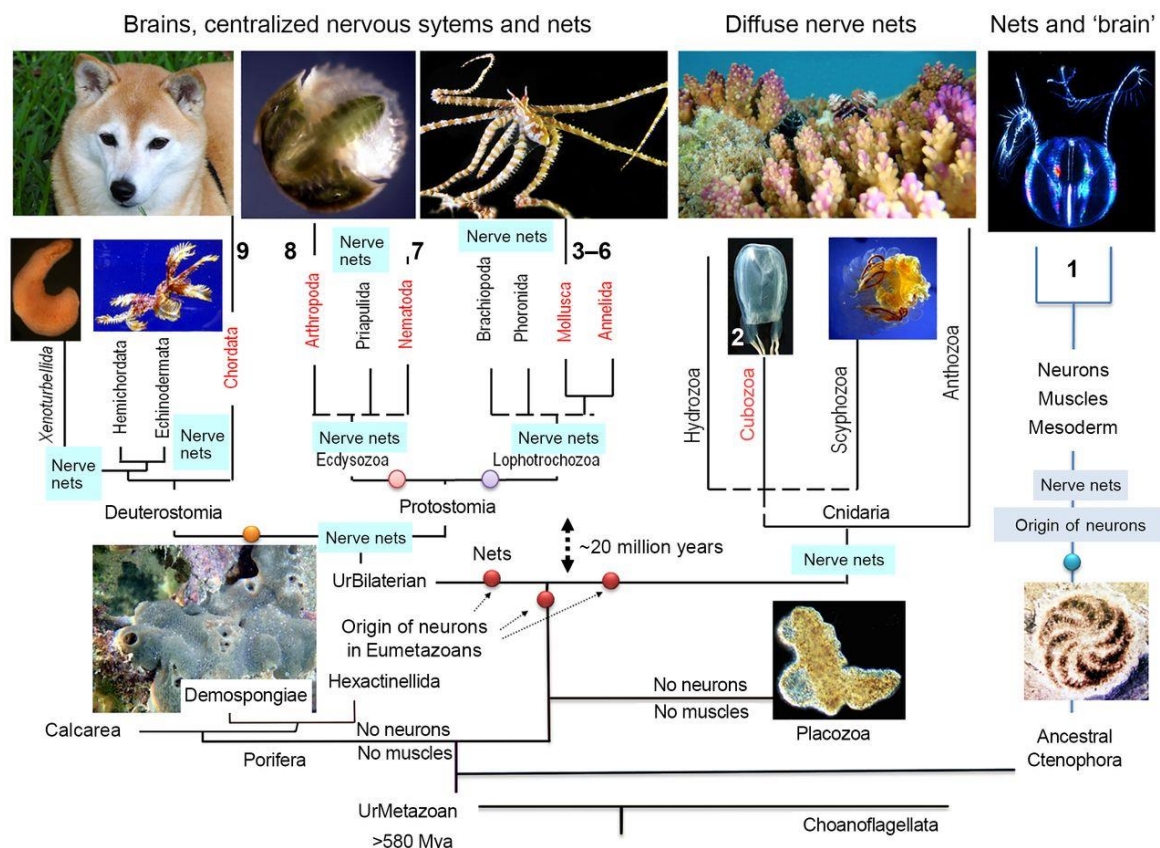


Fig 1: A flow chart representation of the evolution of neurons from 1. Neuron nets to 9. Centralized nervous systems and Brains (Figure adapted from Moroz, 2015).

The increasing brain size in the animal kingdom got selected because it by and large increased the computational capacity of the brain that allowed complex and fast sensory. This capacity has been suggested to have been enabled by the formation of bilateral neurons and inter-hemispheric commissures that connected two homotopic and heterotopic regions of the brain lobes (Suarez et al., 2014). These inter-hemispheric connections are seen across the animal kingdom starting from the lower invertebrates moving up the order to the highest order primates. Commissural development in vertebrates can be seen right from the jawless fish, for example, lampreys and structures such as the anterior commissure and hippocampal commissure are conserved in the higher complex mammals as well (Suarez et al., 2014). The Corpus callosum is the major inter-hemispheric commissure seen in only in eutherian mammals, and hence termed an evolutionary innovation or an evolutionary jump (Aboitiz and Montiel, 2003; Gazzaniga, 2000; Suarez et al., 2014; Wolman, 2012).

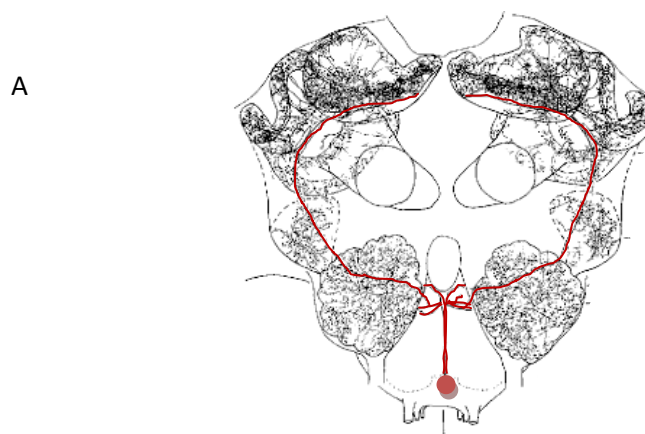
In the invertebrates such as the arthropods example the insects, a number of inter-connecting visual neurons have been well established (de Lussanet and Osse, 2012; Roper et al., 2017; Sanes and Zipursky, 2010). At the locomotor level in insects the central complex, an ellipsoid, fan-shaped body connected to the protocerebral bridge, which is centrally located in the insect brain receives large amounts of variable input from several sensory centers and has been established to play a crucial role in the control and coordination of locomotion in insects (Homberg, 1985; Pfeiffer and Homberg, 2014; Strausfeld and Hirth, 2013; Strauss, 2002). Interesting parallels have been drawn between the central complex of the arthropod system and the basal ganglia of the vertebrates (Strausfeld and Hirth, 2013). With respect to olfaction, the anterior commissure in vertebrates has been known to function in the acquiring and computing olfactory information received from the olfactory bulbs before relaying it to both lobes of the brain (Aboitiz and

Montiel, 2003; Suarez et al., 2014). The insect olfactory circuit, on the other hand, is a unique circuit with most insects exhibiting unilateral olfactory circuits with some interesting exceptions such as *Drosophila* showing the presence of a contralateral commissure, that originates from the glomeruli on one antennal lobe and synapses on to the mirror glomeruli in the contralateral antennal lobe (Kaur et al., 2019). Further, insects such as the desert locust *Schistocerca americana* have been shown to have a cluster of lateral horn neurons that have contralateral arborizations (Gupta and Stopfer, 2012).

Using intracellular recordings from the brain of a species of grasshoppers, *Hieroglyphus banian*, our laboratory has recently provided evidence of a class of odor responsive bilateral neurons that synapse onto contralateral mushroom bodies (Singh and Joseph, 2019). It could be argued that the presence of such bilateral neurons at the olfactory level in insects would enhance their computational abilities and allow for optimal sensory integration. Olfaction is a crucial sense of stimulus required for survival as seen in insects such as the honey bee. However anatomic studies of the honey bee brain have not revealed the presence of olfactory based commissures with connections on both sides of the brain. Extrinsic neurons such as the PE1 have been shown to cross the midline and suggested to play a role in the transferring of olfactory learnt and memory information (Menzel, 2012; Menzel and Benjamin, 2013; Okada et al., 2007; Rybak and Menzel, 1993).

At the gustatory level, the Ventral unpaired median neuron (VUMmx1) in honey bees, with its cell body ventral to the subesophageal ganglion and the maxillary neuromere (Fig 2A), is well documented and has been shown to play a role in reward association by combining the gustatory responses to olfactory learning (Farooqui et al., 2003; Hammer, 1993; Hammer and Menzel,

1998). This neuron has been shown to be octopaminergic (Farooqui et al., 2003) and exogenously applied octopamine in the regions that VUMmx1 arborize can substitute as a reward in honeybees (Hammer and Menzel, 1998). VUMmx1 is bilateral and arborizes in all the known olfactory centers of the honey bee brain such as the MBs, the antennal lobes, and the lateral horns (Hammer, 1993; Hammer and Menzel, 1998; Menzel, 2012). The architecture and function of VUMmx1 would imply its possible role in higher-order conditioning. Higher-order conditioning or second-order conditioning is a process by which the animal can be taught to associate a novel stimulus such as with an already learnt stimulus, using learnt stimulus acts as a reward/punishment. Second-order olfactory conditioning in a PER conditioning paradigm was demonstrated in *Apis mellifera*. The animals were trained to associate a stimulus with a sucrose reward in the first step. In the second step, the trained odor was paired with a novel odor in a forward conditioning paradigm (Hussaini et al., 2007). By the fourth trial, a significant fraction of the animals began responding to the novel odor hence that the odor stimulus that the animal learned in the first step acts as a reward after training (Fig 2B).



B

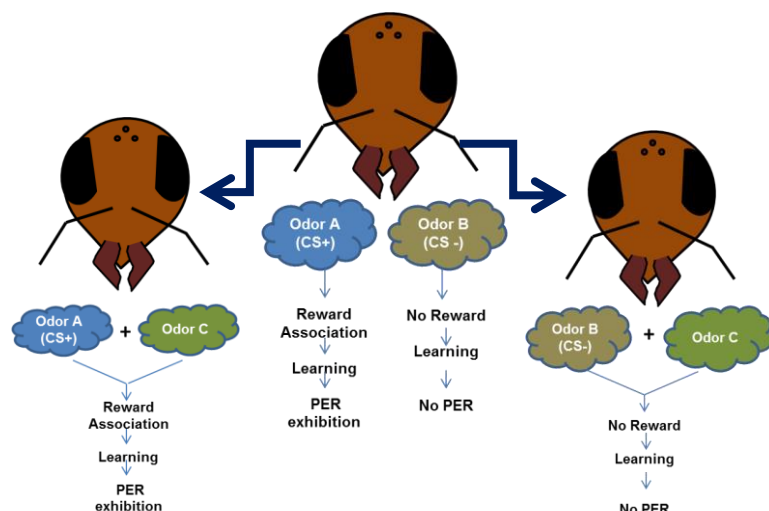


Fig2: A. The anatomy and branches of VUMmx1 neuron, the bilateral tract of the neuron is represented as a red line on both side of the brain, the neuron has been shown with its soma (red dot) located in the maxillary neuromere and extensive bilateral branching into important olfactory centers, namely Antennal Lobe, Lateral Horn, and Mushroom Body, as shown by the black traces, figure adapted from Hammer and Menzel (1998). Principle of the second-order conditioning demonstrated, when the insect is trained in olfactory conditioning with a rewarding odor- Odor A (CS+) (blue cloud) and unrewarded odor- Odor B (CS-) (grey cloud) the insect will first learn to discriminate between the two odors and exhibit PER or a behavioral output for Odor A (CS+) by the end of five trials. The trained rewarded odor itself is now used as a reward to train the bee to a novel odor. Hence when Odor A (CS+) is overlapped with a novel odor- Odor C (green cloud) the animal might learn to associate the rewarded odor with the novel odor and exhibit an increase in PER responses over trials. This increase in responses can be compared to the learning rate in bees when the unrewarded Odor B (CS-) is overlapped with the novel Odor C.

Despite no prominent contralateral olfactory connections in honey bees in the olfactory pathway in *Apis mellifera*, it was reported in a set of behavioral experiments, the possibility of bilateral transfer of olfactory information, wherein when one antenna is isolated from the training

procedure using a plastic wall to separate the two sides and the other antenna used to train the animal to an odor (Fig 3A), the animal was able to transfer this learnt information to the unexposed, isolated side over a period of three hours (Sandoz and Menzel, 2001). It was also reported that, when trained in odor discrimination, the animal was also capable of transferring the odor identity to the unexposed side over a period of three hours (Sandoz and Menzel, 2001). These results in combination with works which showed the increase in memory molecular markers on the unexposed side of the brain after training indicated that there could be a commissure between the olfactory centers of the two sides which could relay olfactory learnt information to the contralateral side of the brain in a temporal manner (Guo et al., 2016). In this set of experiments that showed changes in the molecular markers on the contralateral side to training, one antenna was coated with a silicon paste (Fig 3B) instead of the separation method using a plastic wall as described by Letzkus et al., (2006).

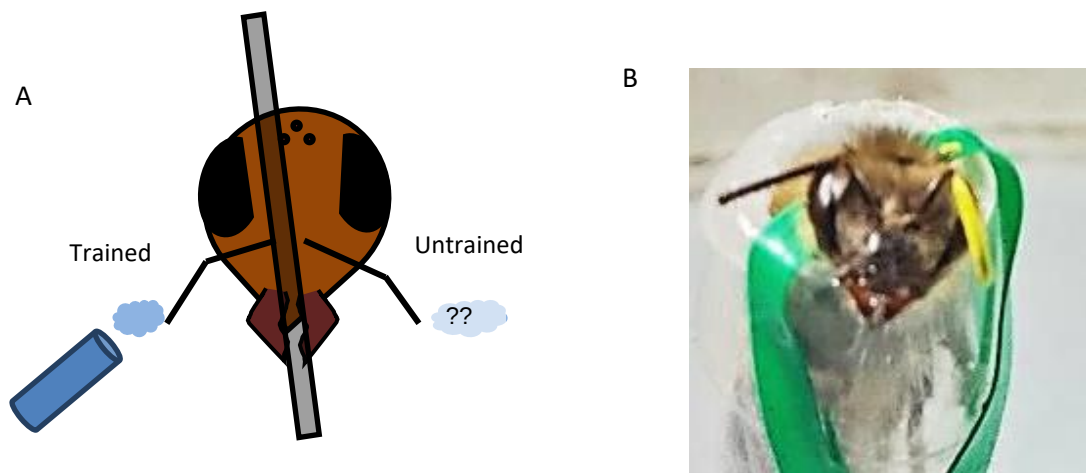


Fig 3: Pictorial representation of the two experimental set ups used to isolate one antenna from the training procedure, A. A plastic wall 40mmx50mm used to separate the two sides and the odor valve is placed on one of the two sides and the animal is trained. Retention is checked on the untrained side post 3 hrs B. A silicon paste is used to cover one antenna (yellow paste on the left antenna in the picture) and the open antenna is used for training the animal.

The above results and experiments laid the foundation for the possibility of finding bilateral connections at the olfactory level in honey bees, which would significantly change the present knowledge of the neuronal circuit map as we know in these animals. The presence of bilateral neurons would also provide us insights into the evolutionary correlates of inter-hemispheric cross-connections in the olfactory pathway. Hence we asked if we could locate these bilateral olfactory neurons in a species of honey bees native to south-east Asia, *Apis dorsata*, and in the Western honey bee *Apis mellifera*, given the behavioral results showing transfer of memory in *A. mellifera*.

Materials and Methods

1. Bee collection

Apis dorsata foragers were collected from seasonal floral sources such as *Turnera subtulata*, *Tecoma stans*, *Eucalyptus globus* at 9 AM. *Apis mellifera* foragers were collected at the entry of the hive at 9 AM. The animals were anesthetized at 4°C for 10 min and harnessed into plastic holders. The training began 2 hrs post harnessing after allowing the animals to normalize.

2. Bee dissection

The heads of the harnessed bees were immobilized using low temperature melting wax and the mandibles proboscis were left untouched. The antennae were isolated from the dissection using a thin strip of insulating tape. Using a fine scissor and forceps the head cuticle was first removed. A drop of bee Ringer's was placed in the exposed region. Using the fine forceps the hypopharyngeal glands, trachea, and membrane were gently dissected out. Bee Ringer's was added amply to keep the animal alive during the recording procedure.

3. Electrophysiology

- i) **Sensilla recordings:** To check for the blocking capacity of acrylic paint we carried out sensilla recordings from the antennae of *A. dorsata*. The harnessed animals with undissected heads were placed in the Faraday cage under a stereomicroscope. The antenna of the animal was immobilized using insulating tape onto a small plastic base which was placed at the level of the bee's head. A glass electrode pulled using the electrode puller (Sutter Instruments) was filled with bee ringer's solution followed by placing the electrode in an electrode holder with a chlorided silver. Once placed in the holder the electrode was guided to the surface of the antenna using a micromanipulator. The signal was amplified by Axon instruments Axoclamp 900A. The impedance of the electrode was between 1-10 M Ω . The signal was filtered between 0.1Hz and 80Hz and the gain was 1000, the amplified signal was digitized using Digiclamp 1440A (Molecular Devices) and pClamp. The data was acquired at a sampling rate of 10 kHz. An oscilloscope helped in the visualization of the signals. Results for this experiment are presented in Appendix A

- ii) **Local Field Potential Recordings :** In order to look for possible bilateral olfactory connections between the two lobes of the brain, LFP recordings were made using an electrode placed in the Mushroom Body (MB) on one side of a bee brain while the antenna on the same side was coated with acrylic paint. The odor delivery tube was placed in front of the bee and the odor (hexanol) delivery was computer-controlled. An exhaust vent placed behind the bee removed the remnant odor molecules post-delivery. Each recording consisted of ten trials of 15 s with the onset of odor at 2 s lasting for 1 s.

We further checked for bilateral connections between the two α -lobes by recording simultaneously from both the α -lobes while sequentially blocking one antenna at a time, then both antennae and finally reopening the blocks on both the antennae. The remaining recording parameters were maintained the same as those for the sensilla recordings. The results for the MB, LFP recordings are displayed in Appendix B and those for the simultaneous recordings from the α -lobes are displayed in Appendix C.

4. **Neuronal Tract Tracing**

We attempted to locate neuronal olfactory contralateral connections between relevant olfactory centers in the brain using fluorescent neuronal tract tracing dyes, Tetramethyl Rhodamine (extinction wavelength 545 Å), and Dextran biotin with secondary Avidin bound to Alexa Fluor (extinction wavelength 633 Å) was used. The cuticle of the bee's head was dissected to expose the brain, small amounts of the dyes were collected on the tips of sharp forceps and gently injected into specific regions. Both the dyes were injected in the same brain but separately in the two regions so as to be able to visualize connections between the injected centers upon processing. The combinations made were, i. Antennal lobe and α -lobe ii. MB and α -lobe iii. Antennal lobe and MB. Post-injection, the animals were kept in the dark for five hours to allow the dye travel. The brains were then dissected out, placed in 1 ml of 4% PFA, and fixed overnight at 4°C. The fixed brains were washed in PBS three times and dehydrated using ascending alcohol series. They were mounted on concave glass slides in methyl salicylate. The mounted brains were imaged using a laser scanning confocal microscope (Leica TCS SP2, Leica Microsystems, Karl Zeiss LSM NLO 710 or Karl Zeiss LSM 780). The z-stacks of the images were analyzed using ImageJ software.

5. **Bee Behavior**

i) To check for bilateral transfer of olfactory learned information in *A. dorsata* and *A. mellifera*:

Harnessed bees were divided into three groups, the experimental group, positive control, and negative control group. The bees were placed under a stereomicroscope and using a toothpick, acrylic paint was coated on one of the two antennae (left or right) for the experimental groups (Fig 4A) and on both the antennae for the negative control group. The antennae of the positive control group were left open. Post-coating the animals were allowed to normalize for two hours. Olfactory conditioning was carried out in accordance with the protocol published by Bitterman et al., (1983). Each bee was placed 3 cm from the odor delivery tube and an air suction vent was placed behind the bee in order to get to remove the odor. The animal was kept on the pedestal for 14s following which a puff of odor (1-hexanol) was released towards the animal. The CS lasted 4 s with a 1 s overlap with the US (30% sucrose), the US was maintained for another two seconds post switching off, of the odor (Fig 4B). A LED signaled the onset and offset of the odor and the time point of presentation of the US to the experimenter. The fraction bees showing proboscis extension reflex-PER was quantified. Retention test was carried out 3 hrs post-training. For the test group, the acrylic paint blocks were removed from the covered antenna and the training antenna was now covered with paint during retention test to check for the transfer of the learned information to the untrained side. For the negative control group both the antennae covers were reopened and retention was checked. For *A. mellifera*, retention was checked using both hexanol and geraniol in the experimental group and a positive control group was not maintained given the high learning rate of *A. mellifera* with one antenna blocked. During the retention test, the

animal was not reinforced with the US, and to check for discrimination, random combinations were made and a puff of either hexanol or geraniol was given followed by either geraniol or hexanol. Five training trials were carried out with a 10 min inter-trial interval- ITI between the trials. To balance sides, an equal number of bees were trained on each side and the final result was calculated after pooling the data for both the left and the right antenna trained bees.

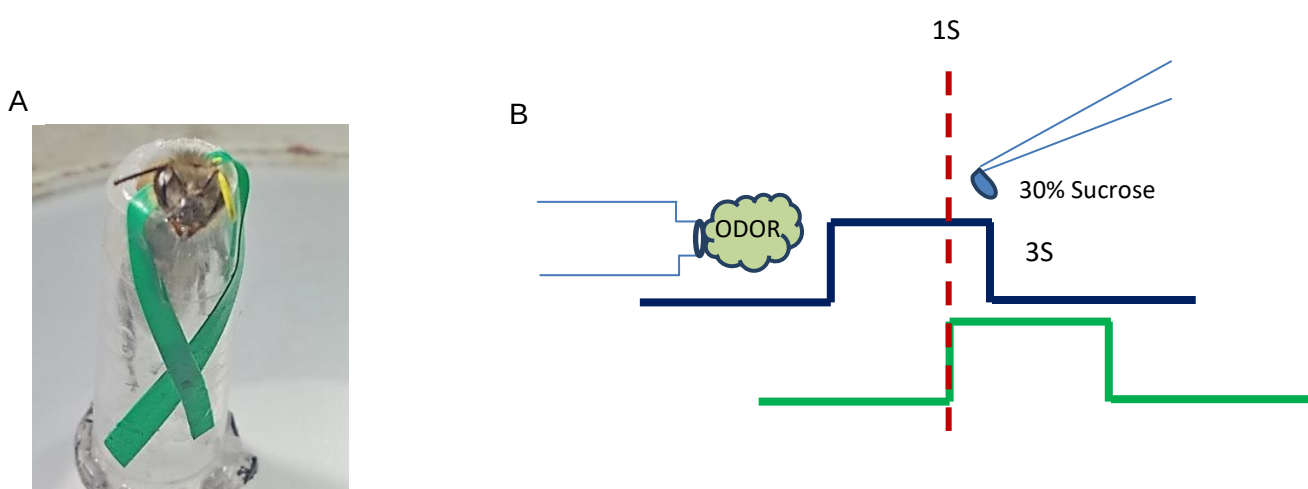


Fig 4: Representation of the A) Harnessed *A. mellifera* bee with the left antenna covered B) conditioning protocol used to train the bees, 4 odor stimulus followed by a 3 s sucrose reward and a 1 s overlap between the two.

ii) To check for retention in the trained antenna in *A. dorsata* and *A. mellifera*

For *A. dorsata*, to check for retention on the trained side, harnessed bees were trained with one antenna and checked for retention at 3 hrs post-training using the training antenna itself. With *A. mellifera*, the bees that were used checking retention on the untrained antenna were used. After the untrained side test, the block from the trained antenna was removed and the bees were replaced on the pedestal. The animals were now

checked for their retention capacity with both the antennae open. With *A. mellifera* discrimination test was also carried out using both hexanol and geraniol.

iii) To check for bilateral transfer of olfactory learned information using the wall setup

We recreated the experimental setup used by Sandoz and Menzel (2001) using *A. mellifera*, to check for the bilateral transfer of olfactory learned information, wherein a plastic wall (40mm x 50mm) was used. A cut-out in the shape of the bee in the holder was made and the plastic wall was placed sagittally over the harnessed bee so as to separate the antennae of the bee. The mandibles and antenna on one side of the bee's face were pushed slightly out, to aid PER conditioning while the other antenna was kept isolated from the training procedure, all the gaps were sealed using dental wax (Fig 5). The odor valve was placed on the side of the bee's face with its mandibles and proboscis free to move, an exhaust vent was placed immediately behind the bee to remove the remnant odor particles and training was carried out in accordance with the conditioning protocol mentioned in section 7 of the "General Materials and Methods" section. Retention test was carried out using the antenna on the untrained side. The retention test was carried out at the end of the training procedure itself, ie. the 6th trial. In a control experiment using the same setup, the antenna used for the training procedure was coated with acrylic paint and training carried out with the antenna blocked. The learning rates were quantified in these experiments.

iv) To check if the acrylic paint might act as a contextual stimulus

The need for the behavioral experiment using the wall was seen as a means to alleviate the possibility of an antennal block such as acrylic paint acting as a contextual stimulus (Sandoz and Menzel, 2001). We carried a set of behavioral experiments to check if the presence of acrylic paint can act as a contextual stimulus. To this end, we trained a set of *A. mellifera* bees with either the left or right antenna blocked. The bees were divided into two groups, one where the cover on the antenna was retained and the second group with the cover removed. The two groups were checked for retention at 3 hrs, both left and right antenna data were pooled and analyzed.

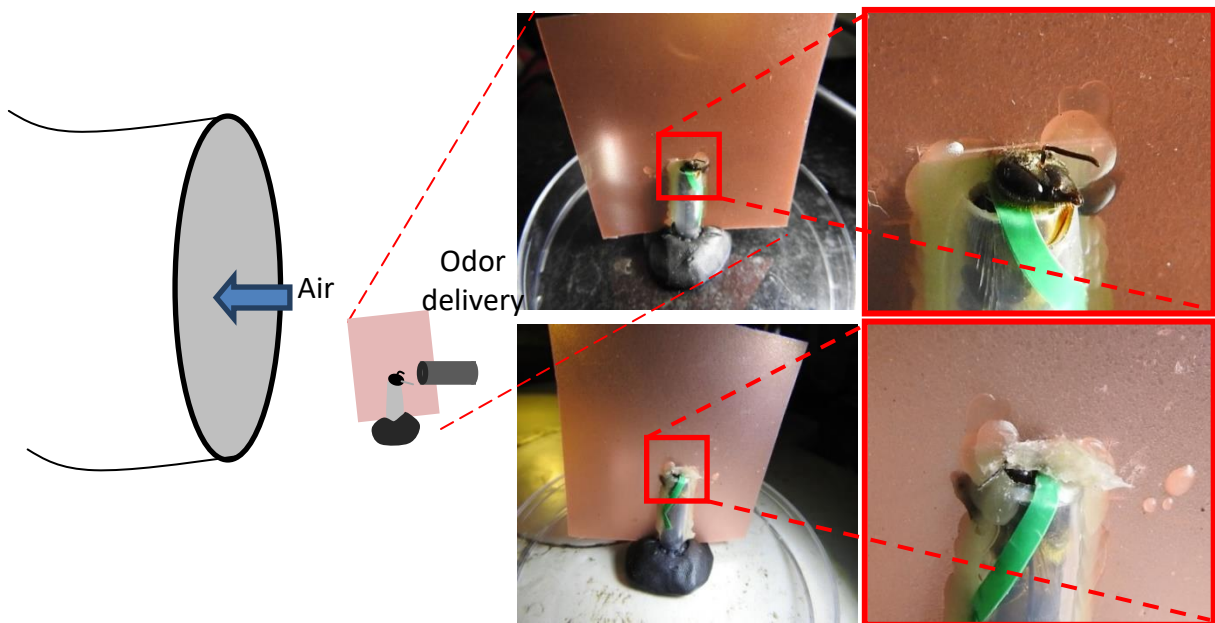


Fig 5: Photographic representation of the setup for the wall experiment. The side with the antenna and mandibles free was used for training (top right panel), the other antenna was isolated from the training procedure (bottom right panel).

v) Second-order conditioning

To qualify and quantify the role played by the reward pathway in the possible bilateral transfer of olfactory learned information *A. mellifera* bees were checked for their capacity to undergo second-order conditioning. The protocol followed was as per Hussaini et al., (2007). Harnessed bees were trained differentially to two odors, odor A was reinforced with a sugar reward and odor B was not reinforced with the sugar reward (Fig 6A). Post-training, second-order conditioning with forward pairing was carried out, with first exposing the animal to a puff of odor C and overlapping it with the rewarded odor A or the unrewarded odor B (Fig 6B). Odor C was delivered for 4 s, with a 1 s overlap with either odor A or odor B which was delivered for 2 s after odor C was turned off. The animals were trained to Odor A and B in every session, with the training starting with either of the two odors but each trial alternating between the two, a 12 min ITI was maintained between the training sessions. Given that the bees exhibited satiety and learning saturation, second-order conditioning was carried out one hour after differential training. The increase in PER in response to odor C over a period of 5 trials was recorded and analyzed. Hexanol, Geraniol and Citral were used in random combinations of odors A, B and C for the experiment.

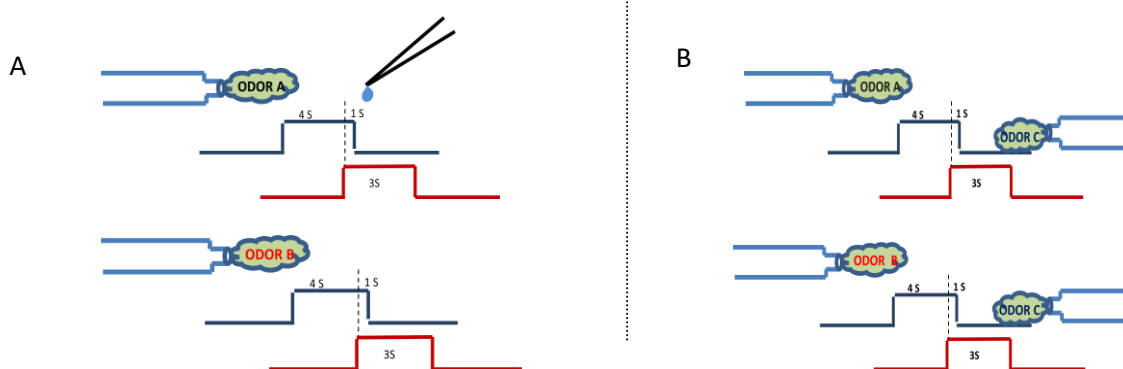


Fig 6: Representation of second-order conditioning, panel A represents the olfactory training procedure for the rewarded “Odor A” and unrewarded “Odor B”. Panel B represents the second-

order conditioning with novel “Odor C” which was delivered for 4S following odor A or B with a 1 s overlap between the novel odor and the two odors.

Results

1. Neuronal tract-tracing revealed no bilateral tracts between olfactory centers

Using Fluorescent dyes we labelled the 1. MB on one side of the brain and the α -lobe on the other (Fig 7) 2. Antennal lobe on one side and α -lobe on the other (Fig 8) 3. MB on one side and Antennal lobe on the other side in *A. dorsata* (Fig 9) (n=7 fills were made with different combinations). No bilateral tracts were revealed between any of the two labelled regions. Analysis revealed that the Kenyon cell tracts leading out of the MB follow a compartmentalized and crystalline pattern, depending on which part of the MB was injected with dye (Fig 7-9). We also characterized the number of cell bodies in the Lateral horn filled by the dyes and their number was found to be similar to that established in *A. mellifera* (35 ± 2).

2. Bee Behavior

i) Bilateral transfer of olfactory memory was not observed in both *A. dorsata* and *A. mellifera*

The analysis of olfactory retention obtained from the untrained side revealed that no transfer of the learned information took place in both species of honey bees (Fig 10). For n= 51 *A. dorsata* bees learning at the fifth trial with one antenna reached 37.7%, the retention from the untrained side showed no significant difference between the retention of the experimental bees and the negative control bees (n= 39), that showed a retention rate of 2 % ($p=0.045$, Cochran's $q= 4$). *A. mellifera* bee's (n=59) learning rate at the fifth

trial was 95% and retention from the untrained side was 10.1% which was not significantly different from the retention rate of the negative control bees (n=45) that showed retention of 6.5% ($p=0.22$, Cochran's $q=1.5$). In addition, the few bees that responded to hexanol also responded to geraniol, hence no odor discrimination was seen in the few bees that showed retention from the untrained side.

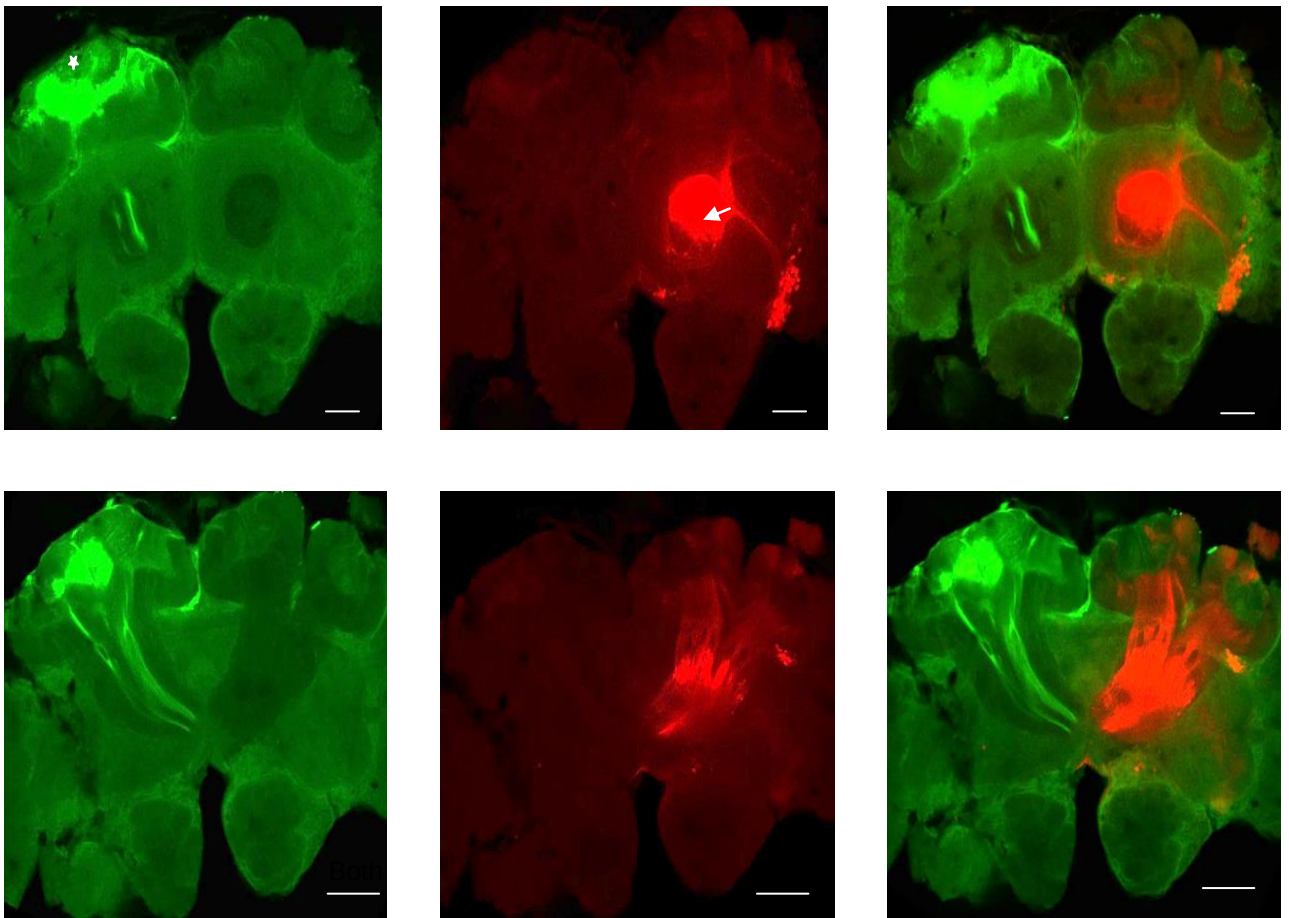


Fig 7: TMR was injected into the α -lobe (red) and Dextran biotin with secondary avidin bound to Alexa fluor 633 (green) was injected into the MB. Tracts are seen leading from the α -lobe to the MB on the same side, however, no tracts are seen leading to the contralateral MB. Panel A and B

represent two depths of the same brain. The star and arrow indicate the points of injection of the dyes. Scale =100 μ , represented by white bar at the right side bottom of the image.

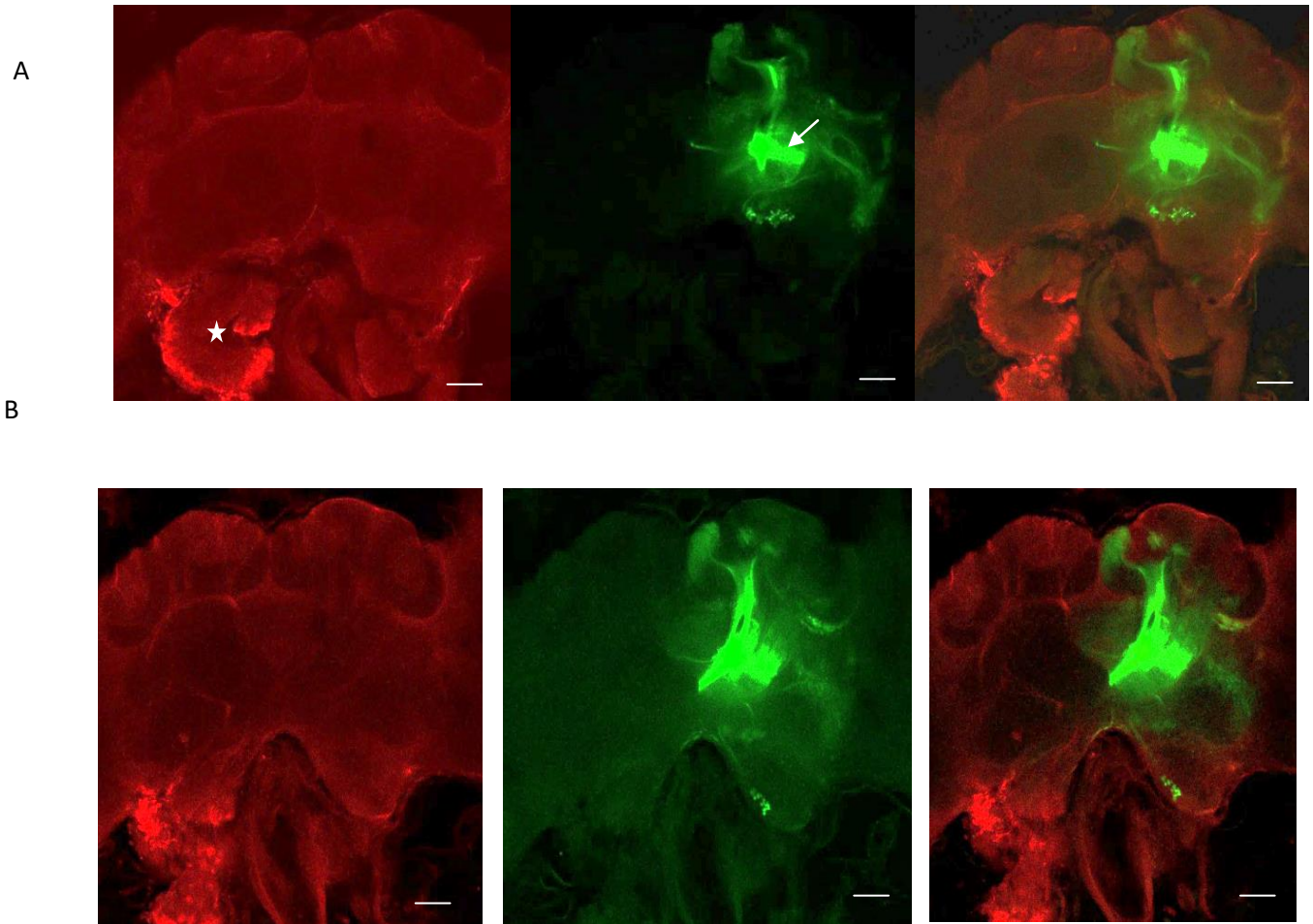


Fig 8: TMR 568 (red) and Dextran biotin with secondary Avidin bound to Alexa fluor 633 (green) were injected into the antennal lobe and α -lobe respectively as indicated by the star and arrow. The dyes travelled to the MBs on each side but not contralateral connections are seen between the two sides. Scale= 100 μ .

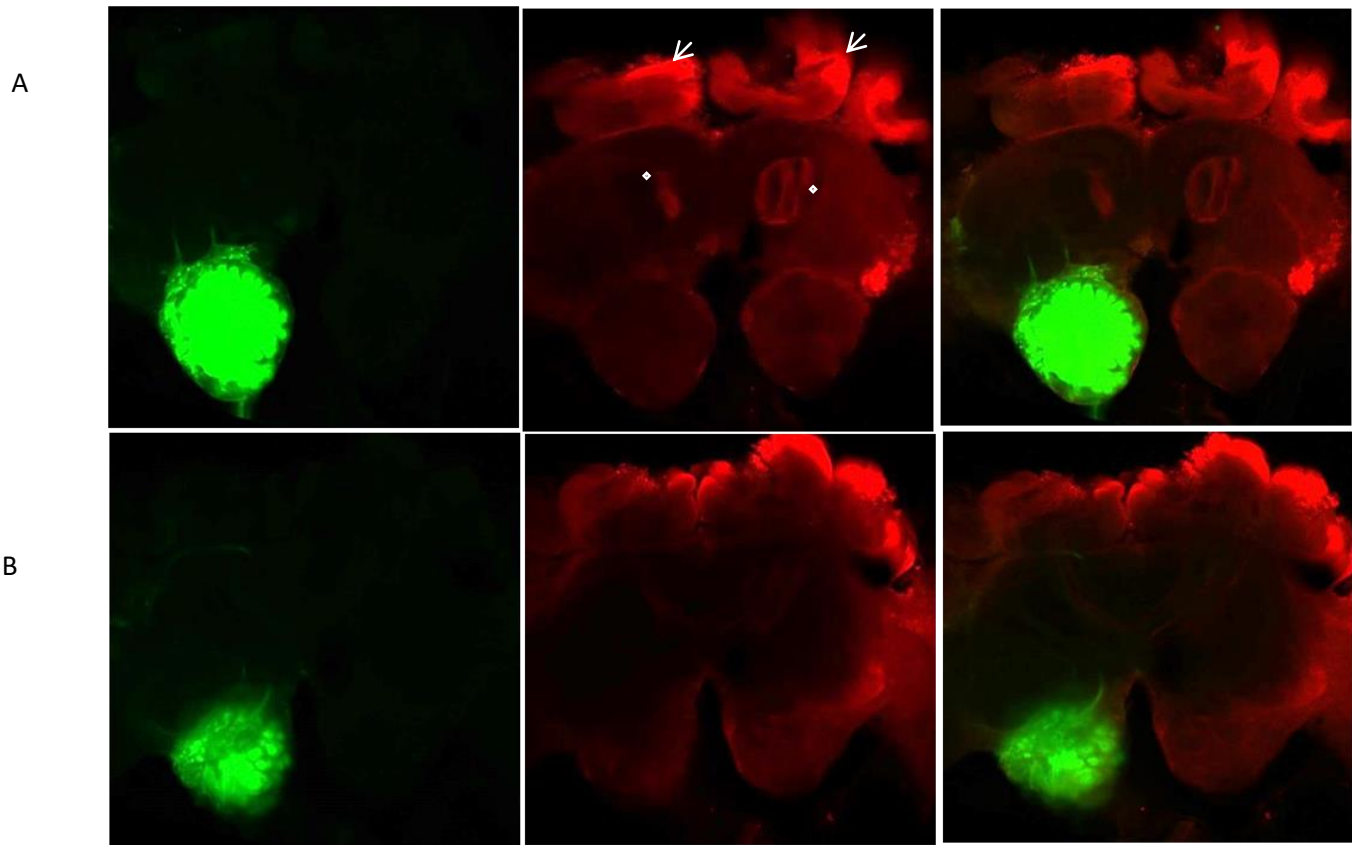


Fig 9: TMR 568 (red) and Dextran biotin with secondary Avidin bound to Alexa fluor 633 (green) were injected into the MB and antennal lobe. Tracts are seen leading to the MB from the antennal lobe and to the lateral horn from the MB on their respective sides, however not cross-connections are seen between the two sides. In addition depending on the point of injection of the dye, TMR is seen travelling from the MB to the pedunculus in a compartmentalized, crystalline manner, indicated by the white dots in the top panel center figure. The scale is 100 μ .

ii) Memory is retained on the trained side in both species

In *A. dorsata* (n=34) learning rate reached 38% by the fifth trial (Fig 11). The retention at 3 hrs was seen to be 92% of the learned rate and was not significantly different from the learning rate at the fifth trial. Bees with both their antennae open (n=25) showed acquisition of 64% and a retention of 87% at the 3rd hour while bees with both antennae

closed showed a learning rate of 0% and retention rate of 2%. No significant difference was seen between the left and right antenna trained groups. We used the *A. mellifera* (n=53) bees that were checked for transfer of learned information to the untrained side. The same bees, that were unable to recall information from the unexposed side, exhibited high memory and significant discrimination when the block from the covered (trained) antenna was removed.

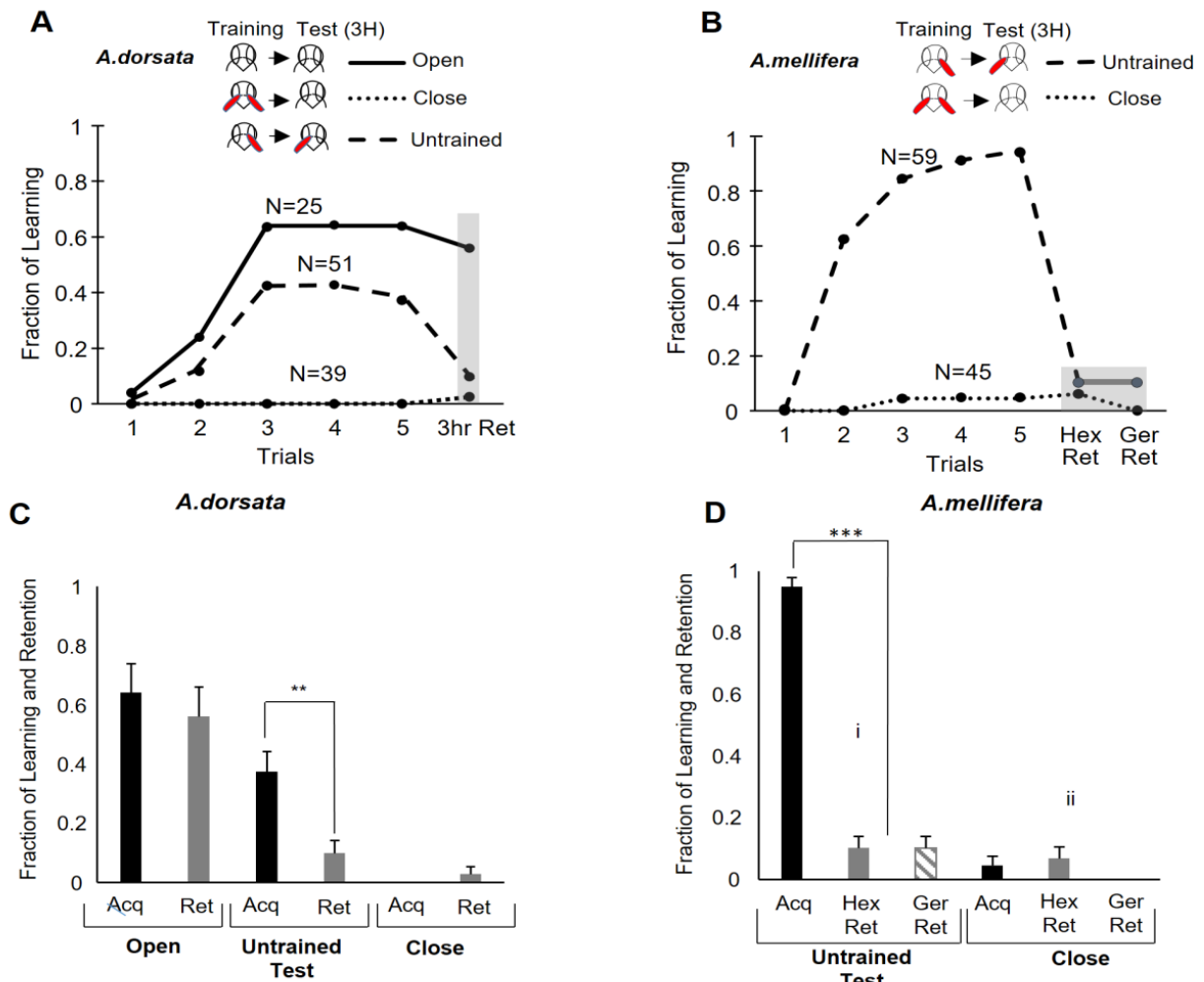


Fig 10: Test for transfer of olfactory learned information in *A. dorsata* and *A. mellifera*. (A,B) The procedure is depicted as a pictorial representation above the graphs in A, B. Acrylic coat is represented by the red color on the antenna. (A) Testing for lateral transfer at 3 hrs (3 hrs Ret) in *A. dorsata* (n=25 open antenna control, n=25 untrained antenna test, n=39 closed antenna control) shows that memory on the untrained side at 3 hrs (gray box) is nearly zero ($P=0.045$, Cochran's $q=4$). (B) *A. mellifera* (n=59 untrained antenna test, n=45 closed antenna control) also did not show any significant lateral transfer at 3 hrs (gray box) ($P=0.22$, Cochran's $q=1.5$), though they had 95% acquisition. The bees that responded to hexanol (Hex Ret) responded to geraniol (Ger Ret), as denoted by the gray line, on the transferred side at 3 hrs indicating no discrimination ($P=1$). (C) *A. dorsata* showed a significant difference between the learning (Acq) and retention (Ret) in the side contralateral to the trained side, and no significant difference between the (i) untrained retention and (ii) closed antenna control group. The closed antenna group showed 0% learning and 2% retention which may indicate the success rate of our method of coating the antenna for blocking. (D) *A. mellifera* showed 95% acquisition but the transfer of memory to (i) the contralateral side was similar to that of the group with (ii) both antenna closed. Bar graphs are means $\pm$ s.e.m. ** $P<0.01$; *** $P<0.001$.

iii) Bilateral transfer of olfactory information was obtained using the wall setup

Upon recreating the behavior experiment using a plastic wall to separate the antennae to check for side-specific transfer of olfactory information we observe in n=10 *A. mellifera* bees that learned the olfactory information on one side exhibited retention from the unexposed antenna at the end of the training session itself (50%) which was at the 6th trial (Fig 12). This result could have been either due to a leak in the experimental setup, preventing proper isolation of the untrained antenna, or that the animal is able to transfer the information within a period of an hour. To verify which of the two possibilities were

responsible for the transfer, we trained $n=19$ *A. mellifera* bees using the wall setup and further covering the training antenna itself with acrylic paint. Despite the odor valve being kept on the side with the covered antenna, the animals learnt to associate the odor with a reward with the learning rate reaching 63 % by the 5th trial.

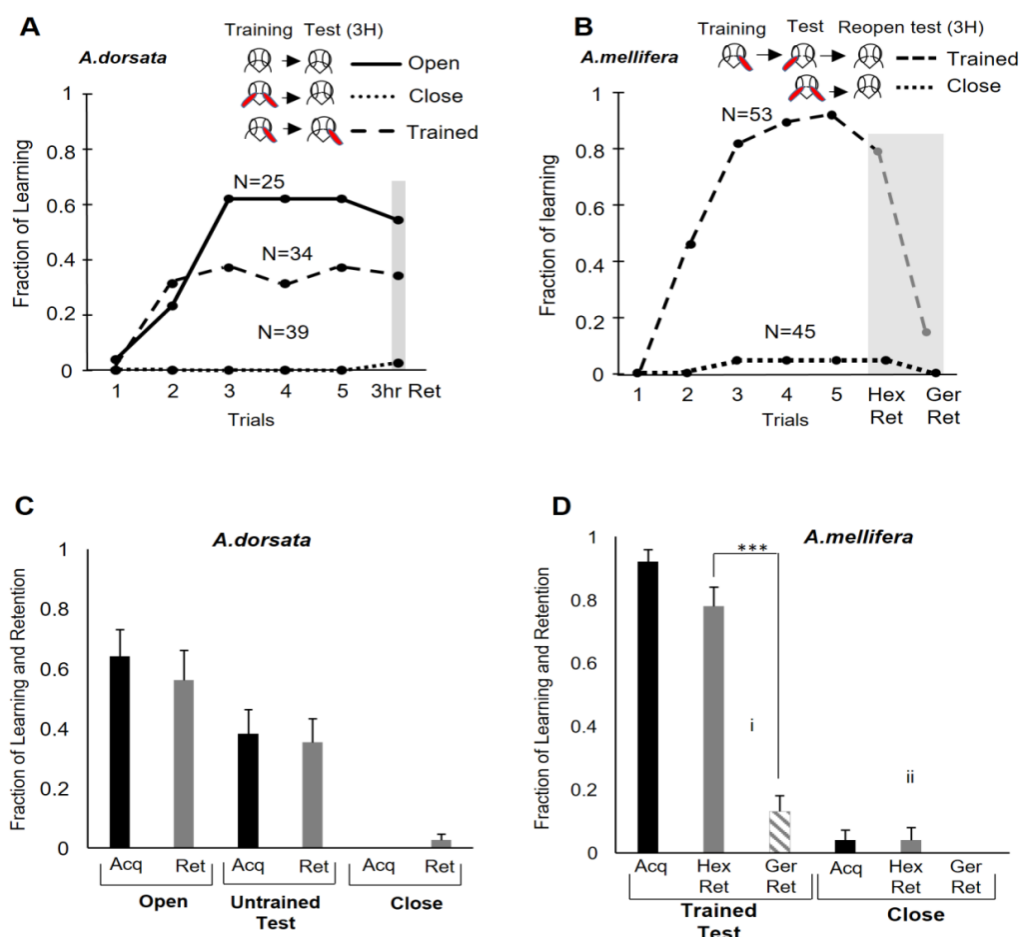


Fig 11: *A. dorsata* and *A. mellifera* retain the learnt information on the trained side. (A,B) The pictorial representation above the panel indicates the procedure and the red paint on the antenna represents the acrylic paint. (A) *A. dorsata* ($n=25$ open antenna control, $n=34$ trained antenna test, $n=39$ closed antenna control) learned and retained memory after 3 hrs (3 hr Ret, gray box) from

the trained antenna, and exhibited significantly higher retention than that of the closed antenna group ($P=8\times 10^{-4}$, Cochran's $q=11.15$). The bees trained with one antenna also showed lower acquisition with one antenna compared with two antennae training, but this was not significant ($P=0.13$, Cochran's $q=2.22$). (B) With *A. mellifera* bees ($n=52$ trained antenna reopened test, $n=45$ closed antenna control), acquired memory was not perturbed by the coating and removal of the coat during the retention test at 3 hrs (gray box). *A. mellifera* displayed 95% acquisition and retention from the same antenna was maintained at 87% at 3 hrs, this retention rate was significantly different from the retention from the untrained antenna ($P=1.8\times 10^{-9}$, Cochran's $q=36.1$). They also showed clear discrimination between hexanol (Hex Ret) and geraniol (Ger Ret), as denoted by the gray dashed line ($P=1.4\times 10^{-8}$, Cochran's $q=32.1$), showing that the covering and uncovering did not stress the bee and cause memory loss. The learning and retention rate of *A. dorsata* (C) and *A. mellifera* (D). In *A. mellifera* the trained antenna, once reopened after the untrained antenna check, showed significant discrimination (i) between the trained odor Hex Ret and the untrained odor, Ger Ret (ii) No discrimination was seen in the set of *A. mellifera* bees with both antennae closed. Bar graphs are means $\pm$ s.e.m. *** $P<0.001$.

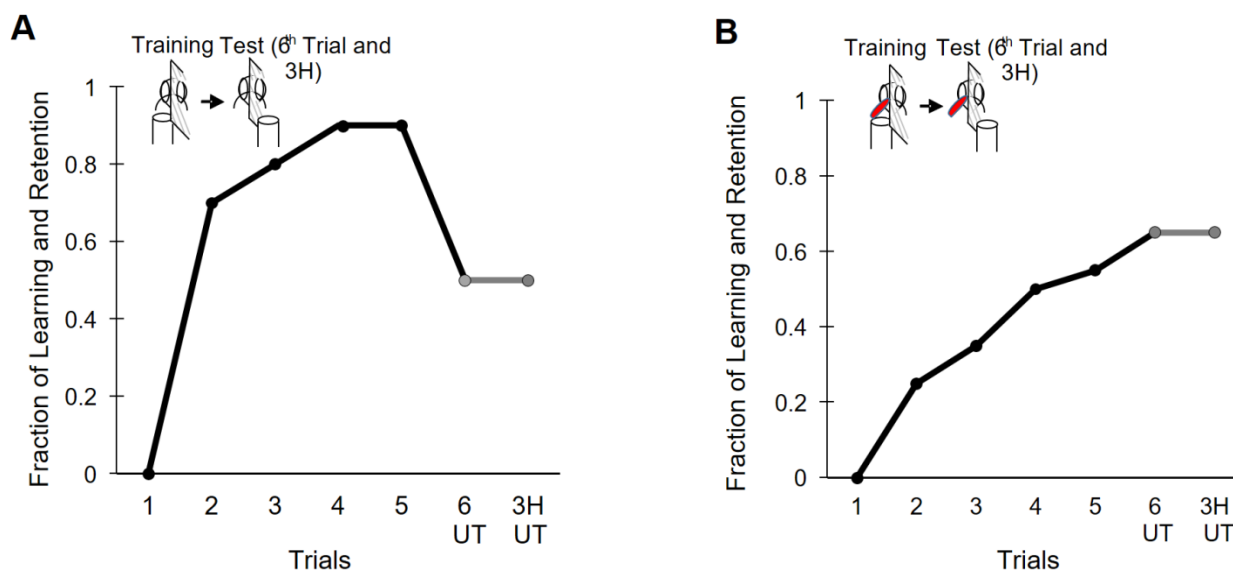


Fig 12: Side-specific training using the wall setup: A. *A. mellifera* (n=10) trained on one side using the wall set up showed a learning rate of 90% and a retention rate of 50% from the unexposed antenna at the Sixth trial (6 UT), this retention was maintained till the 3rd hr (3H UT). B. In n=19 *A. mellifera* bees with the antenna on the training side covered with paint, learning rate achieved at the end of 5 trials was 55%, the retention seen by the unexposed antenna at the 6th trial was 65% (6 UT) and this rate of retention was maintained by the untrained antenna upto 3 hrs (3H UT). The procedure used for both experiments is represented as a picture above the respective graphs.

iv) Acrylic paint does not act as a contextual stimulus when coated on the antenna

In n=37 *A. mellifera* bees, olfactory condition was carried out with one antenna covered in acrylic paint using hexanol as the training odor and geraniol as the discrimination odor (Fig 13). The trained bees were divided into two groups, n=19 bees were checked for retention and discrimination with the acrylic cover removed and n=18 bees were checked for retention with the acrylic cover on. No significant difference was seen in the retention ($p=0.8$, Cochran's $q=0.05$) and discrimination of both the groups ($p=0.1$ Cochran's $q=2.6$). The animals with the paint cover removed, discriminated marginally better than the animals with the cover on. Thus the presence of the acrylic paint did not act as a contextual stimulus for the animals during training.

v) The process of coating and peeling away the coat does not shock the animal into forgetting

Using $n=12$ *A. dorsata* bees we checked for the possibility of the coating and peeling of the paint causing any perturbation or shock to the animal, hence resulting in the animal not recollecting the learnt information (Fig 14 A). The 12 bees were trained with one antenna and a learning rate of 35% was seen. Prior to the 3hr retention test, the testing antenna was coated with the paint allowed to dry and peeled. This process did not perturb the animals into forgetting and 100% retention of information at the 3rd hour was seen.

vi) Coating and un-coating the paint does not interfere with learning

In $n=19$ *A. dorsata* bees the antennae were covered with the acrylic paint and left for an hour. The coating was removed and the animals trained in olfactory conditioning using 1-hexanol, and a learning rate of 76% was obtained (Fig 14 B). There was no significant difference between the learning rate of bees trained after removing the coat and bees trained with both antenna open ($p=0.3$, Cochran's $q=0.8$).

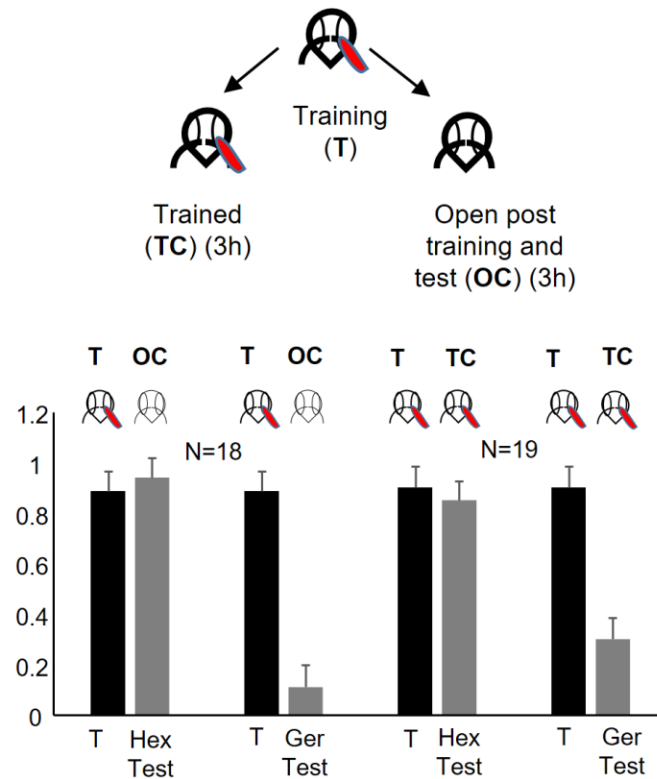


Fig 13: Acrylic paint does not act as a contextual stimulus, The retention and discrimination rates of the two groups of bees, one, trained and checked with paint cover (TC) and the second group with the cover removed and checked for retention (OC) was not significantly different, retention ($p=0.8$, Cochran's $q=0.050$), discrimination ($p=0.1$, Cochran's $q=2.6$). The bar graphs represent the comparison between the learning rate of the trained animals (T) and the retention of the two groups. The procedure is depicted as a picture above the graph.

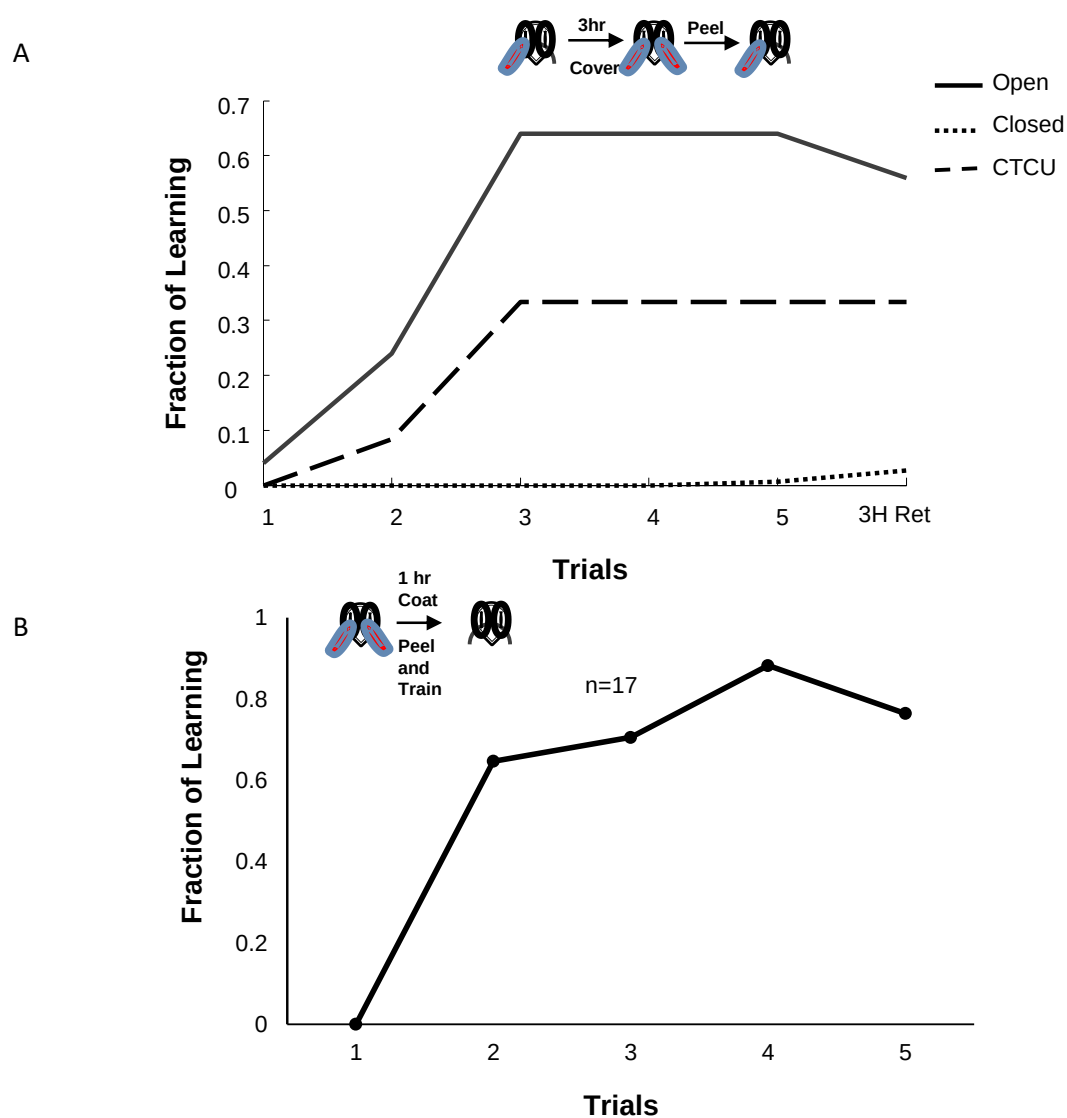


Fig 14: A. The process of coating and uncoating the antenna does not shock the bees into forgetting, nor does the application and removal of the coat, interfere with learning. The top panel shows the learning and retention rates of $n=12$ *A. dorsata* bees that had their antenna coated and uncoated (CTCU) prior to retention test, 100% retention was seen (3H Ret). B. The lower panel shows the learning rate of $n=17$ *A. dorsata* bees whose antennae were covered and were trained 1 hr post coating. A learning rate of 78% was obtained in these bees when the cover was removed.

vii) A mathematical model to predict the retention and retrieval of olfactory learnt information in *A. dorsata*

Given our results, we propose a model that can predict the probability of learning and retention in *A. dorsata*. If the capacity to make the decision to elicit PER were to lie with either of the two brain lobes, the probability can be calculated using the formula,

$$2P-P^2$$

Where, P= Probability of evoking PER when odor presented to only one of the two antennae

Using this model we were able to predict both the learning rate and the retention rate of *A. dorsata* bees, the learning rate of bees with one antenna covered was 38%, using the above formula we get, $2(0.38)-(0.38 \times 0.38) = 0.62$ or 62%, which is close to the learning rate seen in bees with both antennae open, 64%. For the retention we saw that the rate of retention in bees with one antenna covered was 35%, using the above formula we get, $2(0.35)-(0.35 \times 0.35) = 0.55$ or 55%, which is approximately equal to the retention rate we observed in the bees with both their antennae open, 56%. These predictions match the observations, hence proving that each lobe works independently with no transfer of olfactory learned information, both during the training and at 3 hrs.

viii) To check for the role of the reward pathway via second-order conditioning

Second-order olfactory conditioning was carried out using $n=72$ *A. mellifera* bees. Learning rate for the rewarded odor (A^+) reached 90%. The bees responded to the unrewarded odor (B^-) in the first trial at an average rate of 32% and the discrimination improved over five trials with the final percentage of response being 8%. The three odors were used in all the three possible combinations and the data was pooled to generate the

final learning and discrimination curve (Fig 15 A,C). The rewarded odor A training was always conducted before the unrewarded odor training for all the sessions. The trained bees were divided into equal groups for each session and second-order conditioning for the novel odor C was done with both the rewarded and unrewarded odors, A^+ , B^- . An increase in responses was however not observed in the $C A^+$ conditioning with the bees exhibiting 45% response in the first trial itself (Fig 15 B). This response rate gradually dropped to 27% at the 5th trial. For the unrewarded odor conditioning, $C B^-$, response rate at the first trial was 59% and this response rate reduced to 34% at the 5th trial (Fig 15 D). The bees seemed to exhibit a low discrimination capacity from the very first trial and during the second-order conditioning, animals responded to similar odors such as geraniol and citral in the first trial itself. A comparison of the five trials for both $C A^+$ and $C B^-$ showed no significant variation between the two groups using one way ANOVA ($p=0.5$, $F=0.4$, $F\text{-crit}=5.3$). Between the 1st and 2nd trials during the $C A^+$ conditioning, no drop in the responses were observed, however a comparison of the 2nd trial of this group with the 2nd trial of the $C B^-$ conditioning group showed no significant variation ($p=0.6$, Cochran's $q=0.22$). Our results for this experiment were inconclusive and a further comparison between each trial number of $C A^+$ and $C B^-$ conditioning will have to be done with more number of bees to be able to achieve substantial results.

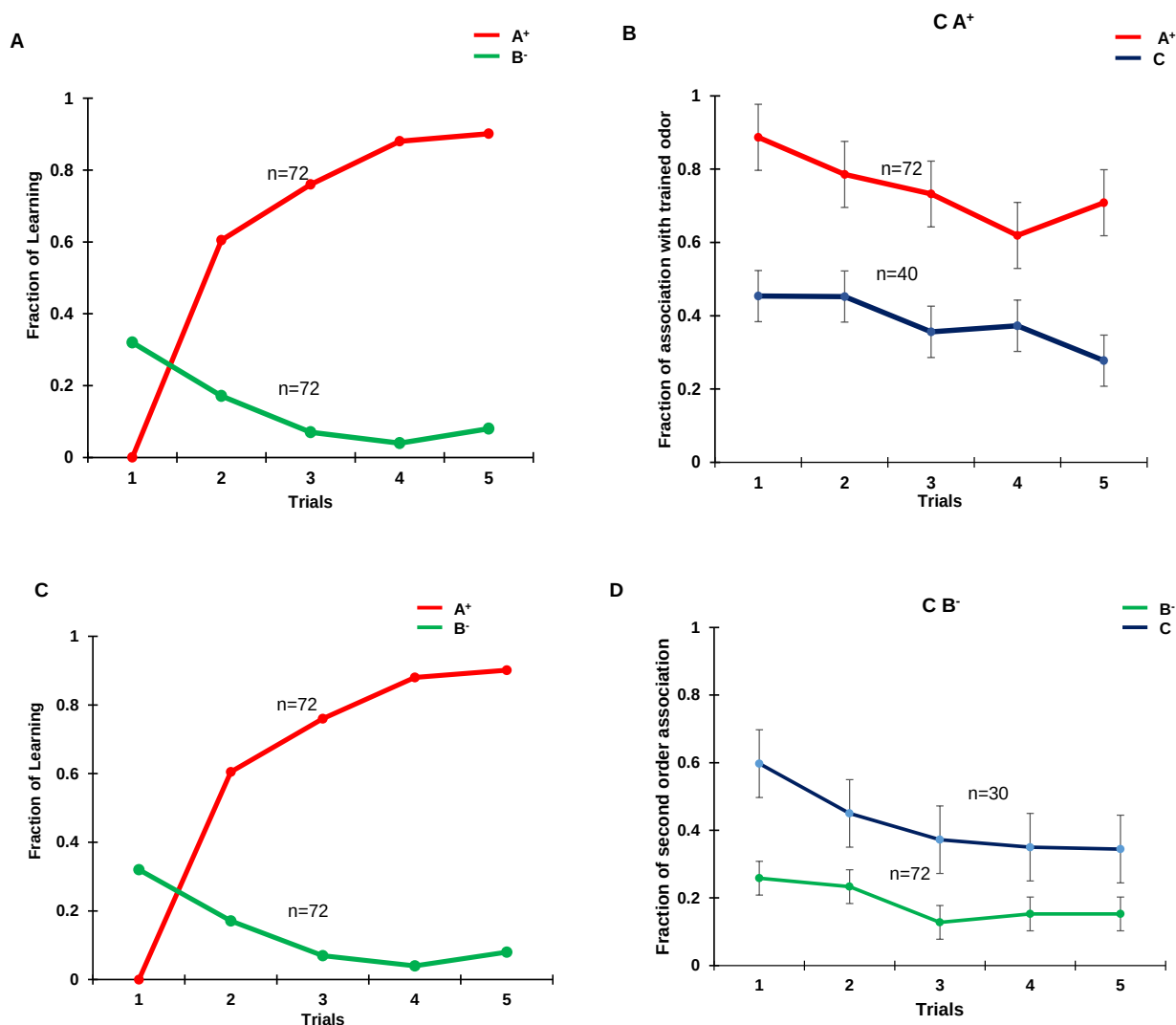


Fig 15: Second-order conditioning in *A. mellifera*. $n=72$ bees were trained in olfactory conditioning panel A, C, a learning rate of 90 % was seen for the rewarded odor, ' A^+ ' and a final response rate of 15% was seen for the unrewarded odor, ' B^- '. Panel B, D represent the results of the second-order association made with the novel odor ' C '. In panel B, the rate of retention for the rewarded odor A^+ is 89% at the 1st trial and drops to 70% at the fifth trial. The rate of response of the animals in the 1st trial to odor C in association with A^+ is 45% and the final response rate is 27%. No increase in responses is seen over the 5 association conditioning trials. The responses to the unrewarded odor B^- gradually reduce from 25% at the first trial to 15% in the 5th trial. The

response rate in the first trial to novel odor 'C' when associated with B⁻ is 60% at the first trial and reduces to 34% in the 5th trial. Comparison of the trials between C A⁺ and C B⁻ showed no significant difference in the responses of the animals to the odor 'C'.

Discussion

Inter-hemispheric communication is said to have evolved in order to increase the computational capacities associated with cortical expansion. It would be intuitive to posit the need for the transfer of learnt information, however studies from split-brain research, clearly show that removal of the corpus callosum does not interfere with the normal functioning in split-brain patients and the absence of this commissure is not lethal (Gazzaniga, 2014; Wolman, 2012). These studies indicate that, though having interhemispheric cross talk would prove advantageous to an animal, the functioning of each lobe on its own for certain stimuli might not be as much a disadvantage as one would anticipate. The bilateral transfer of information would aid in locomotive coordination and rapid decision making, which would be needed in instances such as escaping from predators. At the olfactory level, a number of higher and lower vertebrates exhibit the presence of commissures that aid in the relaying of information from one lobe to the other (Suarez et al., 2014). We attempted to look for the neuronal basis of bilateral transfer of olfactory information in invertebrates using honey bees as our model. Despite no physical evidence of bilateral connections between the olfactory circuits in the two lobes of the brain, behavioral experiments with *A. mellifera* had shown the transfer of olfactory learnt information over a period of 3 hrs (Sandoz and Menzel, 2001). At the transcriptomic level, there has been work done to show the increase in memory molecular markers over a period of 24hrs post-training the honey bee with one antenna (Guo et al., 2016). However given the possibility of multimodal learning during olfactory conditioning, where the bee accumulates visual input and

the anticipation of the sugar reward, along with the odor information, the increase in molecular memory markers on both sides of the brain despite one antenna being blocked might not be the result of olfactory learning alone. Physiological studies of the extrinsic neurons of the MB show the presence of a neuron, PE1 that responds multimodally, has a soma lying ventral to the α -lobe on one side and arborizes around the contralateral α -lobe hence connecting the peduncles (Okada et al., 2007; Rybak and Menzel, 1998). Activity of PE1 has been shown to change during olfactory learning (Rybak and Menzel, 1998.) In addition to the PE1 neuron, the ventral unpaired median neuron of the maxillary neuromere (VUMmx1) has been shown to play a role in memory formation during associative learning (Hammer, 1993; Menzel, 2012). The VUMmx1 in addition to being modulated by octopamine it is also shown to be bilateral with arborizations across the important olfactory centers on both lobes of the honey bee brain (Farooqui et al., 2003; Hammer and Menzel, 1998; Menzel, 2012).

We approached our question by first finding a block for the antennae which was inert, capable of shutting off the response to odor and reversible without causing harm to the antennae. We carried out the electrophysiology experiments using *A. dorsata*. From the sensilla recordings, we confirmed that acrylic paint made for a good block which was inert, reversible, and did not harm the covered antenna. We then looked for the possibility of bilateral connections at the level of the MB, wherein we covered one antenna and recorded from the MB on the covered side. However, we were not able to see any changes in the baseline activity of the MB when the antenna on that side was covered with acrylic paint. If the MB had shown the slightest response despite the antenna on the ipsilateral side being covered, it would have indicated the possibility of olfactory input into the MB from the contralateral side. We also looked for the presence of contralateral connections at the level of the α -lobes, which constitute the fourth-order level of the

olfactory circuit. Upon carrying out simultaneous LFP recordings from both the α -lobes we were unable to establish the presence of any connections between the α -lobes at the olfactory level.

In order to confirm the results we had observed using electrophysiology, we carried out neuronal tract-tracing experiments in parallel in *A. dorsata* bees, between the crucial olfactory centers in the brain lobes. At the anatomical level as well, we were unable to locate any robust contralateral connections between the α -lobe and MB, antennal lobe and α -lobe, and antennal lobe and MB. Analysis of the fills showed the Kenyon cell tracts leading out of the MB follow a clear sectional pattern, with each bunch of tracts leading down the pedunculus and not crossing over across a defined boundary space. Cell bodies in the lateral horn were also filled in the process and the number of cell bodies was found to be 35 ± 2 , which is similar to the number in *A. mellifera*. Despite the lack of contralateral connections observed using the above two methods, the possibility of bilateral transfer of olfactory information had to be determined at the behavioral level. We attempted to recreate the results seen by Sandoz and Menzel (2001) using acrylic paint as our antenna isolator, instead of the plastic wall used in their experiment. Using *A. dorsata* we observed no transfer of olfactory learnt information over a period of 3 hrs. We confirmed that the result we had observed in *A. dorsata* was not a species-specific observation by performing the same experiment using *A. mellifera* and saw no retention of the learnt information at 3hrs from the untrained side. Further, we confirmed in both species that the learnt information was stably represented on the ipsilateral or trained side at 3hrs. Interestingly *A. mellifera* bees that were unable to recall the learnt information from the untrained side, showed significantly high retention and discrimination on the trained side. Control experiments to check whether the animals were shocked into forgetting the learnt information were conducted and the results confirmed that the animals were not perturbed by the process of coating and peeling of the coat

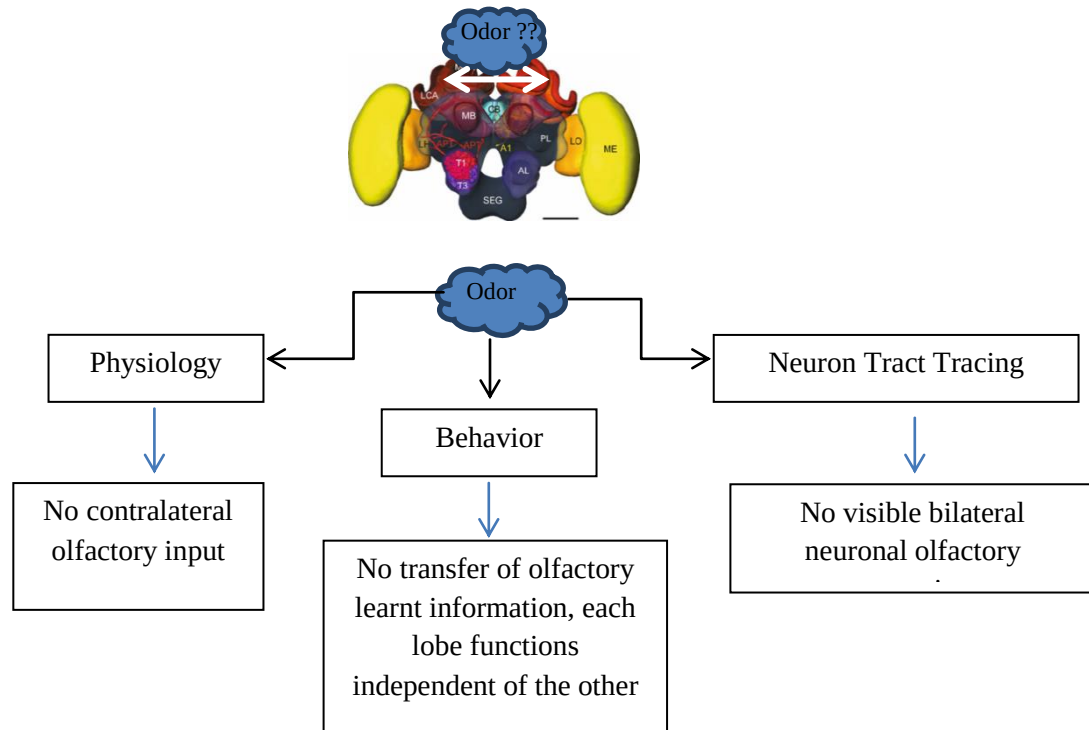
from the antenna. We also demonstrated that the paint when left on the antenna for an hour and then peeled does not interfere with the learning capacity of the honey bees.

The reason cited by Sandoz and Menzel (2001) for not using a paint coat on the antenna, was to avoid the possibility of the coat acting as a contextual stimulus for the animal. We carried out a set of experiments to show that the presence of the acrylic paint coat on the animal's antenna does not act as a contextual stimulus and there is no difference in the retention and discrimination between animals without the paint cover and with the paint cover on their antenna. Given our negative results, we attempted to recreate the setup using a plastic wall to separate the two antennae. We observed that the bees exhibited retention and memory on the untrained side at the end of the training itself. Upon covering the training antenna with acrylic paint and using the plastic wall to separate the antennae, the animals learnt to associate the odor with a reward, suggestive of the fact that this setup is not robust enough to separate the antennae and check for bilateral transfer of olfactory information. Our results were in accordance with the results seen by Masuhr and Menzel (1972). A crucial observation made was, if we are to assume the independent functioning of each brain lobe with respect to olfaction we could predict the probability of the bee learning the information and exhibiting PER as a whole. We calculated and compared the result of learning and retention rates in *A. dorsata* using one antenna and compared it to the animals trained with both their antennae open. Our calculations in *A. dorsata* bees with one antenna used for training, using the formula $2P - P^2$ where P is the probability of evoking PER in either side, gave us a value very close to the observed learning rate in *A. dorsata* bees with both antennae open. The same was true for the retention rate, with the calculated result being very close to the final retention rate in the bees with both their antennae open. This similarity between the observed and calculated result clearly shows the independent working of both the brain lobes with respect to olfaction in honey bees *A. dorsata*. In *A. mellifera* we observed that

the animal was able to achieve a very high learning rate even with one antenna. Why the animal exhibits such high learning and retention rate with one antenna alone is interesting and might have to do with the redundancy of information to increase fitness.

Though at the level of olfactory learning we were not able to establish bilateral transfer of information, the role of the reward pathway in the final decision making needed to be studied. To this end we attempted to carry out second-order conditioning using *A. mellifera* bees. We followed the procedure published by Hussaini et al., 2007. However, our results were inconclusive. Upon exposure to the novel second-order conditioning odor, the animals exhibited a high response rate in the first trial itself. The PER responses gradually decreased and did not increase during any of the trials across the five trials. We observed that the high number of responses in the first trial depended on which odor was used for reward training and second-order conditioning. Eg. When geraniol was used for reward conditioning and citral was used for the second-order conditioning the animal showed a high response rate for citral. This could have been due to how similar geraniol and citral are in terms of odor representation in the bees glomeruli (Laska et al., 1999). When hexanol was used as the rewarded odor and either geraniol or citral as the second-order conditioning odor we observe a very low response rate in the first trial, but no increase in responses across the five trials. Further, we will attempt to analyze our results not only by looking for a significant increase in responses to the novel second-order odor but also any significant decrease in the responses to the novel second-order odor when paired with the unrewarded odor. These results leave the concept of second-order conditioning in honey bees as an open-ended question. We will attempt to increase the number of animals and use a few more combinations of odors to see if we are able to induce and observe second-order conditioning.

Summary



CHAPTER 2

EVALUATION OF EFFECT OF **EXPOSURE TO ACUTE DOSES OF BT** **FORMULATION DORBT-1 ON** **LEARNING AND MEMORY IN APIS** **MELLIFERA**

Chapter 2

Introduction

A study of the evolution of pesticides highlights that they have been in use since 2000 BC, the Egyptians were known to possess the ‘Ebers Papyrus’, a medical document containing 800 recipes for a variety of pesticides and poisons (Banaszkiewicz, 2010). Various pest control methods such as the use of arsenic and sulfur were actively practiced by the Chinese. The world wars saw the rapid advent of inorganic pesticides and Dichloro-Diphenyl Trichloroethane(DDT) began being sprayed along acres of cropland using aircrafts. The year 1994 saw the introduction of neonicotinoids, which are neuro-active chemicals that can act as ligands binding to the acetylcholine receptors and are capable of inducing cognitive dysfunction, a property utilized for the management of field pests (Cresswell, 2014). The mode of action of DDT is via the activation of the Na^+ gated ion channels, resulting in depolarization of the neurons causing them to fire spontaneously which further results in the rapid twitching of the insect muscles. Continuous exposure to the pesticide turns these “DDT jitters” into paralysis resulting from the uncontrolled neuronal excitation and the insect eventually dies (Davies et al., 2007).The evolution of the pesticides has seen the need for a targeted approach since almost all the methods administered thus far have implications on the environment, with water runoffs from the soil carrying these chemicals to the water sources and killing the inhabiting aquatic fauna. In addition, the effect of the pesticides on non-target organisms including humans consuming the crops has been shown to have extremely deleterious effects which include hormonal disruption, neurophysiological disorders, and formation of certain types of cancerous growths(Harada et al., 2016; Matsushima, 2018).

The neonicotinoids such as imidacloprid are still being actively used in different parts of the world, usually in combination with a pyrethroid. In regions of the U.S. Neonicotinoids or “neonics” are considered less toxic, and in India, neonicotinoids are a welcome substitute to the otherwise poisonous organophosphates. One of the biggest shortcomings of neonics is their capacity to harm crucial pollinators and non-target insects visiting the fields and which are important to the ecosystem (Cresswell, 2014). Multiple studies have shown that exposure to sub-lethal doses of neonicotinoids causes severe cognitive dysfunction in pollinators such as honey bees and bumblebees, which in turn kills the visiting forager or deems them incapable of collecting food and nectar for the hive (Lu et al., 2014; Stanley et al., 2015; Tan et al., 2015). The rapid decline in the number of honey bee hives over the past two decades saw the urgent need for pesticides that are active against specific target field pests but do not harm the pollinators.

The bacteria *Bacillus thuringiensis* is a gram-positive bacterium, commonly found dwelling in the soil, water silo dust, leaves of deciduous trees, and a wide range of conifers. During sporulation this bacteria produces two types of δ -endotoxins (i) Cry and (ii) cytosolic toxin Cyt (Ibrahim et al., 2010; Palma et al., 2014). The Cry proteins are parasporal inclusion crystal protein and Cyt is a parasporal protein that exhibits hemolytic activity. Under alkaline conditions in the mid-gut of the target insect species, these two endotoxins undergo proteolytic cleavage via the activity of trypsin and chymotrypsin upon ingestion. The activated toxins can now bind to a number of membrane-bound cell surface G-protein coupled receptors (GPCR) such as aminopeptidase (APN), alkaline phosphatase (ALP) and others (Fig 1). The toxins also bind to cadherins on the cell surface membrane of the midgut epithelial cells and undergo oligomerization upon proteolytic cleavage. The oligomerization allows for these toxins to bore

holes into the cell membrane and form pores which cause the cytosolic contents to spill out and the induction of cell death via necrosis (A. Bravo et al., 2004; Endo et al., 2017; Jurat-Fuentes and Adang, 2007). The insect eventually dies of starvation resulting from the inability to feed.

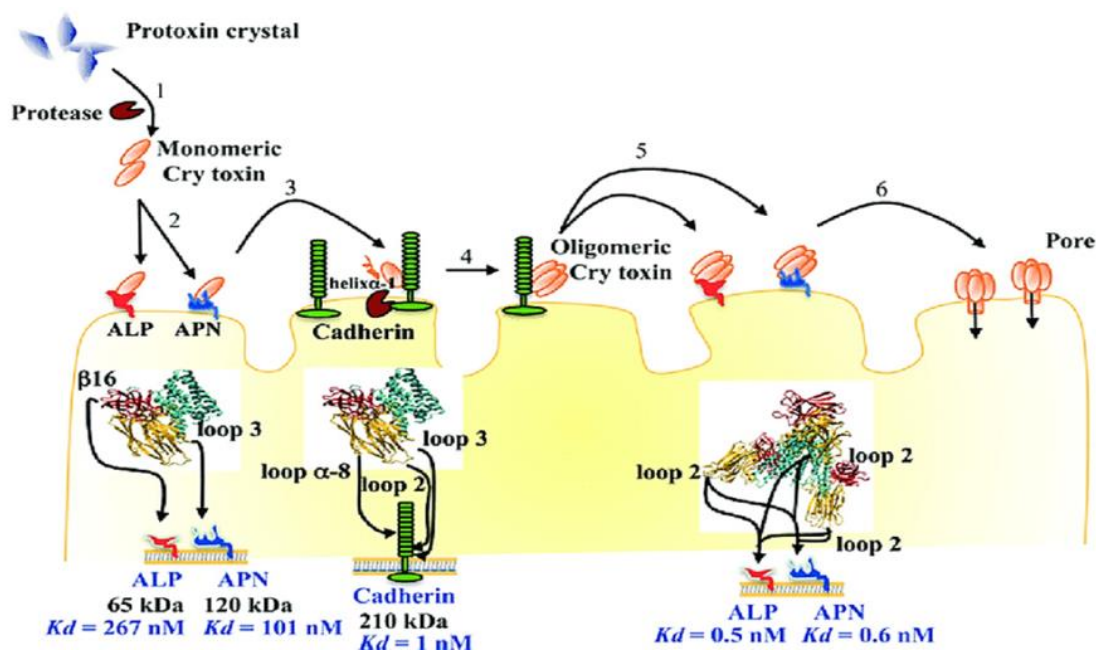


Fig1: A representation of the mode of action of *Bt* toxin: 1. Proteolytic cleavage of the toxin by the midgut protease of insects 2. Monomeric Cry toxin binds with low affinity to the ALP and APN receptors 3. The monomeric Cry protein binds to Cadherin 4. where it undergoes oligomerization, 5. The Cry oligomer binds to APN and ALP with high affinity 6. The Cry oligomers are now capable of making pores in the cell membrane and inducing cell death. (Model adapted from Pardo-López et al., (2013)).

A variety of Cry toxins have been shown to be effective not only against various orders of insects but also against gastropods, nematodes as well as human cancer cells (Fig 2) (Krishnan et al., 2017; Palma et al., 2014). The *Bt* gene has been successfully introduced into a variety of crops including cotton, corn, rice, and a wide variety of vegetables. Though there are no known side effects on humans and the transgenic crops are considered safe for consumption their

capacity to harm non-target insects such as pollinators is still debatable. There are a spectrum of Cry toxins that have been characterized and Cry1A, Cry2A are some of the examples of Cry proteins used commercially to control Lepidopteran insects including castor semi-looper *Achaea janta*, a field pest shown to cause extensive damage to the castor plants (Alejandra Bravo et al., 2007; Schünemann et al., 2014). In India, though transgenic plants other than *Bt* cotton have not been introduced, formulations containing a mixture of the Cry toxins are being used especially in the Southern part of India including the Telangana region to control Lepidopteran pests. The DOR *Bt*-1 formulation is one such example containing both the Cry1 (Cry1 Aa, Cry1Ab, Cry1 Ac) and Cry2 (Cry2 Aa, Cry2 Ab) proteins (Chauhan et al., 2017; Reddy et al., 2012). This formulation has been used to control the damage caused by the castor semi-looper *Achaea janta* in most parts of Telangana, India. There is also an increasing interest in trying to understand the capacity of this target pest to acquire resistance to these formulations and *Bt* toxins in general (Chauhan et al., 2017).

Though *Bt* crops and formulations presently in use have been shown to cause no physiological harm to non-target pollinators and insects a behavioral study in 2007 showed that honey bees exposed to *Bt* toxin Cry 1AC containing sugar feed for six days exhibited lower capacity to return to the feeder once the biopesticide was removed (Fig 3). The authors suggest that this result could have been due to some alteration at the communication level (Ricardo Ramirez-Romero et al., 2005). Communication plays a vital role in the survival and propagation of honey bees, right from the need to communicate the source, location and quality of food to the sister bee, to the need to warn the hive about possible danger. The results of the study did not indicate a perturbation to learning and memory as the number of correct choices made when the foragers visited the flowers did not reduce upon exposure to the *Bt* toxin.

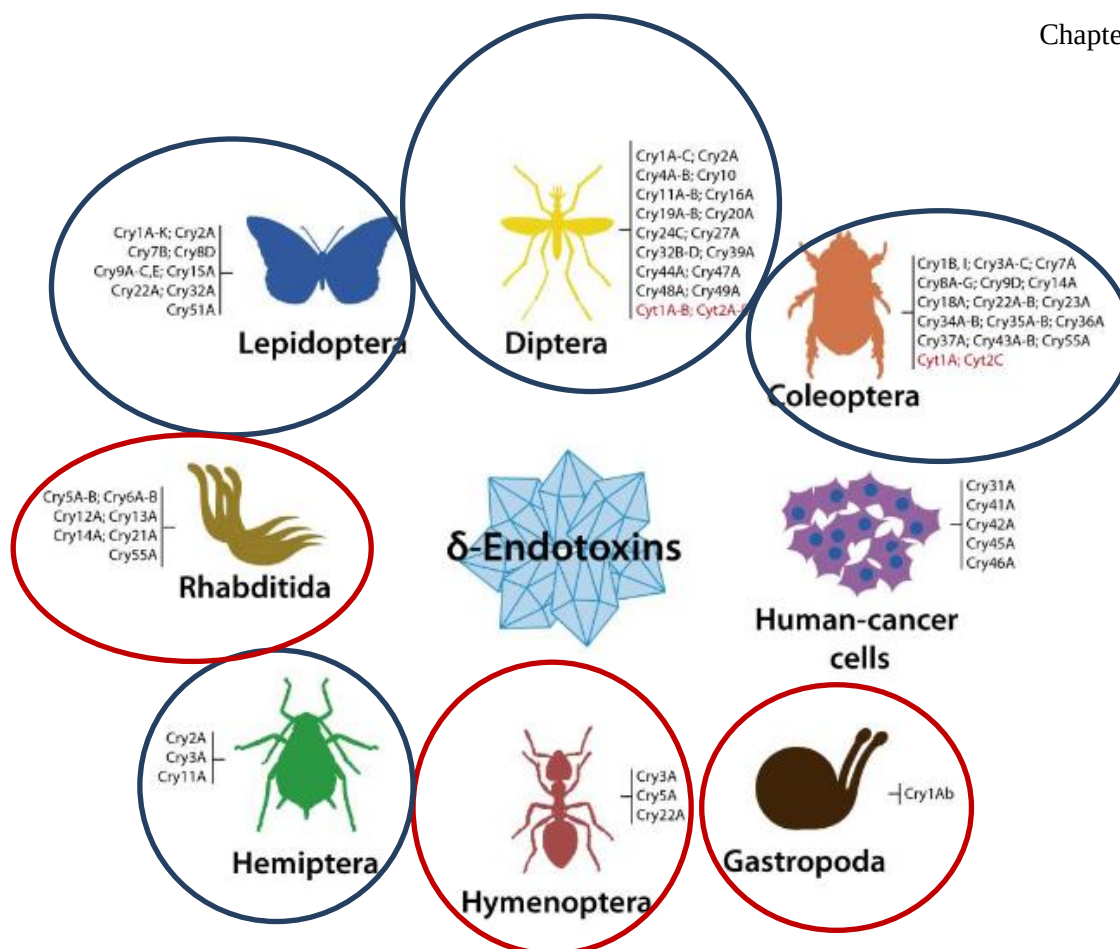


Fig 2: Representation of the spectrum of Cry and Cyt proteins and their respective targets. The Cry toxins against target field pests such as those belonging to the order Lepidoptera are circled in blue and the Cry toxins against non-target agriculturally important species such as Hymenoptera and Gastropoda are circled in red. Picture adapted from Palma et al., 2014.

The group further went on to demonstrate that in high doses of 5000 ppb Cry1Ab affects the learning performances in honey bees with the trained animals exhibiting PER even the absence of the conditioning odor indicative of interference in either the learning or memory formation (Ramirez et al., 2008). Given that the homogeneity of application of formulations such as DOR *Bt*-1 is not usually uniform and very little data being available about the amount of the toxin in the formulation being ingested by honey bees, it becomes imperative to understand the effects

these combinations of toxins might have on the learning, memory, and cognition of honey bees. So far very little work has been done in this regard and we attempted to analyze at the effects of acute exposure to higher and lower concentration doses of DOR *Bt*-1 in honey bees.

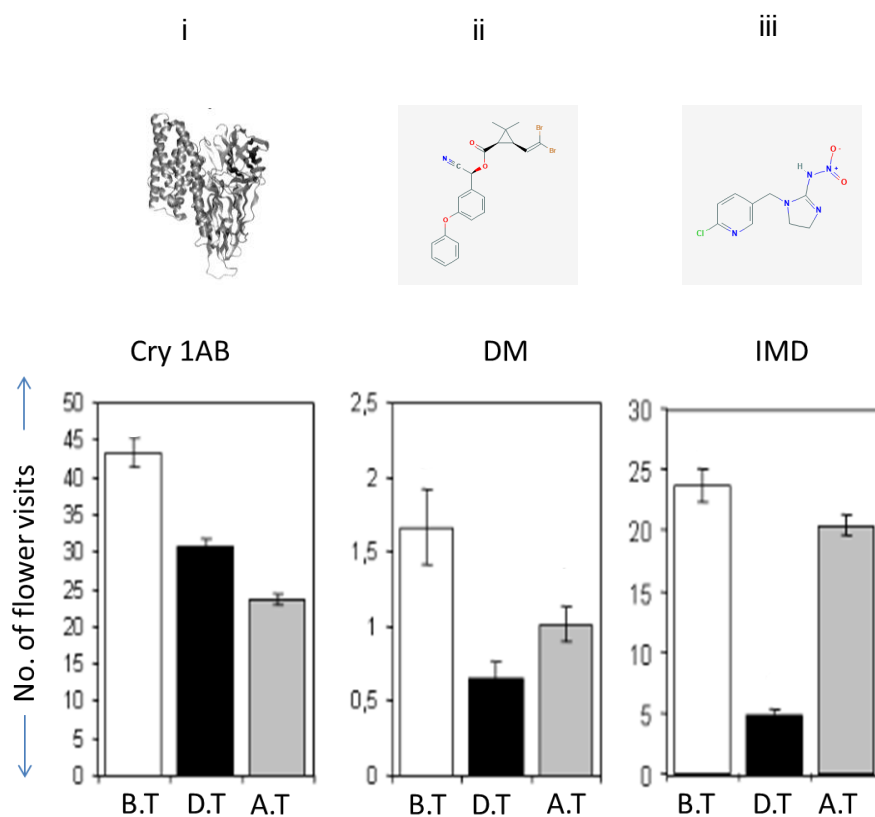


Fig 3: The results of the study highlighting the effect of three toxins i) *Bt* toxin- Cry 1AB ii) Deltamethrin- DM and iii) Imidacloprid- IMD on the learning and memory of honey bee foragers as highlighted by the number of flowers visited on the y=axis, before toxin application- B.T, during toxin application- D.T, after toxin removal- A.T. The number of flower visits by the foragers further reduced post removal of the Cry 1AB toxin as compared to DM and IMD. Figure adapted from Ramirez et al., (2005).

Materials and Methods

1. DOR *Bt*-1 formulation solution preparation for experiments

Honey bees, *Apis mellifera* were exposed to two different concentrations DOR *Bt*-1, which was provided by the Indian Institute of Oils seeds Research, Hyderabad. Two experimental solutions were prepared using this formulation in 30% sucrose solution, one containing 1mg/ml equivalent concentration of the DOR *Bt*-1 formulation in the sucrose solution and the other containing 0.2mg/ml equivalent concentration of DOR *Bt*-1 formulation.

2. *Bt*protoxin production and activation

In order to study the effect of the activated Cry toxin was directly fed to the honey bees, DOR *BT*-1 bacterial isolate was grown in nutrient broth (Himedia) for 72hr with constant agitation at 180 RPM/min in an orbital shaker at 28°C to allow for uniform sporulation. Following the incubation period cells were centrifuged at 1100 X g at 4°C for 10 mins and the pellet obtained was washed three times in distilled water. The crystals were stored at 4°C until further use.

Activation of the protoxin was done by solubilizing the crystal pellet by incubating it in 50mM sodium carbonate/bicarbonate solution (pH 9.5) containing 10mM dithiothreitol (DTT) in a water bath at 37°C for 2hr, 1mM PMSF was added to the solution to inhibit proteolytic activity. The activated toxin was purified using the Sephadex G-100 gel chromatographic column (Sigma-Aldrich USA). The protein content was estimated using Bradford's assay (Bradford, 1976), the presence of the toxin was confirmed using SDS-PAGE gel electrophoresis and immunoblotting with antibodies raised against recombinant DOR *Bt*-1.

3. ***Achaeajanta* culturing and insect maintenance**

Insect eggs were collected from the crops in the agricultural fields of the Telangana region. The collection was made from areas unexposed to the DOR *Bt*-1 formulation. Post hatching, the neonates were transferred onto fresh sterile castor leaves. The larvae were maintained in an insect culture facility under controlled conditions of 14:10 h (light: dark period), and $27 \pm 2^\circ\text{C}$ temperature with $70 \pm 5\%$ RH (Chauhan et al., 2017; Pavani et al., 2015).

4. **Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)**

The procedure was carried out in accordance with the protocol published by Laemmli (1970). 5% stacking gel (2 ml 30% acrylamide mix, 3 ml 0.5 M Tris-HCl pH 6.8, 6.76 ml distilled water, 120 μl 10% SDS, 50 μl 10% of APS and 6 μl of TEMED) and 3/4th of 10 % resolving gel (3.3ml 30% acrylamide mix, 2.5 ml 1.5 M Tris-HCl pH 8.8, 4.1 ml distilled water, 120 μl 10% of SDS, 150 μl 10% APS and 10 μl TEMED) were polymerized and poured in a ratio of 1/4th stacking gel and 3/4th resolving gel respectively, a comb containing the required amount of loading chambers of the desired volume was gently placed into the stacking gel and the gel was allowed to polymerize. Samples of DOR *Bt*1 formulation and active recombinant toxin DOR *BT*-1 were prepared by mixing the protein solutions with a loading buffer which consisted of 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue and heated in a water bath. Electrophoresis was carried out at 60 V initially, till the proteins stacked in the stacking gel and at 100 V for resolving the proteins in the resolving gel. The tracking dye was allowed to run into the tank buffer, and a loading marker (Bio-Rad, USA) was used to help determine the presence of the protein of interest depending on their weight.

5. **Coomassie staining**

In order to visualize the proteins gel staining was carried out using the protocol published by Wilson *et al.*, (1979). The gel containing the separated proteins was placed in a container containing the staining solution (0.025% Brilliantblue- R 250 in 40% methanol and 7% acetic acid), overnight on a rocker. Destaining was carried out the following day using a destaining solution (40% methanol and 10% glacial acetic acid) which revealed the stained proteins and the bands of the protein molecular weight marker.

6. **Western-blotting and immunodetection**

The proteins were electrophoresed and blotted onto a nitrocellulose membrane (Amersham, Germany) the protocol followed was published by Towbin et al(1979). The membrane and the electrophoresed gel were dipped in Towbin's buffer (25 mMTris, 192 mM glycine and 20% methanol) for 10 min. this was followed by the careful overlaying of the gel, the membrane and sandwiching them with stacks of filter papers, the whole set was the carefully placed in a cassette while making sure that no air bubble was present. The entire cassette was placed in transfer unit containing Towbin buffer. The process of transfer was carried out at 25 V with 250 mA current limit for 14hr. Successful transfer of the proteins to the membrane was checked using Ponceau Staining (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid). Once the transfer of proteins to the membrane was confirmed, extensive washes were done with Tris buffered saline (TBS containing 10 mMTris-HCl (pH 7.4), 150 mMNaCl). For immunodetection, immunoblotting of the membrane was carried out by soaking the membrane in 5% blocking solution (5% skim milk powder (w/v) in TBS) or 3% BSA for 3hr to block nonspecific sites or regions of antibody binding on the blot. Three washes of the blotting paper were done for 15 min using TBST (0.1% Tween- 20 in TBS (Sigma, USA). The blot was incubated overnight with a primary antibody

generated in mice against DOR *Bt*-1 toxin (1:1000 dilution) at 4°C, this was followed by incubation with a secondary antibody (1:4000 dilution). Staining was carried out using alkaline phosphatase (ALP) or horseradish peroxidase (HRP) conjugated anti-rabbit IgG diluted in 5% skim milk powder (w/v) dissolved in TBS. The blot was soaked in for 2hrs followed by washes to remove any unbound antibodies and processed for development with BCIP/NBT (G-Biosciences, USA) for ALP or chemiluminescence (Takara Bio Inc, Japan) for HRP conjugated antibodies. The blots were imaged using the Kodak Photo Imager.

7. **Confirmation of activity of recombinant DOR *Bt*-1**

The activity of DOR *Bt*-1 was determined by coating castor leaves with 4.5µg/µl equivalent concentration of the DOR *Bt*-1 protein. *Achaea janta* larvae in the third instar were allowed to feed *ad libitum*, the mortality and cessation of feeding were checked 24hr post-feeding.

8. **Preparation of various control solutions and formulations**

The capacity of the individual components of the DOR *Bt*-1 formulation to affect learning and memory in honey bees also had to be checked. In order to do so, *Bacillus thuringiensis serovar kurstaki* H 3a3b3c strain DOR *Bt*-1 was incubated and grown in a 72% wheat bran nutrient medium for 72hr at 32°C in an orbital shaker. A control solution of 72% wheat bran was also incubated identically. Post incubation the solutions were centrifuged at 10000 X g for 10 min at 4°C, the supernatant and pellet were separated. The supernatant was further filtered through a muslin cloth the liquid filtrate was lyophilized. The pellet and filtered supernatant and lyophilized filtrate were quantified and 5 % equivalent Carboxy methyl cellulose (CMC) was added to each component. The three components were spread onto aluminum foil sheets and allowed to air dry completely. Once dried, the individual components were ground to powder

consistency using liquid nitrogen, and the three sets of powders were then used as control feed for the bees. The procedure was repeated for the incubated control solution of wheat bran, the pure powder obtained was used as a pure wheat bran control feed.

9. **Honey bee collection and toxin ingestion**

Honey bees *Apis mellifera* were collected at the entrance of the hive at 9:00 AM. The bees were anesthetized at 4°C and harnessed. The harnessed bees were kept at 25°C, $\pm$ 60% RH in a dark chamber. The animals were divided into 9 groups, the experimental group was fed 1mg/ml equivalent DOR *Bt*-1 in 30% sucrose, or 0.2mg/ml equivalent DOR *Bt*-1 in 30% sucrose. The seven control groups were prepared in 30% sucrose solution i. 1mg/ml equivalent concentration of lyophilized wheat bran inoculums ;ii. 1mg/ml equivalent lyophilized bacterial pellet; iii. 1mg/ml equivalent lyophilized spent media supernatant; iv. 1mg/ml equivalent lyophilized spent media filtrate ;v. 6% solution of the active DOR *Bt*-1 toxin ; vi. 6% solution of the active toxin buffer, and vii. 30% sucrose solution. The solutions were prepared fresh each day and the animals in each group were fed *ad libitum*. The animals once fed were placed in the dark chamber overnight and training was carried out the following day.

10. **PER conditioning**

The following day the honey bees were trained in olfactory PER conditioning using hexanol and the training odor. A 30% sucrose reward was provided and the learning rate over 5 trials with a 10-minute ITI was recorded. The animals that did not exhibit PER were eliminated from the training. Mid-term memory retention and discrimination were tested at 6hr post-training and long-term memory retention and discrimination was tested 24hr post-training. Discrimination was tested using geraniol.

11. **Statistical analysis**

Analysis was done on MS excel, ANOVA was used as a statistical measure to calculate any significant differences in the learning and retention rates between the groups.

Results

1. **Confirmation of the presence of the activeDOR *Bt*-1 toxin and Cry protein in the *Bt*formulation**

The presence of the *Bt* Cry protein was confirmed using SDS-PAGE gel electrophoresis as a 120 kDa protein band and activated toxin of 65kDa protein (Fig 4A). It was further confirmed by immunoblot with DOR *Bt*-1 antibodies (Fig 4 B) which shows the presence of both protoxin as well as activated toxin.

2. **Confirmation of the activity of activated DOR *Bt*-1**

Protein estimation via Bradford's test revealed the protein content of the activated toxin in the prepared sample to be 4.5µg/µl. Activated DOR *Bt*-1 toxin was coated onto castor leaves and *Achaea janta* larvae in the third instar were allowed to feed on the leaves. 24hr post-exposure, cessation of feeding, and 100% mortality was observed, hence confirming the pest control ability of the generated toxin (Fig 5).

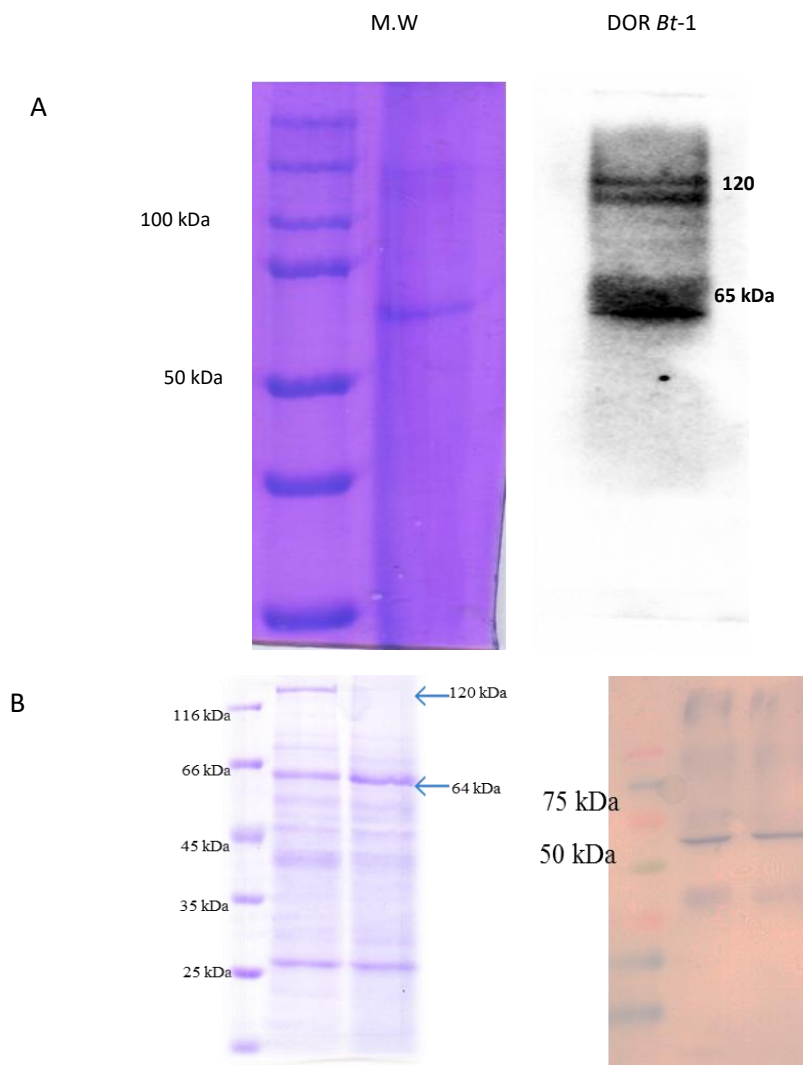


Fig 4: A. Cry proteins were visualized in the SDS-PAGE gel as a 120 kDa band and the activated toxin was seen as a 64 kDa band. B. The presence of the activated DOR *Bt-1* toxin was further confirmed by via immunoblotting .M.W- molecular weight marker.

3. **Acute exposure to *Bt* formulation DOR *Bt-1* does not affect the learning rates of honey bees *Apis mellifera***

We trained a total of n=175 honey bees for this experiment. No significant difference was seen in the learning rates in the groups. The experimental bees acquired information as well as the control and sugar-fed bees. There was no significant difference between the learning rates of the bees fed with toxin and the control sugar-fed group. Sugar fed bees exhibited a learning rate of

65% at the 5th trial while bees fed with 0.2mg/ml and 1mg/ml DOR *Bt*-1 formulation displayed 76% and 64% learning at the 5th trial respectively (Fig 6). This result indicates that in acute doses, both high and low, the *Bt* formulation DOR *Bt*-1 does not affect the learning capacity of honey bees *Api mellifera*.

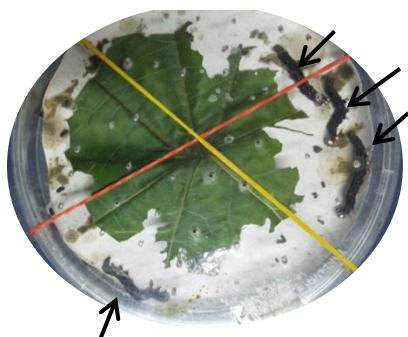


Fig 5: *Achaea janta* larvae fed with the activated toxin died 24hr, as indicated by the black arrows. Cessation of feeding was also observed as seen from the partially eaten leaf.

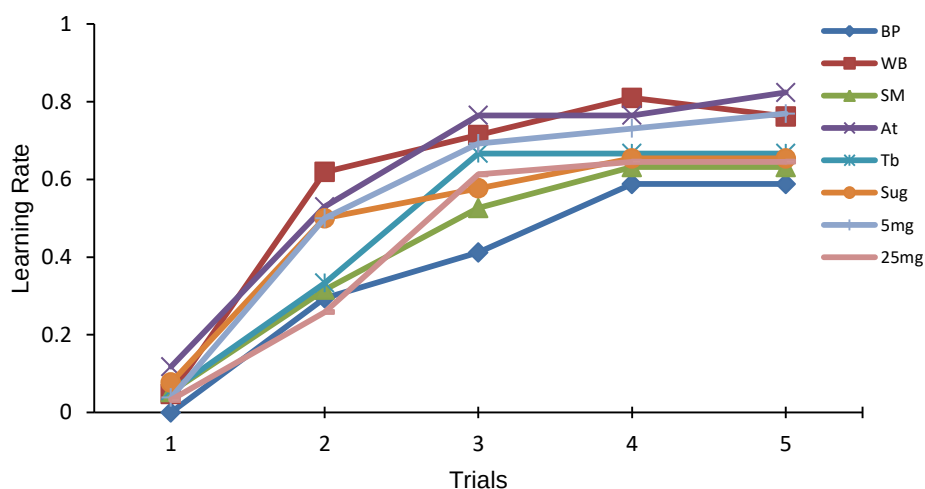


Fig 6: Acute exposure to DOR *Bt*-1 formulation in low and high doses does not affect the animal's capacity to acquire information and learn. The trial number is represented on the X-axis and the learning rate on the Y-axis. The experimental and control groups are represented as colored lines above the graph, BP= Bacterial pellet, WB= Wheat bran, SM= Spent media, At= Activated toxin, Tb= Toxin buffer, Sug= Sugar, 5mg= 0.2mg/ml DOR *BT*-1 formulation, 25mg=

1mg/ml DOR *Bt-1* formulation. No significant difference was seen between the learning rates of the groups.

4. **Acute exposure to DOR *Bt-1* formulation does not affect the mid-term and long-term memory of honey bees**

The result obtained in the present study did not show a significant difference in the memory retention of the honey bees either at 6hr or 24hr (Fig 7). The bees from both the control groups and experimental groups performed identically. Interestingly animals exposed to 0.2mg/ml equivalent concentration of the formulation showed lower memory retention at 24hr compared to their retention rate at 6hr (Cochran's $q=5.4$, $p=0.019$), however, this rate was not significantly different from the retention rate of animals exposed to 1mg/ml DOR *Bt-1*. An average rate of 80% retention to the trained odor 1-hexanol was seen at 6hr and 63% retention was observed at 24hr. Animals exposed to 0.2mg/ml and 1mg/ml formulation exhibited maximum retention of 80% and 70% respectively at 6hr which was not significantly different from the retention of sugar-fed bees, who exhibited retention of 80%. The 0.2mg/ml and 1mg/ml formulation fed bees exhibited 53% and 75% retention at 24hr respectively, while sugar-fed bees exhibited 80%. Despite the drop in retention by the animals exposed to 0.2mg/ml equivalent concentration of formulation at 6hr, the decline was not significantly different from that of the animals fed with 1mg/ml equivalent concentration of spent media. These results indicate that feed containing DOR *Bt-1* formulation does not affect the mid-term and long-term memory in honey bees *Apis mellifera*.

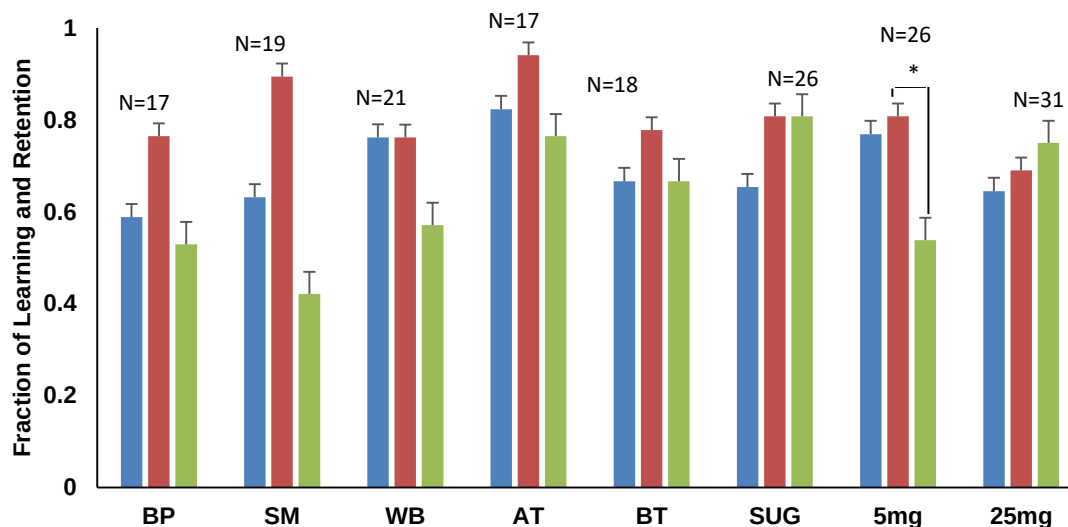


Fig 7: Exposure to DOR *BT-1* formulation does not affect the mid-term and long-term memory in honey bees. No significant difference in memory retention was seen between the groups. The groups represented in the histogram are, BP= Bacterial pellet, WB= Wheat bran, SM= Spent media, At= Activated toxin, Tb= Toxin buffer, Sug= Sugar, 5mg= 0.2mg/ml DOR *BT-1* formulation, 25mg= 1mg/ml DOR *BT-1* formulation. No significant difference was seen between the learning rates of the groups. The blue bar represents the acquisition at the 5th trial, the red bar represents the 6 hr retention and the green bar represents 24hr retention. Error bar=mean±sem
*p<0.05

5. **Discrimination is not affected by acute exposure to DOR *BT-1***

To check if acute exposure via ingestion to DOR *BT-1* affects the bee's capacity to discriminate between the trained odor and a novel odor, retention test was carried out using the trained odor 1-hexanol and a novel odor 1-geraniol (Fig 8). A comparison of the discrimination between sugar-fed bees and DOR *BT-1* formulation fed bees showed no significant difference in the discriminating abilities between the three groups. The animals were able to significantly

discriminate between the trained odor and the novel odor at 6h [$F(1,4)=200$ $p=10^{-3}$] and at 24hr [$F(1,4)= 51.5$, $p=0.001$].

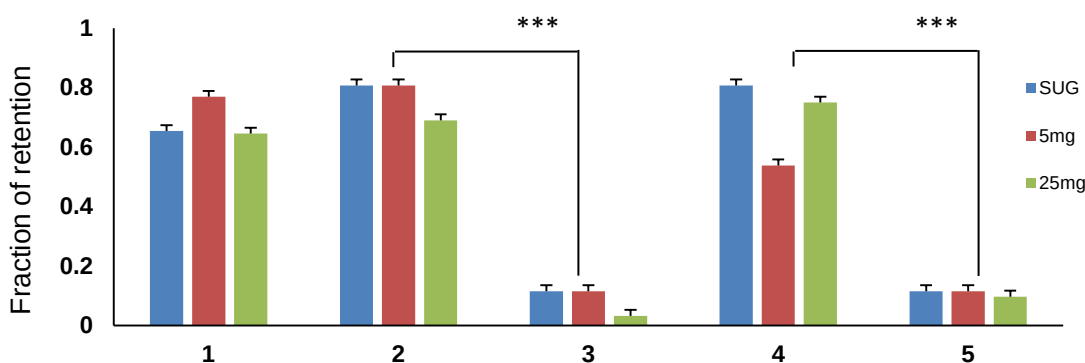


Fig8: Discriminating ability of the honey bees is not affected by acute exposure to the toxin. The X-axis represents the 5 different tests given to the animal to check their discriminating abilities at 6hr (3) and 24hr (5). 1= 5th Trial, 2=6hr Retention, 3=6hr Discrimination, 4=24hr Retention, 5=24hr Discrimination. The discrimination capacity at 6hr (2,3) and 24 hr (4,5) was found to be significant [$F(1,4)=200$ $p=10^{-3}$; $F(1,4)= 51.5$, $p=0.001$]. Sugar fed bees are represented as the blue bar, 0.2mg/ml toxin fed bees as the red bar, and 1mg/ml toxin fed bees as the green bar.

Discussion

Bt based insecticides have been in use since 1958 but gained importance only around 1988 as the demand for eco-friendly pest management and implementation for transgenic, genetically modified crops grew (Ibrahim et al., 2010). The *Bt* based insecticides have a wide range of advantages over organophosphates, glyphosates, other synthetic pesticides, and neonicotinoids. In addition, *B. thuringiensis* has been shown to produce isomers of the Cry and Cyt toxins in a species-specific manner and hence does not affect non-target insects such as pollinators (Palma et al., 2014). In the Telangana region of India, formulations of *Bt* are used to control

Lepidopteran pests such as castor semi-looper *Achaea janta*. The DOR *Bt*-1 formulation is a robust pesticide containing two of the Cry toxins, Cry1, and Cry2 produced by the *Bacillus thuringiensis* serovar *kurstaki* H 3a3b3c strain DOR *Bt*-1(Reddy et al.,2012). *Achaea janta* feeds voraciously on the plant during its third instar phase of development. Upon consumption the protoxin is activated in the animal's mid-gut, under conditions of alkaline pH, the activated toxin binds to the mid-gut epithelium via ALP, APN, and cadherin receptors, followed by oligomerization to produce pores in the epithelial layer or induction of the cell death cascade. The result is death by starvation as the animal ceases to feed and dies within 24hr post-consumption of effective formulations(Bravo et al., 2007; Chauhan et al., 2017; Palma et al., 2014). At the field level, spraying of this formulation provides an uneven coverage, with the upper foliar region of the plant receiving more toxin than the lower stem, branches, and leaves(Chauhan et al., 2017). As a result of this uneven spread, along with factors such as degradation due to UV exposure and wash off resulting from rains, the pests, and insects that target the plant are exposed to lower concentration doses or sub-lethal doses of the formulation. Our laboratory has shown that exposure to these sub-lethal doses does not affect the insect completely, allowing for mid-gut regeneration, which could result in resistance to the toxin over chronic exposure (Chauhan et al., 2017; Dhanial et al., 2019; Tabashnik et al., 2013).

The spectrum of *Bt* toxins normally used for the management of agricultural pests are selected so as to cause no physiological harm to non-target insects such as pollinators like the honey bee and bumblebee and so far the evidence suggests transgenic plants containing the *Bt* gene and formulations containing *Bt* toxins do not have any direct effect on the survival of pollinators (Dai et al., 2012; Rose et al., 2007; Yi et al., 2018). However, the rapid decline in the number of crucial pollinators with the U.S. reporting losses of upto 40% of honey bee hives in 2019, and the

continuing decline in the number of wild bees (Mathiasson and Rehan, 2019) and bumblebees across the globe over the past two decades emphasizes the need to look at other possible effects of pesticides deemed safe for consumption by these animals, (University of Maryland June 19, 2019- Retrieved from <https://www.sciencedaily.com/releases/2019/06/190619142532.htm>). To this end we attempted to look at the effects of acute exposure to both low and high doses of *Bt* formulation DOR-1 in honey bees *Apis mellifera* as this formulation is used extensively in the Telangana region of India for the management of the Lepidopteran pest *Achaea janata*. We observed that in acute doses this formulation does not affect the learning or discriminating abilities of the animal. The mid-term and long term memory are not affected as all the animals retained the learnt information equally. We also exposed the honey bees to the active form of the DOR*Bt*-1 toxin and observed that the animals did not exhibit increased mortality or reduced learning, retention or discrimination abilities. This study hence highlights that the honey bees, even if exposed to the active form of the toxin will not be affected, since the receptors required to bind to the Cry proteins present in the DOR-1*Bt* formulation might be either absent or might be present as different isomers of the receptors that bind to the toxin in the Lepidopteran insect mid-gut. Extensive studies from our lab has shown the presence of multiple isoforms of APN in the larval midgut of *Achaea janata* (Chauhan et al., 2017; Dhanial et al., 2019).

Work published by Ramirez et al., in 2005, showed that chronic exposure to the *Bt* toxin Cry1 Ac, did not affect the honey bees learning and memory capacities, however the animals that returned to the feeder once the toxin was removed were lesser in number than the bees exposed to neonicotinoids. The possible reason suggested by the group for this drop in foraging ability post removal of toxin, was the probable perturbation to their communication and not to the animals learning and memory as no significant change was seen in the right choices of sugar

filled flowers made by the honey bees (Ramirez-Romero et al., 2005). The group went on to demonstrate that in high concentrations Cry1 AC affects the memory of honey bees as the animals make significantly higher wrong choices during the retention test (Ramirez-Romero et al., 2008). Communication is crucial to the eusocial honey bee and the antennae house the organ responsible for communication called the Johnston's organ (JO). The Pedicel at the base of the flagellum contains the scolopale, which are sensitive to vibrations and comprise the sensory unit of the JO (Ai, 2009). The axons projecting out of the JO arborize onto the subesophageal ganglion (SEG) and a number of interneurons originating at the JO arborize onto the posterior protocerebellar lobe, dorsal lobe and dorsal SEG (Ai, 2009). In addition it was demonstrated that one of the interneurons responded to olfaction and vibration when the stimuli were applied simultaneously, indicative of the multi-modality of the interneurons originating at the JO. If communication is being ablated by chronic exposure to the *Bt* toxin, study of the histology and physiology of the JO might lead to some answers. Ramirez et al, (2005) exposed the animals chronically for a period of six days whereas in our experiment we exposed the animal to a single dose of the toxin. It remains to be explored, if chronic exposure to the DOR *Bt-1Bt* formulation might affect the learning and memory in honey bees.

India is home to 4 out of the 7 honey bee species in the world and presently there is very little information available about the number of hives being maintained and if there is any decline in the number of pollinators in the region. There is also very little information available about the bioavailability of field level pesticides, the amount of crop being pollinated by the local pollinators in the region and the effect of seasonal change in the number of pollinators. Despite being deemed safe, there is little work done on the effect of biopesticides on the cognition and behavior of non-target insects. Given the consistent decline of key pollinators and no specific

reason, but a multitude of reasons being the cause for this decline, makes it even more important to study every aspect of physiology and health that might be altered in the animals.

Our observations from the present study indicate that honey bees fed with lower dose of the toxin showed lower memory retention (27% decline) at 24hr in comparison to their mid-term retention but this difference was not found to be significant and a similar decline in the retention rates were seen in control bees fed with spent media. We conclude that acute exposure to both high and low doses of a regularly used *Bt* formulation DOR *Bt*-1 might not have a direct affect the learning and memory in honey bees. However, further in depth studies need to be carried out to test the effect of chronic exposure to the DOR *Bt*-1 formulation and other extensively used *Bt* formulation to really arrive at conclusion.

CHAPTER 3

QUANTIFICATION OF

BEHAVIOR OF HONEY BEES IN

ISOLATED CAPTURE

CHAPTER 3

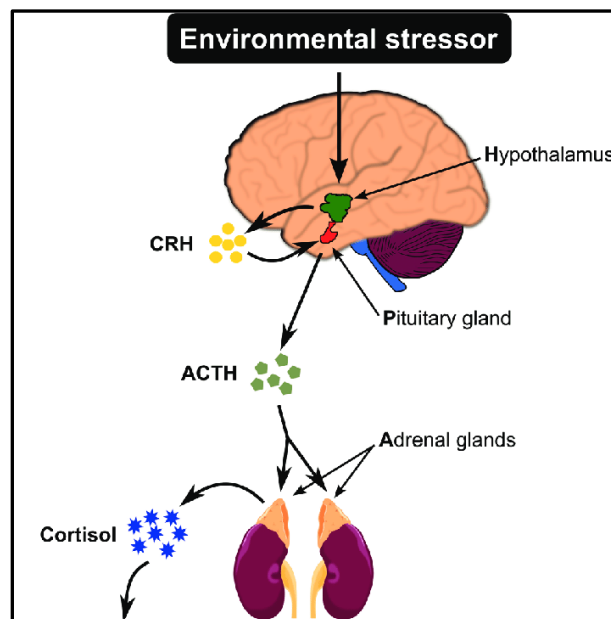
Introduction

Honey bees are eusocial insects that exhibit age-dependent polyethism. The worker caste is divided into the cleaners, nursers, and foragers. The foragers carry out the task of locating and collecting pollen, nectar, water, and propolis for the survival of the hive. An internal compass helps the bees use the sun's polarized light as a reference, while the position and direction to the source of food are communicated via a waggle dance on the vertical surface of the honeycomb which provides the directional information as an azimuth relative to the azimuth of the sun (Evangelista et al., 2014; Kraft et al., 2011). In addition to the navigational cues, olfactory cues play a crucial role in the formation of food source associated memory. Honey bees can travel up to 15 km one way in search of optimal food and water sources. The main sources of energy during these extended flying routines are the hexoses- sucrose, fructose, and trehalose a sugar consisting of two glucose molecules. Metabolomic and proteomic analysis of the flight muscles in foragers ranging from young to old has revealed a significant increase in the level of citrate synthase, and an isoform of troponin, troponin T10A (Schippers et al., 2006). Citrate synthase is an enzyme responsible for the condensation of oxaloacetate and acetyl-CoA to citrate and an age-dependent increase in the levels of this enzyme was observed which was also found to be directly proportional to the foraging performance in the honey bees (Schippers et al., 2006).

The tasks carried out by foragers are highly energy consuming and age-dependent performance is seen amongst the foragers (Blatt and Roces, 2001; Schippers et al., 2006). The foragers also encounter several biotic and abiotic stresses which could perturb them from carrying out their foraging jobs optimally. Stress is defined as an organism's physiological response often thought

to be adaptive to a stimulus called stressor. In mammals, the hypothalamo-pituitary-adrenal axis system (Fig 1A) functions as a stress responder, by boosting the production of catecholamine and corticoid hormones that activate the flight or fight response (Even et al., 2012). In insects (Fig 1B) biogenic amines such as octopamine (OA) and dopamine (DA) have been shown to play a role in mediating stress responses (Even et al., 2012). The neurosecretory cells of the *corpora cardiaca* (CC) induce the mobilization of energy reserves from the fat bodies in the mid-gut epithelium via the production of hormones such as adipokinetic hormone-1 (AKH), corazonin (Crz) and possibly diuretic hormone (DH) (Even et al., 2012). Hormones such as allatostatin-A, insulin-related peptide, and tachykinin related peptide have also been suggested to play a role in the regulation of the secretion of metabolically active hormones by the CC (Even et al., 2012). In insects such as the honey bee, the behavioral output in response to stress might be defensive in nature as seen in the sting extension reflex. Other outputs include escape activity loss of cognitive ability and in extreme cases death.

A



B

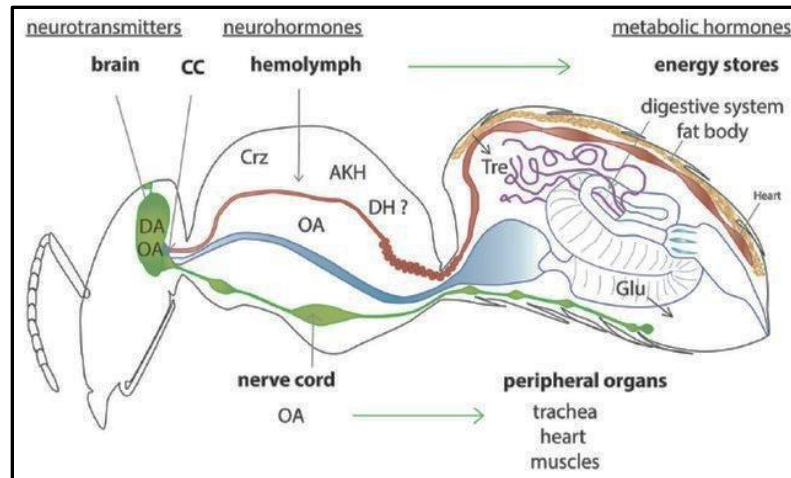


Fig 1: A. Pictorial representation of the stress circuit in humans, in response to a stressor, ACTH- Adrenocorticotrophic hormone, CRH- Corticotropin releasing hormone, induce the production of the cortisol from the adrenal glands which in turn regulate the metabolism and help elicit the flight or fight response (Figure adapted from Lanoix and Plusquellec, 2013) B. Pictorial representation of the stress response in honey bees CC- Corpora cardiaca releases metabolically active hormones such as Crz- corazonin , AKH- Adipokinetic hormone and possibly DH- Diuretic hormone, release of DA- dopamine and OA- Octapamine mediates the activity of the peripheral organs such as the heart trachea and muscles while hormones such as allatostatinA, tachykinin-related and insulin-related peptides released from the peripheral neurosecretory cells modulate gut-activity and may further regulate the activity and release of Crz, AKH and DH from the CC(Figure adapted from Even et al., 2012)

In a mathematical model proposed by Visscher and Dukas in 1997, a type II survivorship curve was found to be the best fit for the honey bee foragers (Visscher and Dukas, 1997). This model demonstrated that honey bee foragers exhibit mortality in an age-independent manner, wherein the animals are more likely to succumb to a variety of environmental pressures including predation and rise in temperature. The study also highlighted that like (senescence which accounted for 21% of mortality, energy depletion, was not a crucial player in general

forager mortality. Foragers have been shown to maintain an optimal energy to cost ratio by not filling their crop load to a maximum during foraging and hence the energy reserves are not depleted to gather more food in each round (Harano, 2020; Schmid-Hempel et al., 1985). The results of these studies indicated that the foragers are more likely to encounter myriad stressors while foraging which could induce mortality and that senescence accounted for just about 21% of the mortality in the foragers.

The role played by the foragers in the preservation and survival of the hive is a crucial one. Without the foragers providing food for the brood and the queen, the hive becomes susceptible to failure. The past two decades have seen a rapid decline in the number of honey bee hives across the globe (Neumann and Carreck, 2010; van Engelsdorp et al., 2009). A phenomenon called the colony collapse disorder was first recorded in the US during the fall and spring from 2006-2007 (Neumann and Carreck, 2010; Ratnieks and Carreck, 2010). It is hypothesized that multiple biotic and abiotic stressors amplify the stress caused to the foragers resulting in their inability to return to the hive post foraging (Fig 2). As the number of foragers returning to the hive dramatically reduces, the unmarked bees in the hive precociously take on the job of the foragers. The brains of the unmarked bee are not developed or wired for the tasks of the foragers, hence the unmarked bees that leave the hive in search of food while not being equipped to do so, inevitably fail at their foraging tasks and only a few young bees return to the hive. Gradually the number of bees in the colony reduce leaving the queen and a few young bees and the brood to fend for themselves, a hive such as this collapses (Clark et al., 2015; Ratnieks and Carreck, 2010; van Engelsdorp et al., 2009). The series of events leading to colony collapse, once triggered could occur anywhere within a few hours to a few days, and saving the colony is close to impossible.

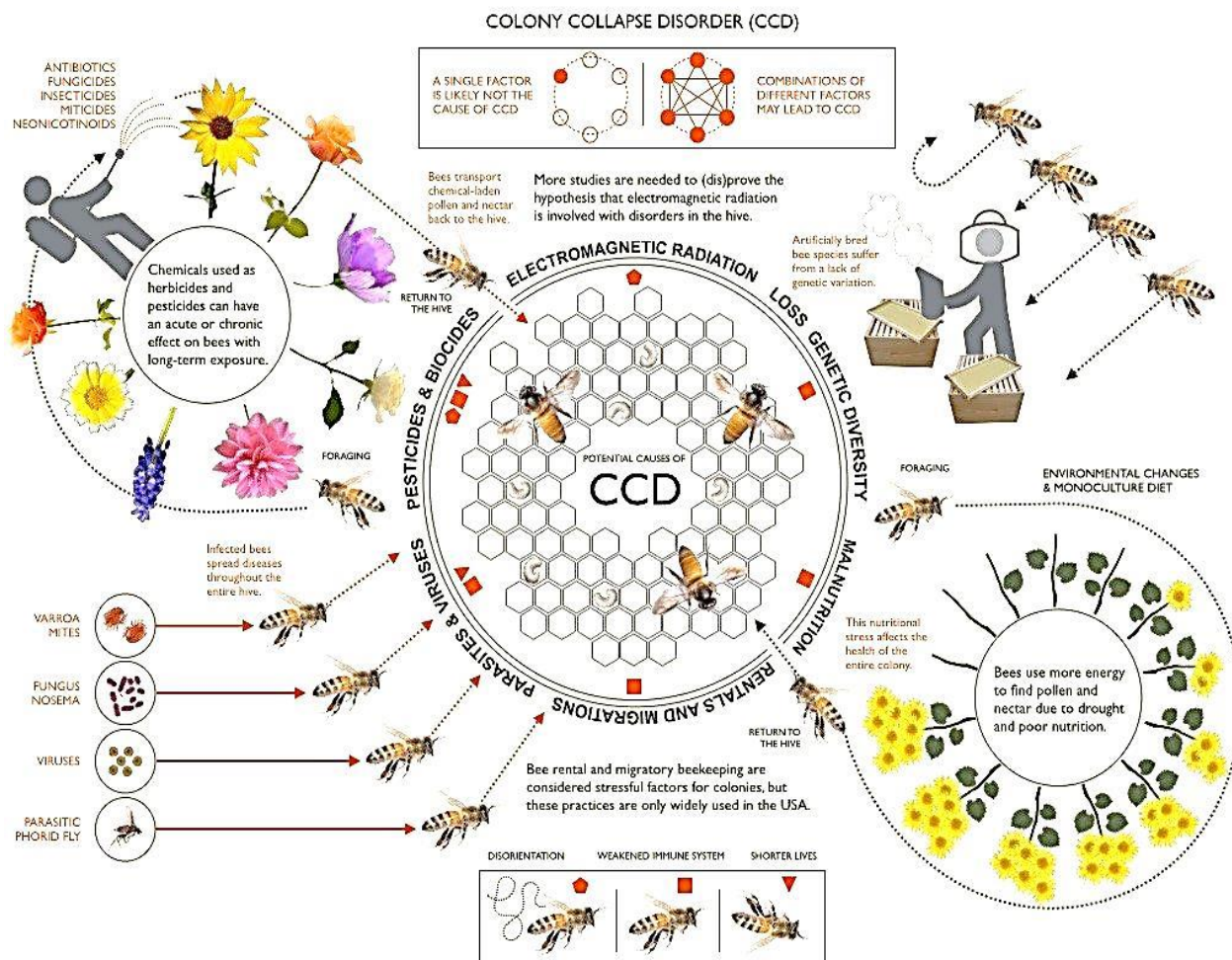
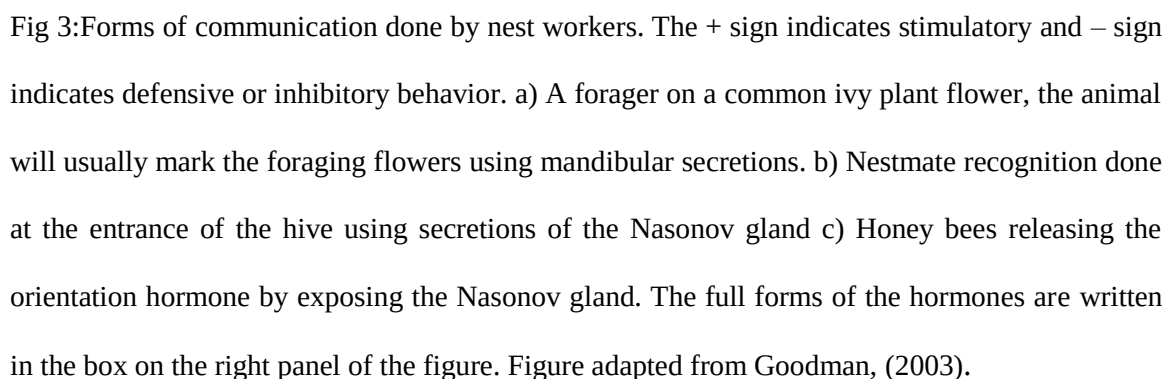


Fig 2: A representation of the factors known to cause colony collapse disorder. Figure adapted from Clark et al., 2015.

Despite extensive research, the main reason behind colony collapse is still to be established. It has been seen that multiple stressors including acres of monoculture farming, the use of pesticides, parasitic infections such as those caused by the mite, *Varroa destructor*, bacterial and viral infections caused by the *Spiroplasma* and *Paenibacillus* species, the Black queen cell virus, the Sacbrood virus, the Israeli acute paralysis virus, together play a role in pushing a colony to collapse (Li et al., 2018). One factor that remains constant is the compromised health of the foragers and the lack of flowering plants, especially the wild ones, has driven both wild and domesticable foragers to travel extensive distances to a foraging location (Mathiasson and

Rehan, 2019). The foraging bees hence succumb to exhaustion which is further exacerbated by the climate changes in recent times. Also very little is known about how foragers behave when stressed.

Molecular and physiological studies have revealed the crucial role played by the ratio of the Juvenile hormone (JH) and vitellogenin in providing immunity to the bees. Proteins such as the heat shock proteins (HSPs), enzymes such as the cytochrome monooxygenases, glutathione synthases, cell signalling kinases such as the c-jun N-terminal kinase (JNK), and transcription factors such as the NF- κ B have been shown to regulate the immune response in bees to both biotic and abiotic stressors (Even et al., 2012; G. Li et al., 2018). Heat shock proteins such as the HSP-70 have been shown to affect the basic cognitive functions in honey bees in a manner similar to humans (Hranitz et al., 2010) and upregulation of HSP-70 has been reported when the animal is exposed to chemicals such as ethanol. Despite having developed and adapted an immunity to individual stresses, it does seem like the foragers are unable to adapt and fend off the plethora of stresses they might experience in today's volatile environment (G. Li et al., 2018). Communication plays a vital role in the survival and propagation of the eusocial honey bee colonies (Bortolotti and Costa, 2014). The Nasonov Gland Pheromones present in the workers has been known to play a role in orientation and recruitment behavior (Fig 3), the tarsal glands in workers and foragers produce pheromones that mark the entrance of the hive, nestmate recognition is done through the production of cuticular hydrocarbons (Bortolotti and Costa, 2014). In addition, the queen mandibular pheromone maintains the equilibrium in the hive by motivating each worker class to carry out their designated jobs. These animals also exhibit advanced social behaviors such as grooming and communication via antennal and proboscis touch.



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made directly from an open *A. dorsata* hive the bees would live for upto 72 hours when supplied with a sucrose feed. This observation led us to ask the following questions about the behavior of foragers in isolation i) Does isolation under capture act as stressor adequate to kill foragers? ii) What is the equivalent stressor the forager experiences in the environment? iii) Is this stressed behavior linked to the memory of the forager? To this end we studied the behavior of foragers and unmarked hive bees when allowed to fly freely in a glass chamber with ample aeration, 25°C ambient temperature and 60% $\pm$ 2% relative humidity and compared their physiology, behavior, and mortality to similarly treated but cooled before being released into the chamber, cooled and harnessed foragers and young hive bees. The results were consistent with our primary observation of foragers having higher mortality than free-flying unmarked hive bees and foragers harnessed and kept in the same conditions. Our results revealed the complex nature of forager behavior and how capture and isolation can act as an abiotic stressor potent enough to kill the animal. This behavior can be mitigated by supplying the captured animal with sucrose. We also observe that exhaustion resulting from active foraging does is not related to the animal's behavior in capture. The harnessed foragers can live on for upto 72 hours indicating an interplay between stress and flight or flight muscle activation.

Materials and Methods

1. Honey bee collection

i) *Apis dorsata* collection

For the experiments, *A. dorsata* foragers bees were collected from floral sources such as Eucalyptus flowers, Hemilia flowers (Fig 4B), and wild alders. Honey bees were also collected from open hives (Fig 4A) using hand made contraptions.

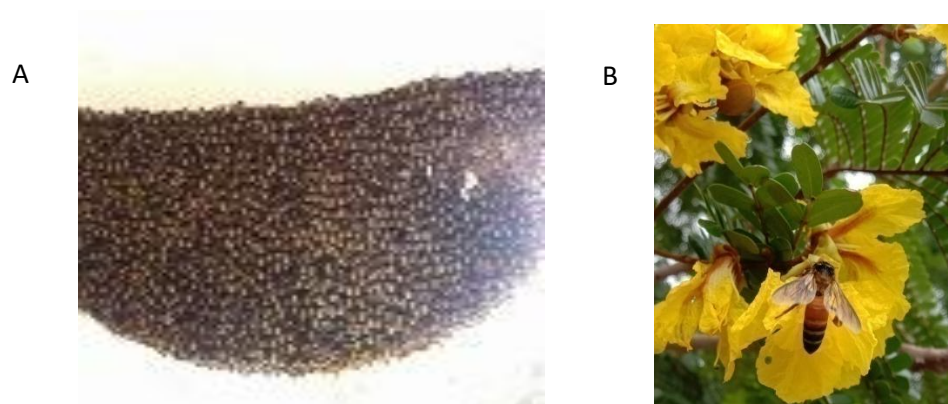


Fig 4: A. An *A. dorsata* hive from where hive bees were collected B. *A. dorsata* forager on a copper pod flower, for the forager collections, foraging bees were either collected with the flowers or without the foraging flower.

ii) *Apis mellifera* collection

In order to obtain a better control on the collection of bees and restrict it to foragers alone, a sucrose feeder containing 30% sucrose was placed approx 3 mts from the bee box (Fig 5 A). A fraction of the visiting bees were tagged with testor paint on their thorax, and these tagged bees were collected from inside the box (Fig 5C). Unmarked hive bees were screened for and only those bees with their bodies immersed in the cells assumed to be cleaning or feeding were selected and collected as the third group since that would increase the probability of collecting a younger hive bee

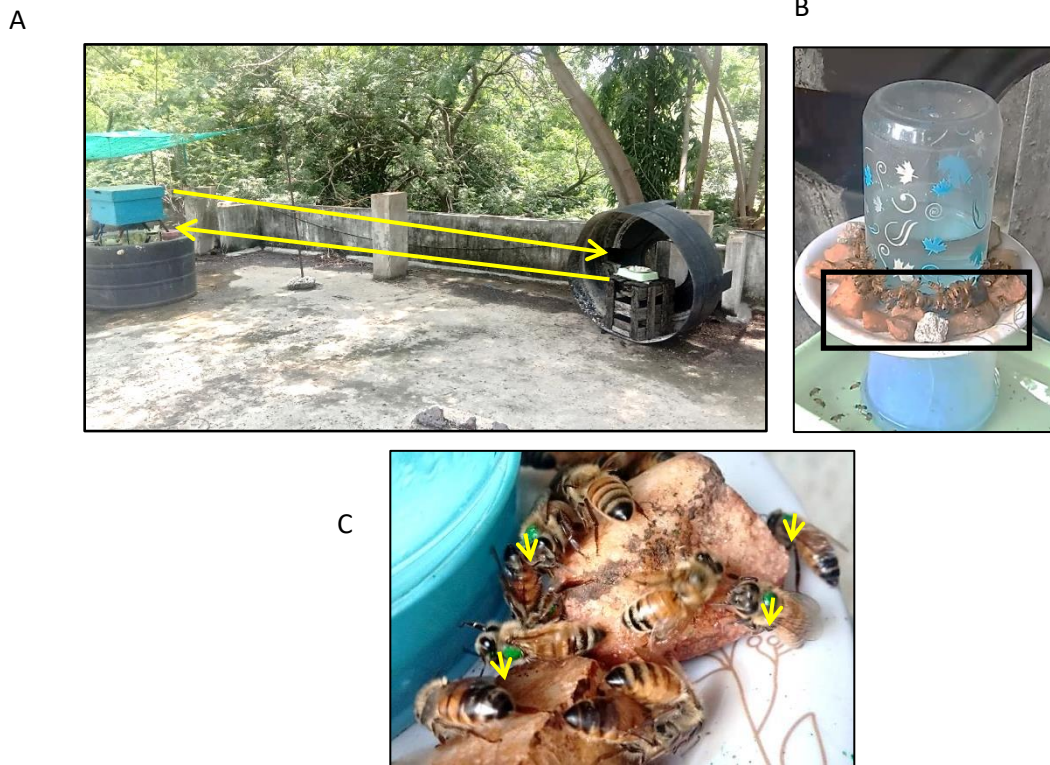


Fig 5: A. Position of the honey bee box (blue) and the feeder, the distance the foragers covered is indicated by the yellow arrows. B. *A. mellifera* foragers visiting the sucrose feeder (black rectangle), rocks were placed to aid the animal's access to the feeder. C. Foragers visiting the feeder were tagged with testor paint (green) as indicated by the yellow arrows on the thorax.

2) Bee Behavior

The collected bees were placed in a glass chamber of dimensions 30cm x 27 cm (Fig 6A). The glass chamber was divided into three compartments using opaque ground glass. A single bee was placed in each section with a damp cotton ball to maintain the humidity in the chamber. The

temperature and relative humidity were maintained at 25°C and $\pm$ 60% RH. An exhaust fan placed above the chamber removed the traces of unwanted odor in the unit.

i) *Apis dorsata*

In one set of experiments foragers were collected from the flowers without or along with the flower, they were foraging on. We observed if the presence of the flower affected the bee's mobility and survival. In the second set of behavior experiments, foragers and honey bees collected directly from the hive were placed in the compartments. In the second set of behavior experiments, foragers and honey bees collected directly from the hive were placed in the compartments. In the third set of experiments animals used were foragers collected from flowers, provided with 30% sucrose feed in the compartment. The animals were transferred directly to the respective chambers post collection with no anesthetization via cooling to avoid any interference with their natural response to capture and isolation. Video recordings were made every hour for a minimum period of 5 minutes. The recordings were made for two hours and video analysis was done by measuring the periods of inactivity for each of the four categories of bees. The survival status of the bees at the end of two hours was also recorded.

ii) *Apis mellifera*

One factor that was unknown in the previous set of experiments using *A. dorsata* was the extent to which the bees were exhausted by flight when we catch them from the foraging location. Given that death was much less in bees collected from the hive, which could be a mixture of nonforagers the exhaustion resulting from flying to the floral sources could be a factor in inducing death.

We were able to circumvent this problem using *A. mellifera* by setting up a feeder close to the hive. With this, we could distinguish foragers from the others in the hive as well because we marked them with paint at the feeding site. In the first set of experiments, we placed a forager collected from the feeder, a forager collected from the hive, and a young hive bee in the glass chambers. In the second set, we placed feeder foragers and feeder foragers that were anesthetized at 4°C for 10 mins before placing them into the chambers. The cooling/anesthetization set of experiments were carried out to check for the role short term memory played in the capture and isolation-induced behavior and mortality in *A. mellifera* foragers (Margulies et al., 2005).

iii) Mounted bees

Using *A. mellifera*, a set of bees foragers and hive and were harnessed into plastic holders (Fig 6B). The mounted bees were kept in the unit where the glass chamber was placed and all the parameters were maintained the same for both the free-flying bees in the glass chamber and the mounted bees. These bees were cooled before being mounted and so could be compared with the bees that were cooled before putting in the chambers.

3) Statistics and data analysis

Video recordings were made for two hours from the time of placing in the glass chamber and analysis was done by measuring the periods of inactivity of each of the four categories of bees. MS Excel spreadsheets were created for the rate of inactivity and 2 min time bins were made for the time span of each video recording. The data was collated and plots were made. One way ANOVA with post hoc t-test assuming equal variances was used to compare the rate of inactivity and the percentage mortality.

4) **Electrophysiology**

Electroretinogram recordings were made from three groups of foragers (Fig 7B), i) Foragers that were collected from the feeder and allowed to free fly in the glass chamber, ii) Foragers collected from the feeder and cooled for ten minutes and then allowed to free fly in the chamber iii) Foragers from the feeder caught and mounted, which has undergone cooling before mounting, ii) feeder foragers cooled for ten minutes and allowed to free fly in the chamber iii) feeder foragers caught and mounted. Animals in the chamber were allowed to fly for 30-45 minutes, following which the free flying bees were collected and mounted. The harnessed bees were kept for 40 mins post mounting before recording commenced. A silver ground wire was inserted into a small incision made in the head capsule and a glass electrode filled with insect saline was guided to the eye lens using a micromanipulator (Fig 7A). The ERG signals were amplified to 200 and digitized using Digidata 1440 at a sampling rate of 10 kHz. A customized LED was placed so as to allow the light to fall on the animal's eye, and a program was custom written in the Axoclamp software which turned the light on at 3 s for 1 s. Ten trials of the program were run and data acquisition was done using the axoclamp software. Axoclamp software was used to adjust and maintain the recording parameters. The electrode impedance was maintained between 2-10 M Ω . An oscilloscope was used to visualize the recording. Data was analyzed using MATLAB software and plots were prepared. The results of this experiment are presented in Appendix D.

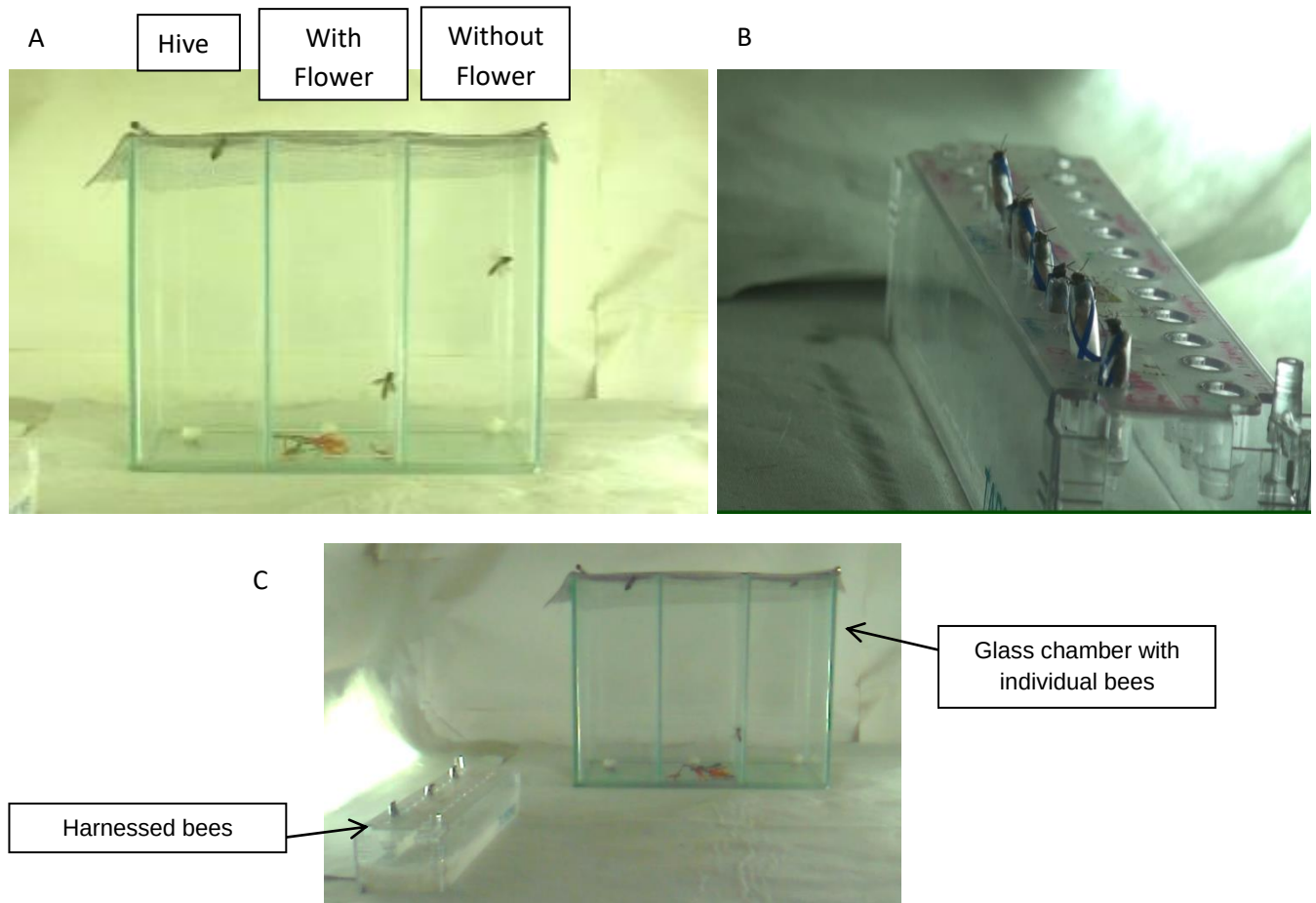


Fig 6: A. *A. dorsata* bees allowed to fly freely in capture isolation. This set up was used for both species of bees and the compartments were randomized for every session B. *A. mellifera* bees, both hive and foragers were also mounted and placed in the same unit as the glass chamber C. The complete set up with the glass chamber and harnessed bees in the same enclosure.

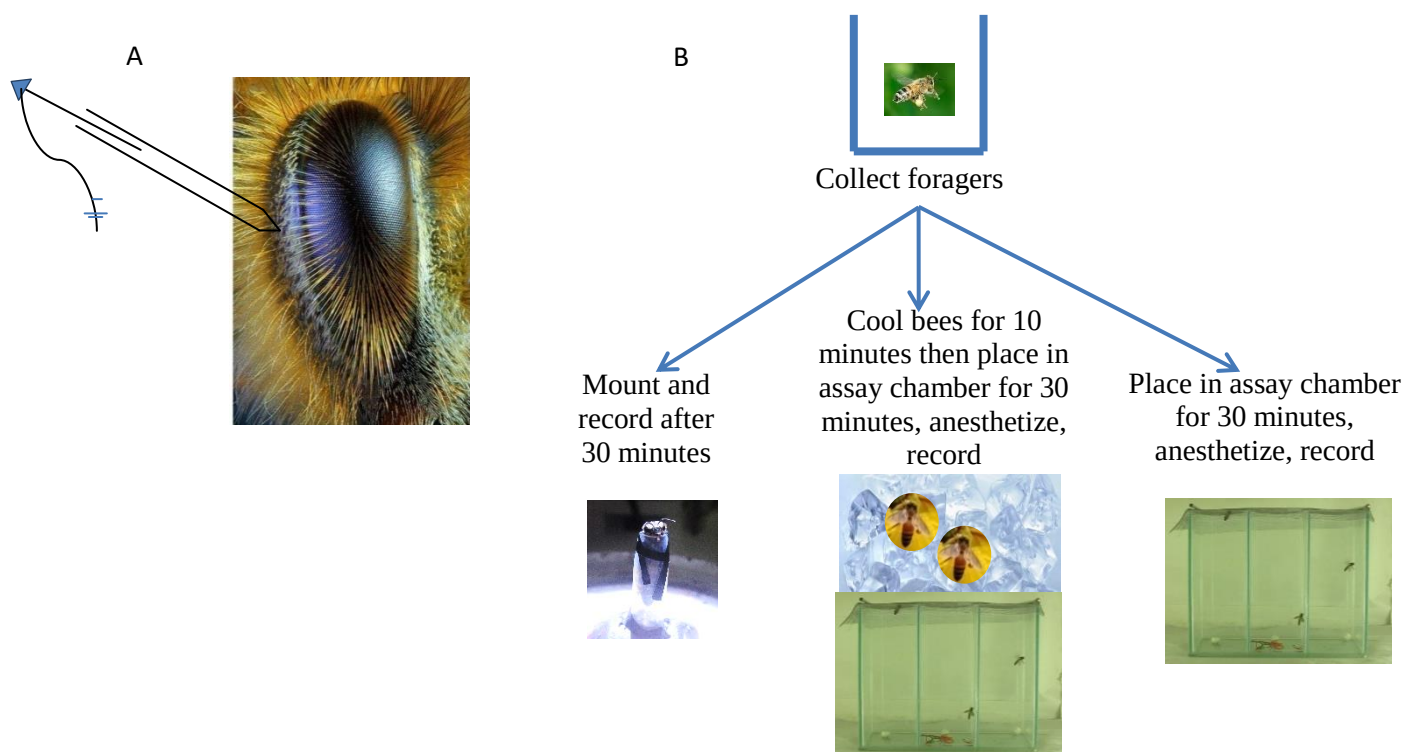


Fig 7: A. Representation of the position of the electrode (black syringe) to carry out electroretinogram recordings B. Flow chart describing the three categories of foragers recordings were made from.

Results

1. *Apis dorsata* foragers exhibit high activity rates upon capture and isolation

Foragers captured at foraging sites and allowed to fly freely in the glass chamber ($n=12$) showed high levels of activity compared to bees collected directly from the hive ($n=7$) and foragers placed with the flower they were foraging on ($n=7$). Total number of bees used for this experiment were $n=26$. We quantified the activity levels as the amount of time the animals flew or walked in the chamber and inactivity time was calculated as the amount of time spent standing or hanging in one position, cleaning, and wing movements while standing were taken as inactivity. The activity rate between the three groups, over the recording period of 2 hrs was

significantly different (Fig 8) with the inactivity rates of hive bees being significantly higher than both groups of foragers [F (2,6)= 49.3, $p= 0.0001$]. Post hoc t-test assuming equal variances revealed that each of the three groups exhibited significant differences in their activity rates, for hive bees ($M= 0.46$, $SD=0.01$) and foragers without flowers ($M=0.31$, $SD=0.01$), $t(4) = 5.96$, $p=0.001$, for foragers with flowers ($M=0.13$, $SD= 0.03$) and without flowers, $t(4)= -4.8$, $p= 0.004$, and for hive bees and foragers with flowers $t(4)= -9.07$, $p= 0.0004$. Our observations indicate that the foragers allowed to fly freely without the flowers exhibited maximum activity rates and the bees collected from the hive exhibited minimum activity rates.

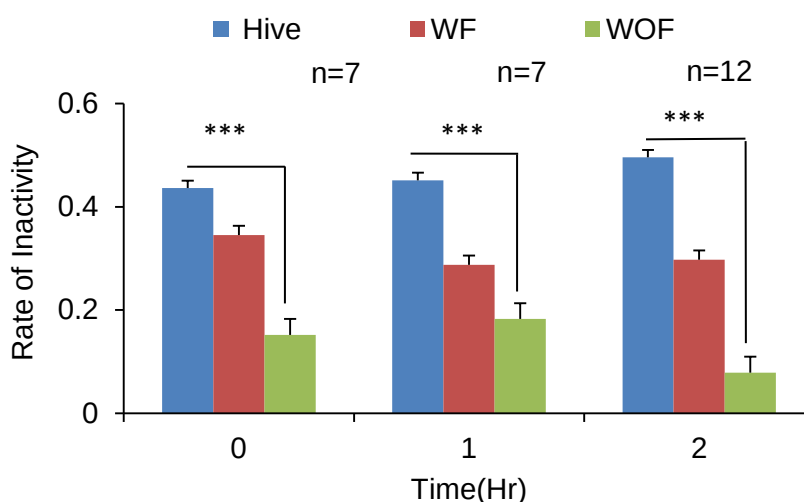


Fig 8: Rate of Inactivity of hive bees, Hive ($n=7$, blue bar), foragers with flower- WF ($n=7$, red bar), and foragers without flower- WOF ($n=12$, green bar) indicated on the y-axis for 2 hrs represented as Time on the x-axis. The level of inactivity was highest for the hive bees at 44% and significantly different from the other two groups (significance represented with three asterisks) and the differences between the three groups was $p=0.0001$. Error bars = mean $\pm$ SEM.

2. Free flying *A. dorsata* foragers exhibit high mortality

Foragers collected from the flowers exhibited higher mortality rates than bees collected from the hives (Fig 9). The foragers collected with the flower on which they were foraging had the highest

percentage mortality at 85% in comparison to the hive bees which showed 14% mortality (Fig 9). The death rate of the hive bees was significantly lesser than that of the foragers left with the foraging flowers [$F(1,12)= 12.5$, $p= 0.004$]. Post hoc t-test also showed the difference between the mortality rates of the foragers with the flowers ($M= 0.85$ $SD=0.07$) and the hive bees ($M=0.14$, $SD=0.07$) was significant, $t(12)= -3.53$, $p=0.002$. The mortality rate of foragers without the foraging flower was not significantly different from that of the foragers with the flowers and the hive bees with percentage mortality of 58%. Maximum onset of death was observed in the second hour post-capture.

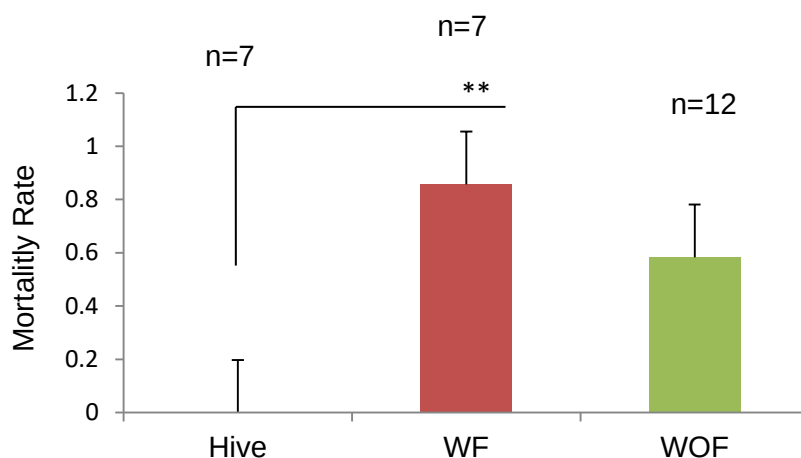
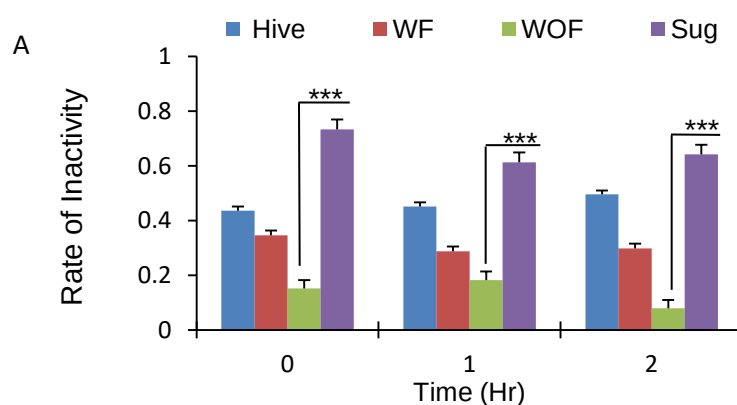


Fig9:A. Mortality Rate of *A. dorsata* foragers placed in the glass chamber with flower- WF (red bar) had the highest mortality at 85 % followed by foragers without flowers - WOF (green bar) at 58% and hive bees - Hive (blue bar) which showed the lowest mortality rate. The mortality rate of WF foragers was significantly higher than the remaining two groups $p=0.004$. Error bars = $\text{mean} \pm \text{SEM}$ ** $p < 0.004$

3. Sucrose substitution reduced the activity and mortality of the *A. dorsata* foragers

In order to observe whether exhaustion and rapid depletion of energy stores could be a reason for the high mortality observed in the foragers, we placed cotton balls dipped in 30% sucrose in the

glass chambers of the foragers allowed to fly freely ($n=7$). The rate of activity and the mortality of foragers supplied with sucrose was compared to that of foragers not supplied with sucrose supplement. Interestingly the foragers supplied with sucrose exhibited lowest activity rates and were significantly more inactive than the hive bees (Fig 10A). One way ANOVA revealed the rate of activity between the four groups was significantly different [$F(3,8)= 68.69$, $p= 4.7 \times 10^{-6}$]. Post hoc t-test showed the inactivity of the sucrose substituted foragers ($M=0.66$, $SD=0.03$) was significantly higher than that of hive bees ($M=0.46$, $SD=0.01$) $t=-4.9$, $p= 0.003$. The mortality rate of the sucrose substituted foragers was lower than that of the two other forager groups and was equal to the mortality rate of the hive bees (Fig 10B). A scatter plot of the mortality of the individual bees in each of the four groups against the inactivity rates of the individual bees in each of the four groups is shown in Fig. 10 C, little correlation was seen between the mortality and inactivity rates. To study whether the inactivity was significantly different between the dead and alive bees in each group we chose the time point of minimum activity observed in the four groups which was the first hour post-capture and we compared the inactivity rates of the dead bees at the end of the recording session to that of the alive bees (Fig. 10D). We observed that the mortality rates were independent of the inactivity rate for the four groups.



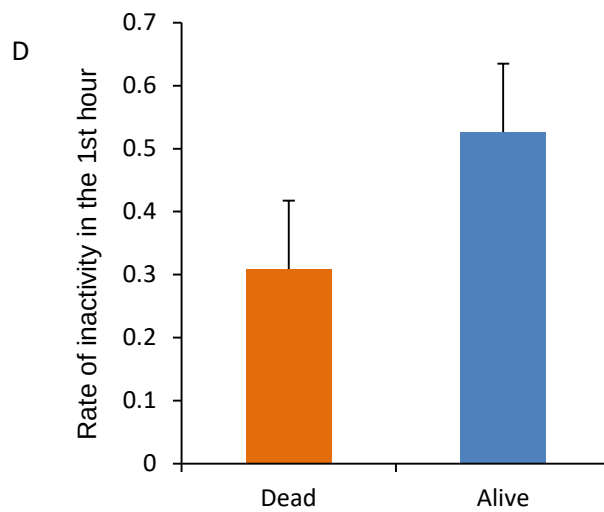
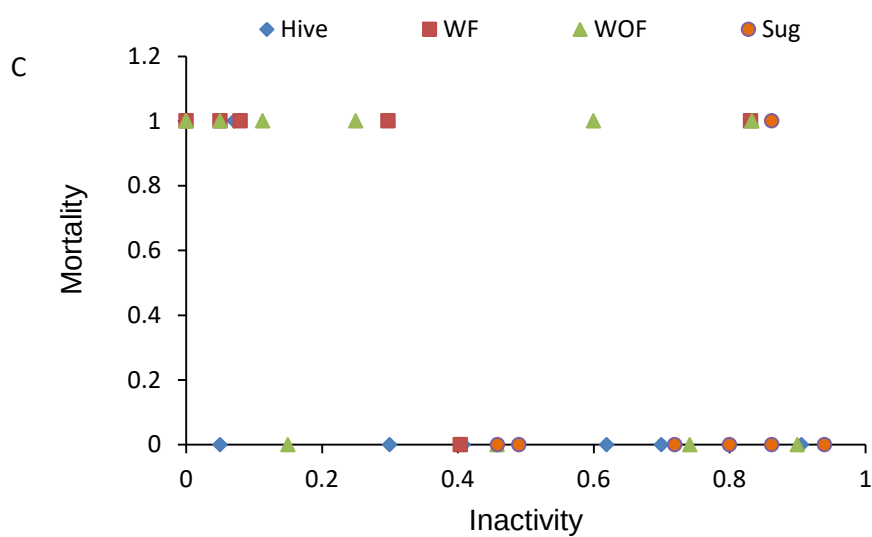
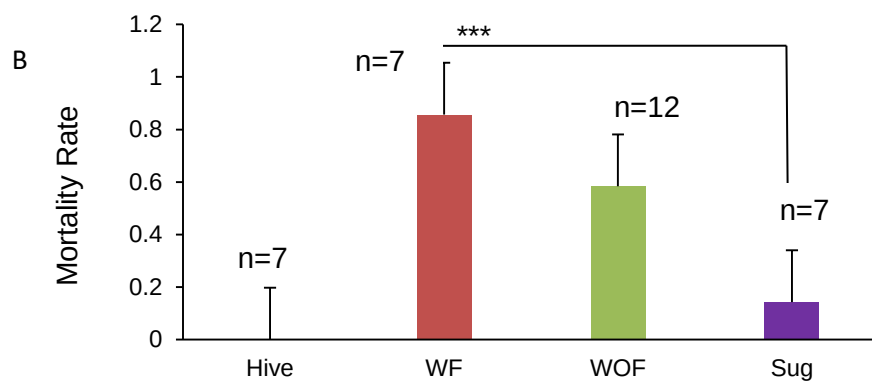


Fig 10: A. On comparing the inactivity rates of the *A. dorsata* sugar fed foragers- Sug (n=7 purple bar) to the three groups previously studied it was observed that the Sug foragers were more inactive than the foragers with flowers- WF(red bar), foragers without flowers- WOF (green bar) and hive bees - Hive (blue bar), $p=4.7 \times 10^{-6}$. B. The sugar fed foragers also had low mortality compared to the mortality of the WF and WOF group and was as much as the Hive bees group C. Scatter plot of the Mortality vs the Inactivity rates for individual bees in each group; Hive (n=7), WF (n=7), WOF (n=12), Sug (n=7). No correlation was seen between the mortality and inactivity of each bee within groups and between groups D. Comparison of the rate of inactivity seen in the 1st hour post capture between the dead bees (Inactivity D)- WF(n=6) , WOF (n=8), Hive(n=0), Sug (n=1) and alive bees (Inactivity A)- WF(n=1) , WOF (n=4), Hive(n=7), Sug (n=6) . No significant difference was seen between the inactivity rates of the dead and alive bees with both types of bees exhibiting similar activity in the 1st hr post capture. Error bars= mean $\pm$ SEM***P<0.001

4. *Apis mellifera* and *Apis dorsata* foragers behave similarly under conditions of capture and isolation

In the experiments using *A. dorsata* bees, we were unable to control a number of parameters, including, distance of hive from the foraging site, the age, and cast of bees collected from the hive. *A. mellifera* provided us with better control over the distance of the feeding site from the hive as we constructed an artificial feeder placed 3mts from the hive box. This allowed us to specifically tag the foragers which gave us the ability to select foragers from inside the hive box. Our behavior results showed that the foragers in the hive box and the foraging foragers had similar activity and mortality rates. Similarly, the unmarked bees collected from inside the hive exhibited significantly higher inactivity and lower mortality compared to the feeder and hive foragers (Fig 11 A) [$F(2,6)= 26.34$, $p= 0.001$]. Post hoc t-test assuming equal variances revealed that the difference between the activity rates of unmarked hive bees ($M=0.3$, $SD=0.02$) was

significantly lower than that of feeder foragers (FF) ($M=0.05$, $SD=0.03$) and hive foragers (HF) ($M=0.01$, $SD=0.01$), $t_{FF} = 4.8$, $p=0.004$ and $t_{HF} = 7.8$, $p= 0.0007$ respectively. The mortality rate of two groups of the free flying category of bees (FF, HF) was significantly higher than that of the same collection of bees harnessed and placed in the unit with the glass chamber [$F(4,58)=16.3$, $p=5.2 \times 10^{-9}$]. Harnessing the bees significantly reduced the mortality rate in both foragers and non foragers (Fig 11 B).

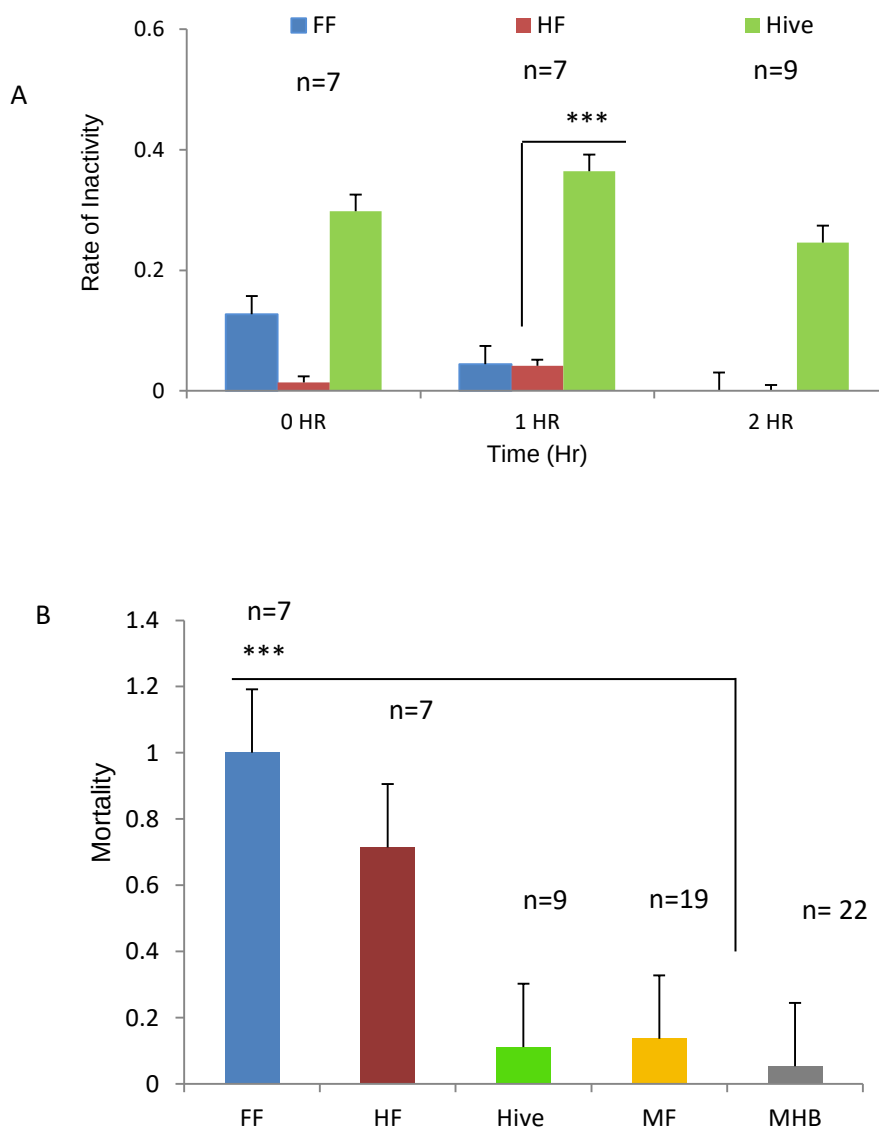


Fig 11: A. Unmarked hive bees- Hive (n=9 green bar) exhibit higher inactivity compared to feeder foragers- FF (n=7 blue bar) and hive foragers- HF (n=7 red bar) as observed over two hours B. A

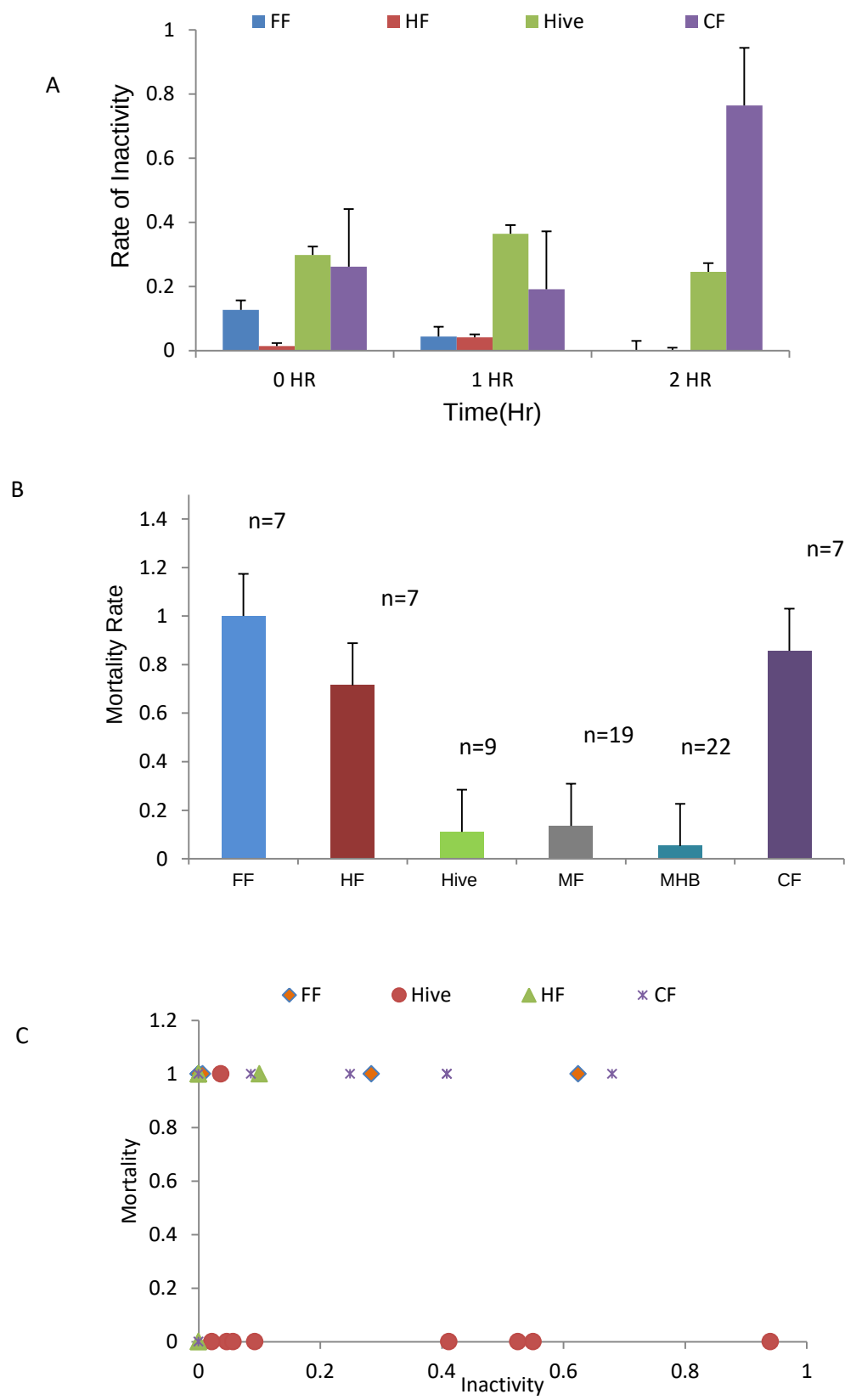
comparison of the mortality rates shows that both mounted foragers- MF (n=19 yellow bar) and mounted hive bees- MHB (n=22 gray bar) had lower mortality rates 13% and 5% respectively than FF and HF which exhibited 100% and 71% mortality respectively. “Hive” bees had a mortality rate of 11%. Error bars= mean $\pm$ SEM ***p<0.001

5. **A. mellifera foragers, cooled and allowed to free fly have high mortality and activity**

We checked whether cooling the foragers at 4°C would alter the activity or mortality. The cooled foragers exhibited activity similar to the feeder foragers and hive foragers. The onset of death in the (n=7) cooled bees occurred by the first half of the second hour hence the activity rates quantified towards the end of the analysis was for one bee. The low activity seen for the cooled forager at the 2nd hr was hence for one bee that stayed alive out of the seven bees (Fig 12A and 12B). A scatter plot of the rates mortality vs the inactivity rates of individual bees in the four categories of free flying bees showed no correlation between the two parameters (Fig 12C). Further in order to look for any significant role played by activity rates we compared the inactivity of the dead bees to that of the alive bees in each of the four groups by choosing the time point of minimum inactivity and lowest mortality (1st hr post-capture) and we observed that both the inactivity rates of both dead and alive bees were not significantly different (Fig 12 D).

Discussion

Our results show that in both species *A. dorsata* and *A. mellifera*, the foragers exhibit high mortality when captured and isolated. The onset of mortality was observed to be within an hour of capture, indicating the rapid activation of the stress pathways and mobilization of the energy



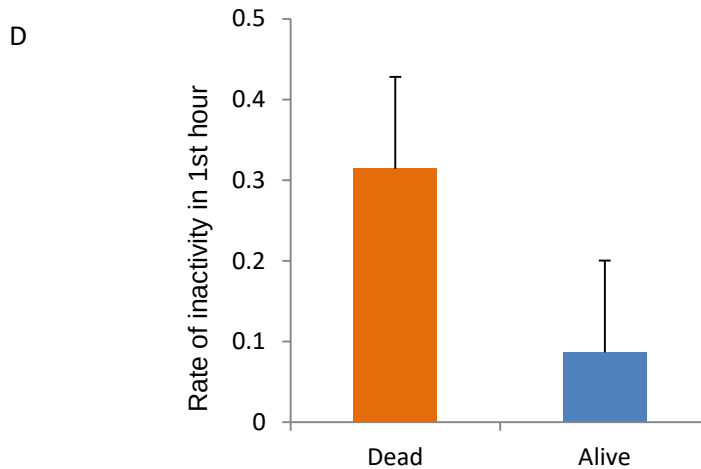


Fig 12: A. Foragers when cooled and allowed to fly freely –CF (n=7 purple bar) were less active than the feeder foragers-FF (n=7 blue bar) and hive foragers- HF(n=7 red bar) placed in the chamber immediately post capture but more active than the unmarked hive bees- Hive (n=9 green bar). The difference in activity of the cooled foragers was not significantly different from the remaining three groups B. The mortality of CF (85%) was as much as the mortality of the FF (100%) and the HF (71%) groups C. The scatter plot of the mortality rates against the inactivity rates of each bee in the four different groups; FF (n=7 orange diamond), HF (n=7 green triangle), Hive (n=9 red dot) and CF (n= 7 blue asterisk). No correlation was observed between the inactivity rates and the mortality D. The comparison of the inactivity rates of the dead bees(Inactivity D- blue bar); FF (n=7) HF(n=6) Hive (n=1)CF (n=6) and alive bees (Inactivity A- red bar); FF (n=0) HF (n=1)Hive (n=8) CF(n=1) was made taking the time point where minimum inactivity was observed (1sthr). There was no significant difference between the inactivity rates of the alive and dead bees in the groups (P=0.23).

resources. The possibility of the foraging flower reducing the stress was ruled out by our behavioral experiment where the foragers when placed in the chamber with the flower they were foraging on, had the same level of mortality as the foragers separated from the flowers during capture. In comparison to the foragers with and without the flowers, the bees collected directly

from the hive exhibited lower mortality. We also looked for the possibility of high levels of activity (flying, escape activity, and walking) being the reason behind the high mortality since constant activity might result in exhaustion and hence death. However, we observed that all the categories of collected bees were active with brief spells of inactivity hence we were unable to find a correlation between activity levels and the high mortality seen in the foragers.

To further understand the behavior of the foragers in isolation we supplied a set of *A. dorsata* foragers with sucrose supplement and interestingly these set of animals exhibited low activity levels and low mortality levels. This result confirmed that the foragers when captured rapidly initiate a series of stress responses that possibly mobilize their energy reserves and cell stress responses which results in the animal's death within two hours post-capture. In the forager studies carried out in *A. dorsata* and we were unable to control a number of parameters including the possibility of the foragers dying from exhaustion given the distance of their hive from the floral sources, and the age of the honey bees collected from the open hives. Our result using *A. dorsata* hive bees indicates age-specific behavior, however, we were unable to differentiate between the unmarked hive bees and older hive bees when collected. These initial results led us to a few hypotheses i) the behavior exhibited by the foragers is an age-dependent behavior ii) the forager is aware of the foraging activity and capture causes the animal to become stressed and want to return to the hive iii) the animal is already exhausted from flying a certain distance to the foraging site and this exhaustion is amplified by the animals flying and motile activity upon capture iv) if that is the case, a forager resting in the hive should behave similarly to a young, well-fed hive-bee.

To answer these open questions we used *A. mellifera* bees. By setting up a feeder close to the hive we eliminated the cause for death in the foragers to be due to exhaustion from flying long

distances. We also tagged the bees visiting the feeder and hence were able to collect resting foragers from the hive combs. Our results using *A. mellifera* were consistent with the results in *A. dorsata*. Despite being captured from a sucrose feeder where the animals were actively foraging and collecting nectar the, captured foragers exhibited a high level of mortality. The foragers collected from inside the hives also had high mortality rates and the activity levels were not significantly different. We concluded that the behavior seen by the foragers when captured and isolated did not vary between forager collected at the feeder and those collected from the hive. The result was further confirmed by the behavior of the young hive bees that were collected from the hive. Their mortality rates were significantly in the young bees low and this class of bees was significantly inactive when placed in capture. We hypothesized that the result we were observing had to do with the animals being allowed to fly freely and hence carried out a mortality check experiment with harnessed foragers collected at the feeder. As predicted the harnessed foragers had low mortality rates and stayed alive for as long as the harnessed young hive bees, even without being provided sucrose. To check if the inactivity rates of the bees that died and the bees that remained alive could be correlated to the mortality, we pooled the inactivity rates of the dead bees in all the groups and that of the alive bees in all the groups, from a time point when maximum insects remained alive (1st hr post-capture). Our results showed that there was no correlation between the inactivity rates of the dead and alive bees, hence it would seem unlikely that energy depletion solely from overactivity or flying in the chamber could be a reason for the high mortality seen in the foragers of both species.

Physiology studies using foragers have shown that this caste of bees has varying levels of utilizable energy sources compared to young hive bees (Blatt and Roces, 2001). Studies have also shown that learning and memory in honey bees are energetically demanding processes (Jaumann

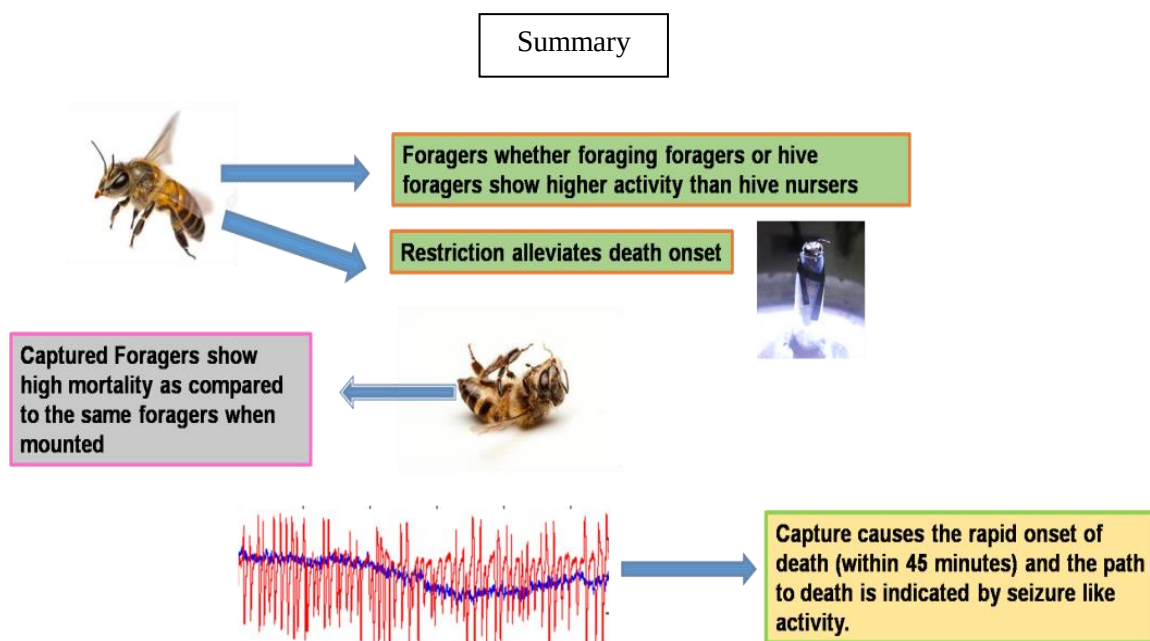
et al., 2013). We then attempted to study the relationship between short term memory and the behavior we had observed in the foragers so far. To do this we cooled the foragers for ten minutes and released the cooled foragers into the glass chamber. Cooling the honey bees for variable periods has been shown to affect the animal's cognitive abilities, short term memory and reduces the stress of capture and experiment preparation (Chen et al., 2014). Disruption of short term memory did not seem to interfere with their behavior in capture as these set of animals displayed activity and mortality levels as much as the two other sets of foragers. Through our behavior experiments, we were able to conclude that foragers when captured exhibit high levels of activity, which seems stressed in nature and eventually succumb to capture. The series of physiological and behavioral events leading to the rapid onset of death were circumvented by harnessing the animals indicative of the vital role played by the wings and flight in the activation and propagation of the stress in these foragers. Cooling the animal does not alter or modify the effect capture has on the foragers.

Electroretinogram recordings were made from the eye lens of i) harnessed foragers, ii) foragers allowed to fly freely immediately post-capture, and iii) foragers cooled then allowed to fly freely. The recordings showed seizure-like deflections in the baseline activity of foragers that were allowed to fly freely immediately post-capture. However, these deflections were not significantly different from the baseline activity of the other groups of foragers. We will be able to arrive at a conclusion post carrying out electroretinogram recordings from young hive bees and comparing the results to the forager recordings. From these results, however, we were unable to conclude whether the activity we observed was forager specific or stress-related. Metabolomic analysis of the hemolymph of the different sets of bees might also yield answers about the physiological state of the animals under capture stress.

The immunoprotective role played by the JH:Vitellogenin (Vg) ratio in honey bees is well established (Corona et al., 2007; Even et al., 2012). Foragers are known to have higher levels of JH to Vg and these ratios are reversed in unmarked hive bees. JH has been shown to play a role in the job differentiation of the honey bees and high levels of this hormone are crucial to the functioning of honey bee foragers whereas high levels of Vg has been suggested to be immunoprotective for the unmarked workers and queen bees (Corona et al., 2007). A low nutritional state can also activate insulin-like protein receptors in honey bees which reduces the levels of vitellogenin and increases the levels of JH. The high level of JH has been shown to play a role in the mobilization of sugars such as trehalose and is also responsible for increasing the neural plasticity to aid the foraging behaviors of this caste of worker bees (Fahrbach and Robinson, 1996; Wang et al., 2012). The forager's tasks tend to drive them into a state of high energy demand, one of the side effects of high rates of oxidation is the production of reactive oxygen species, which in a regular forager is sequestered out using the general anti-oxidation mechanisms (Li et al., 2018), however, very few studies provide a clear picture of how the stress hormones and the antioxidants are regulated in foragers especially in response to abiotic stressors such as capture.

Honey bees are eusocial in nature and display very complex social behaviors which are very crucial to the survival and functioning of the hive. Each class of honey bees has their form of communication which is usually done via the production of pheromones, proboscis interaction, and antennal touches. The hive is kept organized and functional with each class doing its job mainly by the production of the queen's, queen mandibular pheromone. A forager is primed to fly from the hive to a floral source, return to the hive with the feed, pass on this food, water or propolis to a nurser and communicate the position of the floral source to the foragers in the hive

by doing a waggle dance. The results of our study highlight that when a forager's path is perturbed the perturbation can be potent enough to kill the animal. Colony collapse disorder is one of the prominent reasons for the dramatic loss of honey bee hives. This disorder results from a number of stressors and stimuli, both biotic and abiotic in nature. In the US, thousands of hives are carted to almond fields every year for the purpose of pollination and this process has been suggested to cause a loss in honey bees as the animals usually die in the field and during the transport process. Most of the forager deaths in the field happen due to exhaustion and in isolation. Through this study, we have attempted to understand the behavior and physiology of foragers when they are captured and isolated. We see that the foragers are highly susceptible to being perturbed from their regular pattern and this perturbation results in high mortality. Whether the observed results can be modified by placing more than one animal in capture needs to be studied. Further studies also need to be carried out to understand the interplay between the stress hormones, neurotransmitters, and reactive oxygen species (ROS) production in stressed honey bees. Much work needs to be done to study the change in behavior of foragers when faced with an abiotic stressor.



SUMMARY AND CONCLUSION

Summary and Conclusion

Honey bees are vital to our ecosystem, providing us with important pollination services that are responsible for pollinating nearly 70% of our crops. Honey bee foragers have been established as a useful learning and memory model that under controlled laboratory conditions can be trained in variations of Pavlovian conditioning. In honey bees, olfactory conditioning is a highly reproducible and robust form of Pavlovian conditioning. Bees trained in this way exhibit all three variations in the retention of the learnt information, namely short-term memory, mid-term memory, and long-term memory. At the neuronal level, the olfactory circuit of the honey bee *A. mellifera* is well established and a digital atlas of the honey bee standard brain is available (Brandt et al., 2005). While foraging the honey bee encounters myriad smells and must navigate towards the odors that lead them to the right floral patches. The forager must also form a long term memory of the patch and the odors associated so as to be able to revisit the patch. In the present study, we attempted to examine facets of honey bee behavior at the behavioral, neuronal, and physiological levels. We also looked at aspects that could act as abiotic stressors for foraging honey bees.

Each brain lobe plays a crucial role in the processing of odor information and memory storage. Neuronal tract-tracing and fills of the honey bee olfactory circuit have shown no contralateral connections between the two lobes (Rössler and Brill, 2013). However behavioral studies had shown that upon olfactory conditioning with one antenna, transfer of both the olfactory information and the odor identity takes place temporally to the untrained side (Sandoz and Menzel, 2001). Using electrophysiology and behavior we attempted to look for the bilateral transfer of olfactory information in two species of honey bees, *A. dorsata* and, *A. mellifera*. We were able to show at the level of behavior and physiology no transfer of olfactory information

takes place in both species. Further, each lobe works independent of the other when it comes to olfactory information processing. We also provide evidence that *A. dorsata* exhibits a behavioral response (PER) that can be predicted by the learning and retention capacity of each brain lobe when it functions independently.

The past 2 decades have seen a dramatic decline in the number of honey bee hives across the globe. Multiple stressors such as pesticides have been shown to interfere with the normal functioning of the foraging activities of the bees, especially at the neuronal level. To this end, we studied the effect of acute doses of a biopesticide formulation DOR *Bt-1* on the learning and memory of the honey bees. This formulation is used extensively in the Telangana region of the country and very little information about the effect this toxin has on honey bee cognition is available. The DOR *Bt-1* formulation neutralized a target pest *Achea janata* but caused no mortality to the honey bee foragers fed high (1mg/ml) and low (0.2mg/ml) doses of the toxin. The foragers exposed to the two levels of toxin exhibited normal learning and retention. DOR BT-1 did not interfere with the normal learning, mid-term memory and long-term memory processes in *A. mellifera* foragers.

Abiotic stressors such as sudden climate change, pollution have been shown to play a role in the decline in the hive numbers. We studied the effect of capture and isolation on *A. dorsata* and *A. mellifera* honey bees. In both species, we observed high mortality in the captured foragers in comparison to younger hive bees. The mortality rate was significantly reduced by harnessing the foragers. However, the activity levels of the groups of bees were not significantly different leading us to ask why the foragers alone exhibit high mortality upon capture and isolation? The foragers provided with a sucrose substitute in the chamber showed low mortality equal to the younger hive bees and harnessed bees. This indicates that the captured foragers allowed to fly

freely are driven into an energy-depleted state and are replenished by the sucrose substitute. ERG recordings (Appendix D) revealed an increase of deflections in the baseline activity of the captured foragers, however this change in the baseline rate was not found to be significant enough to arrive at a conclusion. It is plausible that the foragers enter a seizure-like state before the onset of death in isolated capture.

Conclusion

The independent working of each brain lobe when it comes to olfactory information processing is evolutionarily conserved in two species of honey bees *A. dorsata* and *A. mellifera*. The significance of have two independently functioning brain lobes might have to do with the theory of redundancy and reliability (Glassman, 1987). Our study shows that in acute doses biopesticides might have an advantage over the regularly used field pesticides such as neonicotinoids. Whether chronic exposure to the biopesticides and locally used BT formulations can affect the learning and memory in honey bee foragers still needs to be examined. Further how does chronic exposure to the biopesticide formulation affect native, wild species of honey bees such as *A. dorsata*? Our observations from the capture and isolation experiments show that honey bee foragers are not robust enough to combat simple abiotic stressors such as perturbation from their navigation path. The reasons behind this high mortality rate might also have to do with the hive environment and the role communication plays in the health and survival of honey bees. The study highlights the need to understand forager behavior in response to abiotic stressors, as this could provide a guideline to the understanding of whether a hive might be susceptible to collapse.

REFERENCES

References

- Aben, B., Stapert, S., and Blokland, A.(2012).**About the Distinction between Working Memory and Short-Term Memory.*Front. Psychol*, 3. <https://doi.org/10.3389/fpsyg.2012.00301>
- Aboitiz, F., and Montiel, J. (2003).** One hundred million years of interhemispheric communication: The history of the corpus callosum. *Braz. J. Med. Biol. Res*, 36(4), 409–420. <https://doi.org/10.1590/S0100-879X2003000400002>
- Ache, B. W., and Young, J. M. (2005).** Olfaction: Diverse Species, Conserved Principles. *Neuron*, 48(3), 417–430. <https://doi.org/10.1016/j.neuron.2005.10.022>
- Agarwal, M., Guzmán, M. G., Morales-Matos, C., Díaz, R. A. D. V., Abramson, C. I., and Giray, T. (2011).**Dopamine and Octopamine Influence Avoidance Learning of Honey Bees in a Place Preference Assay. *PLoS.One*, 6(9), e25371. <https://doi.org/10.1371/journal.pone.0025371>
- Ai, H. (2009).**Vibration-processing interneurons in the honeybee brain.*Front. Syst. Neurosci.* <https://doi.org/10.3389/neuro.06.019.2009>
- Arien, Y., Dag, A., Zarchin, S., Masci, T., and Shafir, S. (2015).** Omega-3 deficiency impairs honey bee learning. *Proc. Natl. Acad. Sci. U.S.A*, 112(51), 15761–15766. <https://doi.org/10.1073/pnas.1517375112>
- Aso, Y., Sitaraman, D., Ichinose, T., Kaun, K. R., Vogt, K., Belliart-Guérin, G., Plaçais, P.-Y., Robie, A. A., Yamagata, N., Schnaitmann, C., Rowell, W. J., Johnston, R. M., Ngo, T.-T. B., Chen, N., Korff, W., Nitabach, M. N., Heberlein, U., Preat, T., Branson, K. M., Tanimoto, H., Rubin, G. M. (2014).**Mushroom body output neurons encode valence and guide memory-based action selection in *Drosophila*. *eLife*, 3, e04580. <https://doi.org/10.7554/eLife.04580>

- Atkinson, R. C., and Shiffrin, R. M. (1968).** Human Memory: A Proposed System and its Control Processes. *Psychol. Learn. Motiv*, 2, 89–195. Academic Press. [https://doi.org/10.1016/S0079-7421\(08\)60422-3](https://doi.org/10.1016/S0079-7421(08)60422-3)
- Baars, B. J., and Gage, N. M. (Eds.).(2013).** Chapter 9 — Learning and memory. *Fundamentals of Cognitive Neuroscience A beginner's guide*, 253–288. Elsevier Academic Press. <https://doi.org/10.1016/B978-0-12-415805-4.00009-6>
- Baddeley, A. (2010).** Working memory. *Curr. Biol*, 20(4), R136–R140. <https://doi.org/10.1016/j.cub.2009.12.014>
- Baddeley, A. D., and Hitch, G. (1974).** Working Memory. In G. H. Bower (Ed.), *Psychol. Learn. Motiv*, 8, 47–89. Elsevier Academic Press. [https://doi.org/10.1016/S0079-7421\(08\)60452-1](https://doi.org/10.1016/S0079-7421(08)60452-1)
- Banaszkiewicz T. (2010).** Evolution of pesticide use. In: Skibniewska KA, editor. *Influence of the pesticide dump on the environment*. 5. Olsztyn, Poland: University of Warmia and Mazury, 7–18
- Barron, A. B., and Plath, J. A. (2017).** The evolution of honey bee dance communication: A mechanistic perspective. *J. Exp. Biol*, 220(23), 4339–4346. <https://doi.org/10.1242/jeb.142778>
- Barron, A. B., Søvik, E., and Cornish, J. L. (2010).** The Roles of Dopamine and Related Compounds in Reward-Seeking Behavior Across Animal Phyla. *Front. Behav. Neurosci*, 4. <https://doi.org/10.3389/fnbeh.2010.00163>
- Beeman, D. (2013).** Hodgkin-Huxley Model. In D. Jaeger and R. Jung (Eds.), *Encyclopedia of Computational Neuroscience*, 1–13. Springer. https://doi.org/10.1007/978-1-4614-7320-6_127-3
- Belsky, J., and Joshi, N. K. (2019).** Impact of Biotic and Abiotic Stressors on Managed and Feral Bees. *Insects*, 10 (8). <https://doi.org/10.3390/insects10080233>

- Bitterman, M. E., Menzel, R., Fietz, A., and Schäfer, S. (1983).** Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol* , 97 (2), 107–119.
- Blatt, J., and Roces, F. (2001).** Haemolymph sugar levels in foraging honey bees (*Apis mellifera carnica*): Dependence on metabolic rate and in vivo measurement of maximal rates of trehalose synthesis. *J. Exp. Biol*, 204(15), 2709–2716.
- Boitard, C., Devaud, J. M., Isabel, G., and Giurfa, M. (2015).** GABAergic feedback signaling into the calyces of the mushroom bodies enables olfactory reversal learning in honey bees. *Front. Behav. Neurosci*, 9. <https://doi.org/10.3389/fnbeh.2015.00198>
- Bortolotti L, Costa C. Chemical Communication in the Honey Bee Society. (2014).** Chapter-5 In: Mucignat-Caretta C, (Ed.), *Neurobiology of Chemical Communication*. Boca Raton (FL): CRC Press/Taylor and Francis. <https://www.ncbi.nlm.nih.gov/books/NBK200983/>
- Bradford, M. M. (1976)** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*, 72, 248-254. doi:10.1006/abio.1976.9999
- Brandt, R., Rohlfing, T., Rybak, J., Krofczik, S., Maye, A., Westerhoff, M., Hege, H. C., and Menzel, R. (2005).** Three-dimensional average-shape atlas of the honey bee brain and its applications. *J. Comp. Neurol* , 492(1), 1–19. <https://doi.org/10.1002/cne.20644>
- Bravo, A., Gomez, I., Conde, J., Munoz-Garay, C., Sánchez, J., Miranda, R., ...and Soberon, M. (2004).** Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin taminopeptidase N receptor leading to insertion into membrane microdomains. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1667: 38-46.
- Bravo, A., Gill, S. S., and Soberón, M. (2007).** Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon*, 49(4), 423–435. <https://doi.org/10.1016/j.toxicon.2006.11.022>

- Bucher, D., and Anderson, P. A. V. (2015).** Evolution of the first nervous systems – what can we surmise? *J. Exp. Biol*, 218(4), 501–503. <https://doi.org/10.1242/jeb.111799>
- Carter, M., and Shieh, J. C. (2010).** Chapter 4—Electrophysiology. In M. Carter and J. C. Shieh (Eds.), *Guide to Research Techniques in Neuroscience*, 91–118. Elsevier Academic Press. <https://doi.org/10.1016/B978-0-12-374849-2.00004-5>
- Cervantes-Sandoval, I., Martin-Peña, A., Berry, J. A., and Davis, R. L. (2013).** System-Like Consolidation of Olfactory Memories in *Drosophila*. *J. Neurosci*, 33(23), 9846–9854. <https://doi.org/10.1523/JNEUROSCI.0451-13.2013>
- Chai, W. J., Abd Hamid, A. I., and Abdullah, J. M. (2018).** Working Memory From the Psychological and Neurosciences Perspectives: A Review. *Front. Psychol*, 9. <https://doi.org/10.3389/fpsyg.2018.00401>
- Chauhan, V. K., Dhanial, N. K., Chaitanya, R. K., Senthilkumaran, B., and Dutta-Gupta, A. (2017).** Larval Mid-Gut Responses to Sub-Lethal Dose of Cry Toxin in Lepidopteran Pest *Achaea janata*. *Front. Physiol.*, 8, 662. <https://doi.org/10.3389/fphys.2017.00662>
- Chen, Y. M., Fu, Y., Hu, J., and Wang, J. H. (2014).** Effects of cold narcosis on memory acquisition, consolidation and retrieval in honeybees (*Apis mellifera*). *Zool. Res*, 35(2), 118–123. <https://doi.org/10.11813/j.issn.0254-5853.2014.2.118>
- Chittka, L., Geiger, K., and Kunze, J. (1995).** The influences of landmarks on distance estimation of honey bees. *Anim. Behav*, 50(1), 23–31. <https://doi.org/10.1006/anbe.1995.0217>
- Chittka, L., Giurfa, M., and Riffell, J. A. (2019).** Editorial: The Mechanisms of Insect Cognition. *Front. Psychol*, 10. <https://doi.org/10.3389/fpsyg.2019.02751>

- Chou, Y. H., Spletter, M. L., Yaksi, E., Leong, J. C. S., Wilson, R. I., and Luo, L. (2010).** Diversity and Wiring Variability of Olfactory Local Interneurons in the *Drosophila* Antennal Lobe. *Nat. Neurosci*, 13(4), 439–449. <https://doi.org/10.1038/nn.2489>
- Christensen, T. A., Waldrop, B. R., Harrow, I. D., and Hildebrand, J. G. (1993).** Local interneurons and information processing in the olfactory glomeruli of the moth *Manduca sexta*. *J. Comp. Physiol. A*, 173(4), 385–399. <https://doi.org/10.1007/BF00193512>
- Clark, E. by K. A., Shaul, T. R., Clark, B. H. L. and E. by K. A., Shaul, T. R., and Lower, B. H. (2015).** 5.4 The Mysterious Case of Colony Collapse Disorder. In *Environmental Science Bites*. The Ohio State University. <https://ohiostate.pressbooks.pub/sciencebites/chapter/the-mysterious-case-of-colony-collapse-disorder/>
- Clark, R. E. (2018).** A History and Overview of the Behavioral Neuroscience of Learning and Memory. *Curr. Top. Behav. Neurosci*. https://doi.org/10.1007/7854_2017_37
- Collett, T. S. (2019).** Path integration: How details of the honeybee waggle dance and the foraging strategies of desert ants might help in understanding its mechanisms. *J. Exp. Biol*, 222(11). <https://doi.org/10.1242/jeb.205187>
- Corona, M., Velarde, R. A., Remolina, S., Moran-Lauter, A., Wang, Y., Hughes, K. A., and Robinson, G. E. (2007).** Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proc. Natl. Acad. Sci. U.S.A.*, 104(17), 7128–7133. <https://doi.org/10.1073/pnas.0701909104>
- Cowan, N. (2008).** What are the differences between long-term, short-term, and working memory? *Prog. Brain. Res*, 169, 323–338. [https://doi.org/10.1016/S0079-6123\(07\)00020-9](https://doi.org/10.1016/S0079-6123(07)00020-9)
- Cresswell, J. (2014).** On the natural history of neonicotinoids and bees. *Funct. Ecol*, 28(6), 1311–1312. <https://doi.org/10.1111/1365-2435.12319>

- Dacks, A. M., Reisenman, C. E., Paulk, A. C., and Nighorn, A. J. (2010).** Histamine-immunoreactive local neurons in the antennal lobes of the Hymenoptera. *J. Comp. Neurol.*, 518(15), 2917–2933. <https://doi.org/10.1002/cne.22371>
- Dai, P. L., Zhou, W., Zhang, J., Jiang, W. Y., Wang, Q., Cui, H. J., Sun, J. H., Wu, Y. Y., and Zhou, T. (2012).** The effects of Bt Cry1Ah toxin on worker honeybees (*Apis mellifera ligustica* and *Apis cerana cerana*). *Apidologie*, 43(4), 384–391. <https://doi.org/10.1007/s13592-011-0103-z>
- Das, A., Sen, S., Lichtneckert, R., Okada, R., Ito, K., Rodrigues, V., and Reichert, H. (2008).** *Drosophila* olfactory local interneurons and projection neurons derive from a common neuroblast lineage specified by the empty spiracles gene. *Neural. Dev.*, 3(1), 33. <https://doi.org/10.1186/1749-8104-3-33>
- Demmer, H., and Kloppenburg, P. (2009).** Intrinsic Membrane Properties and Inhibitory Synaptic Input of Kenyon Cells as Mechanisms for Sparse Coding? *J. Neurophysiol.*, 102(3), 1538–1550. <https://doi.org/10.1152/jn.00183.2009>
- Deseyn, J., and Billen, J. (2005).** Age-dependent morphology and ultrastructure of the hypopharyngeal gland of *Apis mellifera* workers (Hymenoptera, Apidae). *Apidologie*, 36(1), 49–57. <https://doi.org/10.1051/apido:2004068>
- Devaud, J. M., Papouin, T., Carcaud, J., Sandoz, J. C., Grünewald, B., and Giurfa, M. (2015).** Neural substrate for higher-order learning in an insect: Mushroom bodies are necessary for configural discriminations. *Proc. Natl. Acad. Sci. U.S.A.*, 112(43), E5854–E5862. <https://doi.org/10.1073/pnas.1508422112>
- Dhanial, N. K., Chauhan, V. K., Chaitanya, R. K., and Dutta-Gupta, A. (2019).** RNA-Seq analysis and de novo transcriptome assembly of Cry toxin susceptible and tolerant *Achaea janata* larvae. *Sci. Data*, 6(1), 1–6. <https://doi.org/10.1038/s41597-019-0160-0>

- Doublet, V., Labarussias, M., Miranda, J. R. de, Moritz, R. F. A., and Paxton, R. J. (2015).** Bees under stress: Sublethal doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle. *Environ. Microbiol.*, 17(4), 969–983. <https://doi.org/10.1111/1462-2920.12426>
- Ebbinghaus H. (2013).** Memory: a contribution to experimental psychology. *Ann. Neurosci.*, 20(4), 155–156. <https://doi.org/10.5214/ans.0972.7531.200408>
- Endo, H., Azuma, M., Adegawa, S., Kikuta, S. and Sato, R. (2017) .** Water influx via aquaporin directly determines necrotic cell death induced by the *Bacillus thuringiensis* Cry toxin. *FEBS letters*, 591(1), 56–64.
- Erber, J., Masuhr, T., and Menzel, R. (1980).** Localization of short-term memory in the brain of the bee, *Apis mellifera*. *Physiol. Entomol.*, 5(4), 343–358. <https://doi.org/10.1111/j.1365-3032.1980.tb00244.x>
- Evangelista, C., Kraft, P., Dacke, M., Labhart, T., and Srinivasan, M. V. (2014).** Honeybee navigation: Critically examining the role of the polarization compass. *Philos. Trans. R. Soc. B*, 369(1636), 20130037. <https://doi.org/10.1098/rstb.2013.0037>
- Even, N., Devaud, J. M., and Barron, A. B. (2012).** General Stress Responses in the Honey Bee. *Insects*, 3(4), 1271–1298. <https://doi.org/10.3390/insects3041271>
- Fahrbach, S. E., and Robinson, G. E. (1996).** Juvenile hormone, behavioral maturation, and brain structure in the honey bee. *Dev. Neurosci.*, 18(1–2), 102–114. <https://doi.org/10.1159/000111474>
- Farooqui, T., Robinson, K., Vaessin, H., and Smith, B. H. (2003).** Modulation of Early Olfactory Processing by an Octopaminergic Reinforcement Pathway in the Honeybee. *J. Neurosci.*, 23(12), 5370–5380. <https://doi.org/10.1523/JNEUROSCI.23-12-05370.2003>

- Farris, S. M., Robinson, G. E., and Fahrbach, S. E. (2001).** Experience- and Age-Related Outgrowth of Intrinsic Neurons in the Mushroom Bodies of the Adult Worker Honeybee. *J. Neurosci*, 21(16), 6395–6404. <https://doi.org/10.1523/JNEUROSCI.21-16-06395.2001>
- Filla, I., and Menzel, R. (2015).** Mushroom body extrinsic neurons in the honeybee (*Apis mellifera*) brain integrate context and cue values upon attentional stimulus selection. *J. Neurophysiol*, 114(3), 2005–2014. <https://doi.org/10.1152/jn.00776.2014>
- Gage, S. L., Kramer, C., Calle, S., Carroll, M., Heien, M., and DeGrandi-Hoffman, G. (2018).** *Nosema ceranae* parasitism impacts olfactory learning and memory and neurochemistry in honey bees (*Apis mellifera*). *J. Exp. Biol*, 221(4), jeb161489. <https://doi.org/10.1242/jeb.161489>
- Gauthier, M., and Grünewald, B. (2012).** Neurotransmitter Systems in the Honey Bee Brain: Functions in Learning and Memory. In C. G. Galizia, D. Eisenhardt, and M. Giurfa (Eds.), *Honeybee Neurobiology and Behavior*, 155–169. Springer Netherlands. https://doi.org/10.1007/978-94-007-2099-2_13
- Gazzaniga, M. S. (2000).** Cerebral specialization and interhemispheric communication, Does the corpus callosum enable the human condition? *Brain*, 123(7), 1293–1326. <https://doi.org/10.1093/brain/123.7.1293>
- Gazzaniga, M. S. (2014).** The split-brain: Rooting consciousness in biology. *Proc. Natl. Acad. Sci. U.S.A*, 111(51), 18093–18094. <https://doi.org/10.1073/pnas.1417892111>
- Ghirlanda, S., Frasnelli, E., and Vallortigara, G. (2009).** Intraspecific competition and coordination in the evolution of lateralization. *Philos. Trans. R. Soc. B*, 364(1519), 861–866. <https://doi.org/10.1098/rstb.2008.0227>
- Giese, K. P., and Mizuno, K. (2013).** The roles of protein kinases in learning and memory. *Learn. Mem*, 20(10), 540–552. <https://doi.org/10.1101/lm.028449.112>

- Giurfa, M. (2012).** Social learning in insects: A higher-order capacity? *Front. Behav. Neurosci.*, 6. <https://doi.org/10.3389/fnbeh.2012.00057>
- Giurfa, M., and Sandoz, J. C. (2012).** Invertebrate learning and memory: Fifty years of olfactory conditioning of the proboscis extension response in honey bees. *Learn. Mem.*, 19(2), 54–66. <https://doi.org/10.1101/lm.024711.111>
- Glassman, R. B. (1987).** An hypothesis about redundancy and reliability in the brains of higher species: Analogies with genes, internal organs, and engineering systems. *Neurosci. Biobehav. Rev.*, 11(3), 275–285. [https://doi.org/10.1016/S0149-7634\(87\)80014-3](https://doi.org/10.1016/S0149-7634(87)80014-3)
- Gómez-Moracho, T., Heeb, P., and Lihoreau, M. (2017).** Effects of parasites and pathogens on bee cognition. *Ecol. Entomol.*, 42(S1), 51–64. <https://doi.org/10.1111/een.12434>
- Goulson, D., Nicholls, E., Botias, C., and Rotheray, E. L. (2015).** Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, 347(6229), 1255957–1255957. <https://doi.org/10.1126/science.1255957>
- Grünewald, B. (2003).** Differential expression of voltage-sensitive K⁺ and Ca²⁺ currents in neurons of the honey bee olfactory pathway. *J. Exp. Biol.*, 206(1), 117–129. <https://doi.org/10.1242/jeb.00053>
- Guo, Y., Wang, Z., Li, Y., Wei, G., Yuan, J., Sun, Y., Wang, H., Qin, Q., Zeng, Z., Zhang, S., and Chen, R. (2016).** Lateralization of gene expression in the honeybee brain during olfactory learning. *Sci. Rep.*, 6(1), 34727. <https://doi.org/10.1038/srep34727>
- Gupta, N., and Stopfer, M. (2012).** Functional Analysis of a Higher Olfactory Center, the Lateral Horn. *J. Neurosci.*, 32(24), 8138–8148. <https://doi.org/10.1523/JNEUROSCI.1066-12.2012>
- Gupta, N., and Stopfer, M. (2014).** A temporal channel for information in sparse sensory coding. *Curr. Biol.*, 24(19), 2247–2256. <https://doi.org/10.1016/j.cub.2014.08.021>

- Gutiérrez-Gálvez, A., and Marco, S. (2013).** Study of the Coding Efficiency of Populations of Olfactory Receptor Neurons and Olfactory Glomeruli. In K. C. Persaud, S. Marco, and A. Gutiérrez-Gálvez (Eds.), *Neuromorphic Olfaction*. CRC Press/Taylor and Francis. <http://www.ncbi.nlm.nih.gov/books/NBK298826/>
- Haehnel, M., and Menzel, R. (2012).** Long-term memory and response generalization in mushroom body extrinsic neurons in the honeybee *Apis mellifera*. *J. Exp. Biol*, 215(3), 559–565. <https://doi.org/10.1242/jeb.059626>
- Hammer, M. (1993).** An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature*, 366(6450), 59–63. <https://doi.org/10.1038/366059a0>
- Hammer, M., and Menzel, R. (1998).** Multiple Sites of Associative Odor Learning as Revealed by Local Brain Microinjections of Octopamine in Honeybees. *Learn. Mem*, 5(1), 146–156.
- Hanslmayr, S., Staudigl, T., and Fellner, M. C. (2012).** Oscillatory power decreases and long-term memory: The information via desynchronization hypothesis. *Front. Hum. Neurosci*, 6. <https://doi.org/10.3389/fnhum.2012.00074>
- Harada, T., Takeda, M., Kojima, S., and Tomiyama, N. (2016).** Toxicity and Carcinogenicity of Dichlorodiphenyltrichloroethane (DDT). *Toxicol. Res* 32(1), 21–33. <https://doi.org/10.5487/TR.2016.32.1.021>
- Harano, K. (2020).** Honeybee colonies provide foragers with costly fuel to promote pollen collection. *J. Comp. Physiol. A*, 206(4), 587–595. <https://doi.org/10.1007/s00359-020-01427-5>
- Hejnol, A., Obst, M., Stamatakis, A., Ott, M., Rouse, G. W., Edgecombe, G. D., Martinez, P., Baguña, J., Bailly, X., Jondelius, U., Wiens, M., Müller, W. E. G., Seaver, E., Wheeler, W. C., Martindale, M. Q., Giribet, G., and Dunn, C. W. (2009).** Assessing the root of bilaterian animals with scalable phylogenomic methods. *Proc. R. Soc. B*, 276(1677), 4261–4270. <https://doi.org/10.1098/rspb.2009.0896>

- Heylen, K., Gobin, B., Billen, J., Hu, T.-T., Arckens, L., and Huybrechts, R. (2008).** Amfor expression in the honeybee brain: A trigger mechanism for nurse-forager transition. *J. Insect Physiol*, 54(10–11), 1400–1403. <https://doi.org/10.1016/j.jinsphys.2008.07.015>
- Holló, G., and Novák, M. (2012).** The manoeuvrability hypothesis to explain the maintenance of bilateral symmetry in animal evolution. *Biol. Direct*, 7, 22. <https://doi.org/10.1186/1745-6150-7-22>
- Homberg, U. (1985).** Interneurons of the central complex in the bee brain (*Apis mellifera*, L.). *J. Insect. Physiol*, 31(3), 251–264. [https://doi.org/10.1016/0022-1910\(85\)90127-1](https://doi.org/10.1016/0022-1910(85)90127-1)
- Horne, J. A., Langille, C., McLin, S., Wiederman, M., Lu, Z., Xu, C. S., Plaza, S. M., Scheffer, L. K., Hess, H. F., and Meinertzhagen, I. A. (2018).** A resource for the *Drosophila* antennal lobe provided by the connectome of glomerulus VA1v. *eLife*, 7, e37550. <https://doi.org/10.7554/eLife.37550>
- Hourcade, B., Muenz, T. S., Sandoz, J. C., Rössler, W., and Devaud, J. M. (2010).** Long-Term Memory Leads to Synaptic Reorganization in the Mushroom Bodies: A Memory Trace in the Insect Brain? *J. Neurosci*, 30(18), 6461–6465. <https://doi.org/10.1523/JNEUROSCI.0841-10.2010>
- Hranitz, J. M., Abramson, C. I., and Carter, R. P. (2010).** Ethanol increases HSP70 concentrations in honeybee (*Apis mellifera* L.) brain tissue. *Alcohol*, 44(3), 275–282. <https://doi.org/10.1016/j.alcohol.2010.02.003>
- Hussaini, S. A., Komischke, B., Menzel, R., and Lachnit, H. (2007).** Forward and backward second-order Pavlovian conditioning in honeybees. *Learn. Mem*, 14(10), 678–683. <https://doi.org/10.1101/lm.471307>
- Ibrahim, M. A., Griko, N., Junker, M., and Bulla, L. A. (2010).** *Bacillus thuringiensis*. *Bioeng. Bugs*, 1(1), 31–50. <https://doi.org/10.4161/bbug.1.1.10519>

- Jarriault, D., Fuller, J., Hyland, B. I., and Mercer, A. R. (2018).** Dopamine release in mushroom bodies of the honey bee (*Apis mellifera* L.) in response to aversive stimulation. *Sci. Rep*, 8(1), 1–12. <https://doi.org/10.1038/s41598-018-34460-1>
- Jaumann, S., Scudelari, R., and Naug, D. (2013).** Energetic cost of learning and memory can cause cognitive impairment in honeybees. *Biol. Lett*, 9(4), 20130149. <https://doi.org/10.1098/rsbl.2013.0149>
- Jenkins, P. M., McEwen, D. P., and Martens, J. R. (2009).** Olfactory Cilia: Linking Sensory Cilia Function and Human Disease. *Chem. Senses*, 34(5), 451–464. <https://doi.org/10.1093/chemse/bjp020>
- Jia, H. R., Geng, L. L., Li, Y. H., Wang, Q., Diao, Q. Y., Zhou, T., and Dai, P. L. (2016).** The effects of Bt Cry1Ie toxin on bacterial diversity in the midgut of *Apis mellifera ligustica* (Hymenoptera: Apidae). *Sci. Rep*, 6(1), 1–8. <https://doi.org/10.1038/srep24664>
- Jurat-Fuentes, J. L., and Adang, M. J. (2007).** A proteomic approach to study Cry1Ac binding proteins and their alterations in resistant *Heliothis virescens* larvae. *J. Invertebr. Pathol*, 95(3), 187–191. <https://doi.org/10.1016/j.jip.2007.01.008>
- Kaur, R., Surala, M., Hoyer, S., Grössmann, N., Grimm, A., Timaeus, L., Kallina, W., and Hummel, T. (2019).** Pioneer interneurons instruct bilaterality in the *Drosophila* olfactory sensory map. *Sci. Adv*, 5(10), eaaw 5537. <https://doi.org/10.1126/sciadv.aaw5537>
- Kay, L. M. (2014).** Circuit oscillations in odor perception and memory. *Prog. Brain. Res*, 208, 223–251. <https://doi.org/10.1016/B978-0-444-63350-7.00009-7>
- Kessler, S., Tiedeken, E. J., Simcock, K. L., Derveau, S., Mitchell, J., Softley, S., Stout, J. C., and Wright, G. A. (2015).** Bees prefer foods containing neonicotinoid pesticides. *Nature*, 521(7550), 74–76. <https://doi.org/10.1038/nature14414>

- Kirschner, S., Kleineidam, C. J., Zube, C., Rybak, J., Grünewald, B., and Rössler, W. (2006).** Dual olfactory pathway in the honeybee, *Apis mellifera*. *J. Comp. Neurol*, 499(6), 933–952. <https://doi.org/10.1002/cne.21158>
- Kloppenburg, P., and Nawrot, M. P. (2014).** Neural Coding: Sparse but On Time. *Curr. Biol*, 24(19), R957–R959. <https://doi.org/10.1016/j.cub.2014.08.041>
- Komischke, B., Sandoz, J.-C., Malun, D., and Giurfa, M. (2005).** Partial unilateral lesions of the mushroom bodies affect olfactory learning in honeybees *Apis mellifera* L. *European J. Neurosci*, 21(2), 477–485. <https://doi.org/10.1111/j.1460-9568.2005.03879.x>
- Kraft, P., Evangelista, C., Dacke, M., Labhart, T., and Srinivasan, M. V. (2011).** Honeybee navigation: Following routes using polarized-light cues. *Philos. T. R. Soc. B*, 366(1565), 703–708. <https://doi.org/10.1098/rstb.2010.0203>
- Kristan, W. B. (2016).** Early evolution of neurons. *Curr. Biol*, 26(20), R949–R954. <https://doi.org/10.1016/j.cub.2016.05.030>
- Kropf, J., Kelber, C., Bieringer, K., and Rössler, W. (2014).** Olfactory subsystems in the honeybee: Sensory supply and sex specificity. *Cell. Tissue. Res*, 357(3), 583–595. <https://doi.org/10.1007/s00441-014-1892-y>
- Kropf, J., and Rössler, W. (2018).** In-situ recording of ionic currents in projection neurons and Kenyon cells in the olfactory pathway of the honeybee. *PLoS ONE*, 13(1). <https://doi.org/10.1371/journal.pone.0191425>
- Lanoix, D., and Plusquellec, P. (2013).** Adverse effects of pollution on mental health: The stress hypothesis. *OA Evidence-Based Medicine*, 1(1). <https://doi.org/10.13172/2053-2636-1-1-572>

- Laska, M., Galizia, C. G., Giurfa, M., and Menzel, R. (1999).** Olfactory Discrimination Ability and Odor Structure–Activity Relationships in Honeybees. *Chem. Senses*, 24(4), 429–438. <https://doi.org/10.1093/chemse/24.4.429>
- Leadbeater, E., and Chittka, L. (2009).** Bumble-bees learn the value of social cues through experience. *Biol. Lett.*, 5(3), 310–312. <https://doi.org/10.1098/rsbl.2008.0692>
- Li, G., Zhao, H., Liu, Z., Wang, H., Xu, B., and Guo, X. (2018).** The Wisdom of Honeybee Defenses Against Environmental Stresses. *Front. Microbiol.*, 9. <https://doi.org/10.3389/fmicb.2018.00722>
- Li, Q., Ha, T. S., Okuwa, S., Wang, Y., Wang, Q., Millard, S. S., Smith, D. P., and Volkan, P. C. (2013).** Combinatorial Rules of Precursor Specification Underlying Olfactory Neuron Diversity. *Curr. Biol.*, 23(24), 2481–2490. <https://doi.org/10.1016/j.cub.2013.10.053>
- Lichtneckert, R., and Reichert, H. (2007).** 1.19—Origin and Evolution of the First Nervous System. In J. H. Kaas (Ed.), *Evolution of Nervous Systems*, 289–315. Academic Press. <https://doi.org/10.1016/B0-12-370878-8/00157-9>
- Lin, A. C., Bygrave, A., de Calignon, A., Lee, T., and Miesenböck, G. (2014).** Sparse, Decorrelated Odor Coding in the Mushroom Body Enhances Learned Odor Discrimination. *Nat. Neurosci.*, 17(4), 559–568. <https://doi.org/10.1038/nn.3660>
- Lin, T., Li, C., Liu, J., Smith, B. H., Lei, H., and Zeng, X. (2018).** Glomerular Organization in the Antennal Lobe of the Oriental Fruit Fly *Bactrocera dorsalis*. *Front. Neuroanat.*, 12. <https://doi.org/10.3389/fnana.2018.00071>
- Liu, C., Plaçais, P. Y., Yamagata, N., Pfeiffer, B. D., Aso, Y., Friedrich, A. B., Siwanowicz, I., Rubin, G. M., Preat, T., and Tanimoto, H. (2012).** A subset of dopamine neurons signals reward for odour memory in *Drosophila*. *Nature*, 488(7412), 512–516. <https://doi.org/10.1038/nature11304>

- Lu, C., Warchol, K. M., and Callahan, R. A. (2014).** Sub-lethal exposure to neonicotinoids impaired honey bees winterization before proceeding to colony collapse disorder. *Bull. Insectology*, 67, 125.
- Malun, D. (1998).** Early Development of Mushroom Bodies in the Brain of the Honeybee *Apis mellifera* as Revealed by Brd U Incorporation and Ablation Experiments. *Learn. Mem*, 5(1), 90–101.
- Malun, D., Giurfa, M., Galizia, C. G., Plath, N., Brandt, R., Gerber, B., and Eisermann, B. (2002).** Hydroxyurea-induced partial mushroom body ablation does not affect acquisition and retention of olfactory differential conditioning in honeybees. *J. Neurobiol*, 53(3), 343–360. <https://doi.org/10.1002/neu.10119>
- Margulies, C., Tully, T., and Dubnau, J. (2005).** Deconstructing Memory in *Drosophila*. *Curr Biol*, 15(17), R700–R713. <https://doi.org/10.1016/j.cub.2005.08.024>
- Mathiasson, M. E., and Rehan, S. M. (2019).** Status changes in the wild bees of north-eastern North America over 125 years revealed through museum specimens. *Insect. Conserv. Diver*, 12(4), 278–288. <https://doi.org/10.1111/icad.12347>
- Matsumoto, Y., Matsumoto, C. S., and Mizunami, M. (2018).** Signaling Pathways for Long-Term Memory Formation in the Cricket. *Front. Psychol*, 9. <https://doi.org/10.3389/fpsyg.2018.01014>
- Matsushima, A. (2018).** A Novel Action of Endocrine-Disrupting Chemicals on Wildlife; DDT and Its Derivatives Have Remained in the Environment. *Int. J. Mol. Sci*, 19(5). <https://doi.org/10.3390/ijms19051377>
- Mattila, H. R., and Smith, B. H. (2008).** Learning and memory in workers reared by nutritionally stressed honey bee (*Apis mellifera* L.) colonies. *Physiol. Behav*, 95(5), 609–616. <https://doi.org/10.1016/j.physbeh.2008.08.003>

- Menzel, R. (2001).** Searching for the Memory Trace in a Mini-Brain, the Honeybee. *Learn. Mem.*, 8(2), 53–62. <https://doi.org/10.1101/lm.38801>
- Menzel, Randolph. (2012).** The honeybee as a model for understanding the basis of cognition. *Nat. Rev. Neurosci.*, 13(11), 758–768. <https://doi.org/10.1038/nrn3357>
- Menzel, R and Benjamin,P. R. (Eds.) (2013).** *Invertebrate Learning and Memory*. Elsevier Associative Press. Amsterdam, London, Heidelberg.
- Menzel, Randolph, Galizia, G., Müller, D., and Szyszka, P. (2005).** Odor Coding in Projection Neurons of the Honeybee Brain. *Chem. Senses*, 30 (suppl_1), i301–i302. <https://doi.org/10.1093/chemse/bjh234>
- Mihrshahi, R. (2006).** The corpus callosum as an evolutionary innovation. *J. Exp. Zool* 306 B (1), 8–17. <https://doi.org/10.1002/jez.b.21067>
- Mizunami, M., Nemoto, Y., Terao, K., Hamanaka, Y., and Matsumoto, Y. (2014).** Roles of Calcium/Calmodulin-Dependent Kinase II in Long-Term Memory Formation in Crickets. *PLoS.One*, 9(9), e107442. <https://doi.org/10.1371/journal.pone.0107442>
- Moroz, L. L. (2015).** Convergent evolution of neural systems in ctenophores. *J. Exp. Biol.*, 218(4), 598–611. <https://doi.org/10.1242/jeb.110692>
- Murre, J. M. J., and Dros, J. (2015).** Replication and Analysis of Ebbinghaus' Forgetting Curve. *PLoS.One*, 10(7). <https://doi.org/10.1371/journal.pone.0120644>
- Namiki, S., and Kanzaki, R. (2016).** Comparative Neuroanatomy of the Lateral Accessory Lobe in the Insect Brain. *Front. Physiol* ,7. <https://doi.org/10.3389/fphys.2016.00244>
- Neumann, P., and Carreck, N. L. (2010).** Honey bee colony losses. *J. Apic. Res.*, 49(1), 1–6. <https://doi.org/10.3896/IBRA.1.49.1.01>
- Nilsen, K.-A., Ihle, K. E., Frederick, K., Fondrk, M. K., Smedal, B., Hartfelder, K., and Amdam, G. V. (2011).** Insulin-like peptide genes in honey bee fat body respond differently to

- manipulation of social behavioral physiology. *J. Exp. Biol*, 214(9), 1488–1497.
<https://doi.org/10.1242/jeb.050393>
- Okada, R., Rybak, J., Manz, G., and Menzel, R. (2007).** Learning-Related Plasticity in PE1 and Other Mushroom Body-Extrinsic Neurons in the Honeybee Brain. *J. Neurosci*, 27(43), 11736–11747. <https://doi.org/10.1523/JNEUROSCI.2216-07.2007>
- Pahl, M., Zhu, H., Tautz, J., and Zhang, S. (2011).** Large Scale Homing in Honeybees. *PLoS.One*, 6(5), e19669. <https://doi.org/10.1371/journal.pone.0019669>
- Palma, L., Muñoz, D., Berry, C., Murillo, J., and Caballero, P. (2014).** *Bacillus thuringiensis* Toxins: An Overview of Their Biocidal Activity. *Toxins*, 6(12), 3296–3325. <https://doi.org/10.3390/toxins6123296>
- Pardo-López, L., Soberón, M., and Bravo, A. (2013).** *Bacillus thuringiensis* insecticidal three-domain Cry toxins: Mode of action, insect resistance and consequences for crop protection. *FEMS.Microbiol. Rev*, 37(1), 3–22. <https://doi.org/10.1111/j.1574-6976.2012.00341.x>
- Pavani, A., Chaitanya, R.K., Chauhan, V.K., Dasgupta, A. and Dutta-Gupta, A., 2015.** Differential oxidative stress responses in castor semilooper, *Achaea janata*. *J. Invertebr. Pathol*, 132, pp.157-164.
- Pfeiffer, K., and Homberg, U. (2014).** Organization and Functional Roles of the Central Complex in the Insect Brain. *Annu.Rev. Entomol*, 59(1), 165–184. <https://doi.org/10.1146/annurev-ento-011613-162031>
- Popov, T., and Szyszka, P. (2020).** Alpha oscillations govern interhemispheric spike timing coordination in the honey bee brain. *Proc. Royal. Soc. B* 287(1921), 20200115. <https://doi.org/10.1098/rspb.2020.0115>
- Ramirez-Romero, R., Desneux, N., Decourtye, A., Chaffiol, A., and Pham-Delègue, M. H. (2008).** Does Cry1Ab protein affect learning performances of the honey bee *Apis mellifera* L.

(Hymenoptera, Apidae)? *Ecotoxicol. Environ. Saf*, 70(2), 327–333.

<https://doi.org/10.1016/j.ecoenv.2007.12.002>

Ramirez-Romero, Ricardo, Chaufaux, J., and Pham-Delègue, M.-H. (2005). Effects of Cry1Ab protoxin, deltamethrin and imidacloprid on the foraging activity and the learning performances of the honeybee *Apis mellifera*, a comparative approach. *Apidologie*, 36(4), 601–611. <https://doi.org/10.1051/apido:2005039>

Ratnieks, F. L. W., and Carreck, N. L. (2010). Clarity on Honey Bee Collapse? *Science*, 327(5962), 152–153. <https://doi.org/10.1126/science.1185563>

Reddy V. P., Rao N. N., Devi P. S. V., Narasu M. L., Kumar V. D. (2012). PCR-based detection of cry genes in local *Bacillus thuringiensis* DOR Bt-1 Isolate. *Pest. Technol*, 6, 79–82.

Reitmayer, C. M., Ryalls, J. M. W., Farthing, E., Jackson, C. W., Girling, R. D., and Newman, T. A. (2019). Acute exposure to diesel exhaust induces central nervous system stress and altered learning and memory in honey bees. *Sci. Rep*, 9(1), 5793. <https://doi.org/10.1038/s41598-019-41876-w>

Rose, R., Dively, G. P., and Pettis, J. (2007). Effects of Bt corn pollen on honey bees: Emphasis on protocol development. *Apidologie*, 38(4), 368–377. <https://doi.org/10.1051/apido:2007022>

Rössler, W., and Brill, M. F. (2013). Parallel processing in the honeybee olfactory pathway: Structure, function, and evolution. *J. Comp. Physiol. A*, 199(11), 981–996. <https://doi.org/10.1007/s00359-013-0821-y>

Ruiz-Trillo, I., and Paps, J. (2016). Acoelomorpha: Earliest branching bilaterians or deuterostomes? *Org. Divers. Evol*, 16(2), 391–399. <https://doi.org/10.1007/s13127-015-0239-1>

Russell, C., Wessnitzer, J., Young, J. M., Armstrong, J. D., and Webb, B. (2011). Dietary Salt Levels Affect Salt Preference and Learning in Larval *Drosophila*. *PLoS.One*, 6(6), e20100. <https://doi.org/10.1371/journal.pone.0020100>

- Rybak, Jürgen. (2012).** The Digital Honey Bee Brain Atlas. In C. G. Galizia, D. Eisenhardt, and M. Giurfa (Eds.), *Honeybee Neurobiology and Behavior: A Tribute to Randolph Menzel*, 125–140. Springer Netherlands. https://doi.org/10.1007/978-94-007-2099-2_11
- Rybak, J., and Menzel, R. (1998).** Integrative properties of the Pe1 neuron, a unique mushroom body output neuron. *Learn. Mem* ,5(1-2), 133–145.
- Rybak, Jürgen, and Menzel, R. (1993).** Anatomy of the mushroom bodies in the honey bee brain: The neuronal connections of the alpha-lobe. *J. Comp. Neurol*,334(3), 444–465. <https://doi.org/10.1002/cne.903340309>
- Sachse, S., and Galizia, C. G. (2002).** Role of inhibition for temporal and spatial odor representation in olfactory output neurons: A calcium imaging study. *J. Neurophysiol*, 87(2), 1106–1117. <https://doi.org/10.1152/jn.00325.2001>
- Sandoz, J.-C. (2001).** Side-Specificity of Olfactory Learning in the Honeybee: Generalization between Odors and Sides. *Learn. Mem*, 8(5), 286–294. <https://doi.org/10.1101/lm.41401>
- Schäfer, S., and Bicker, G. (1986).** Distribution of GABA-like immunoreactivity in the brain of the honeybee. *J. Comp. Neurol*, 246(3), 287–300. <https://doi.org/10.1002/cne.902460302>
- Scheiner, R., Plückhahn, S., Oney, B., Blenau, W., and Erber, J. (2002).** Behavioural pharmacology of octopamine, tyramine and dopamine in honey bees. *Behav. Brain. Res*, 136(2), 545–553. [https://doi.org/10.1016/s0166-4328\(02\)00205-x](https://doi.org/10.1016/s0166-4328(02)00205-x)
- Schippers, M.-P., Dukas, R., Smith, R. W., Wang, J., Smolen, K., and McClelland, G. B. (2006).** Lifetime performance in foraging honeybees: Behaviour and physiology. *J. Exp. Biol*, 209(19), 3828–3836. <https://doi.org/10.1242/jeb.02450>
- Schmid-Hempel, P., Kacelnik, A., and Houston, A. I. (1985).** Honeybees maximize efficiency by not filling their crop. *Behav. Ecol. Sociobiol*,17(1), 61–66. <https://doi.org/10.1007/BF00299430>

- Schünemann, R., Knaak, N., and Fiuza, L. M. (2014).** Mode of Action and Specificity of *Bacillus thuringiensis* Toxins in the Control of Caterpillars and Stink Bugs in Soybean Culture. *ISRN Microbiology*. <https://doi.org/10.1155/2014/135675>
- Schwiening, C. J. (2012).** A brief historical perspective: Hodgkin and Huxley. *J. Physiol*, 590(11), 2571–2575. <https://doi.org/10.1113/jphysiol.2012.230458>
- Singh, S., and Joseph, J. (2019).** Evolutionarily conserved anatomical and physiological properties of olfactory pathway through fourth-order neurons in a species of grasshopper (*Hieroglyphus banian*). *J. Comp. Physiol. A*, 205(6), 813–838. <https://doi.org/10.1007/s00359-019-01369-7>
- Squire, L. R., Genzel, L., Wixted, J. T., and Morris, R. G. (2015).** Memory Consolidation. *Cold. Spring. Harb. Perspect. Biol*, 7(8). <https://doi.org/10.1101/cshperspect.a021766>
- Stanley, D. A., Garratt, M. P. D., Wickens, J. B., Wickens, V. J., Potts, S. G., and Raine, N. E. (2015).** Neonicotinoid pesticide exposure impairs crop pollination services provided by bumblebees. *Nature*, 528(7583), 548–550. <https://doi.org/10.1038/nature16167>
- Stopfer, M., Bhagavan, S., Smith, B. H., and Laurent, G. (1997).** Impaired odour discrimination on desynchronization of odour-encoding neural assemblies. *Nature*, 390(6655), 70–74. <https://doi.org/10.1038/36335>
- Strausfeld, N. J., and Hirth, F. (2013).** Deep Homology of Arthropod Central Complex and Vertebrate Basal Ganglia. *Science*, 340(6129), 157–161. <https://doi.org/10.1126/science.1231828>
- Strauss, R. (2002).** The central complex and the genetic dissection of locomotor behaviour. *Curr. Opin. Neurobiol*, 12(6), 633–638. [https://doi.org/10.1016/S0959-4388\(02\)00385-9](https://doi.org/10.1016/S0959-4388(02)00385-9)
- Strube-Bloss, M. F., Nawrot, M. P., and Menzel, R. (2011).** Mushroom Body Output Neurons Encode Odor-Reward Associations. *J. Neurosci*, 31(8), 3129–3140. <https://doi.org/10.1523/JNEUROSCI.2583-10.2011>

- Strube-Bloss, Martin F., and Rössler, W. (2018).**Multimodal integration and stimulus categorization in putative mushroom body output neurons of the honeybee. *R. Soc. Open. Sci.*, 5(2), 171785. <https://doi.org/10.1098/rsos.171785>
- Suárez, R., Gobius, I., and Richards, L. J. (2014).**Evolution and development of interhemispheric connections in the vertebrate forebrain. *Front. Hum. Neurosci.*, 8. <https://doi.org/10.3389/fnhum.2014.00497>
- Szyszkka, P., Ditzen, M., Galkin, A., Galizia, C. G., and Menzel, R. (2005).**Sparsening and Temporal Sharpening of Olfactory Representations in the Honeybee Mushroom Bodies. *J. Neurophysiol.*, 94(5), 3303–3313. <https://doi.org/10.1152/jn.00397.2005>
- Tabashnik, B. E., Brévault, T., and Carrière, Y. (2013).**Insect resistance to Bt crops: Lessons from the first billion acres. *Nat. Biotechnol.*, 31(6), 510–521. <https://doi.org/10.1038/nbt.2597>
- Tan, K., Chen, W., Dong, S., Liu, X., Wang, Y., and Nieh, J. C. (2015).** A neonicotinoid impairs olfactory learning in Asian honey bees (*Apis cerana*) exposed as larvae or as adults. *Sci. Rep.*, 5(1), 10989. <https://doi.org/10.1038/srep10989>
- Traniello, I. M., Chen, Z., Bagchi, V. A., and Robinson, G. E. (2019).**Valence of social information is encoded in different subpopulations of mushroom body Kenyon cells in the honeybee brain. *Proc. R. Soc. B*, 286(1910), 20190901. <https://doi.org/10.1098/rspb.2019.0901>
- Ueno, T., Takeuchi, H., Kawasaki, K., and Kubo, T. (2015).**Changes in the Gene Expression Profiles of the Hypopharyngeal Gland of Worker Honeybees in Association with Worker Behavior and Hormonal Factors. *PLoS.One*, 10(6), e0130206. <https://doi.org/10.1371/journal.pone.0130206>
- Krishnan, V., Domanska, B., Elhigazi, A., Afolabi, F., West, M. J., Crickmore, N. (2017)** The human cancer cell active toxin Cry41Aa from *Bacillus thuringiensis* acts like its insecticidal counterparts. *Biochem.J.*, 474(10):1591-1602. doi:10.1042/BCJ20170122

- van Middendorp, J. J., Sanchez, G. M., and Burridge, A. L. (2010).** The Edwin Smith papyrus: A clinical reappraisal of the oldest known document on spinal injuries. *Eur. Spine. J*, 19(11), 1815–1823. <https://doi.org/10.1007/s00586-010-1523-6>
- Vance, J. T., Williams, J. B., Elekonich, M. M., and Roberts, S. P. (2009).** The effects of age and behavioral development on honey bee (*Apis mellifera*) flight performance. *J. Exp. Biol*, 212(16), 2604–2611. <https://doi.org/10.1242/jeb.028100>
- vanEngelsdorp, D., Evans, J. D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B. K., Frazier, M., Frazier, J., Cox-Foster, D., Chen, Y., Underwood, R., Tarpy, D. R., and Pettis, J. S. (2009).** Colony Collapse Disorder: A Descriptive Study. *PLoS. ONE*, 4(8), e6481. <https://doi.org/10.1371/journal.pone.0006481>
- Visscher, P. K., and Dukas, R. (1997).** Survivorship of foraging honey bees. *Insectes. Soc*, 44(1), 1–5. <https://doi.org/10.1007/s000400050017>
- Vitzthum, H., Müller, M., and Homberg, U. (2002).** Neurons of the Central Complex of the Locust *Schistocerca gregaria* are Sensitive to Polarized Light. *J. Neurosci*, 22(3), 1114–1125. <https://doi.org/10.1523/JNEUROSCI.22-03-01114.2002>
- Waddell, S. (2013).** Reinforcement signalling in *Drosophila*; dopamine does it all after all. *Curr. Opin. Neurobiol*, 23(3), 324–329. <https://doi.org/10.1016/j.conb.2013.01.005>
- Wang, Y., Brent, C. S., Fennern, E., and Amdam, G. V. (2012).** Gustatory Perception and Fat Body Energy Metabolism Are Jointly Affected by Vitellogenin and Juvenile Hormone in Honey Bees. *PLoS. Genet*, 8(6). <https://doi.org/10.1371/journal.pgen.1002779>
- Webb, B. (2012).** Cognition in insects. *Philos. Trans. R. Soc. Lond. B. Biol. Sci*, 367(1603), 2715–2722. <https://doi.org/10.1098/rstb.2012.0218>
- Wolman, D. (2012).** The split brain: A tale of two halves. *Nature*, 483(7389), 260. <https://doi.org/10.1038/483260a>

- Wright, G. A., Mustard, J. A., Simcock, N. K., Ross-Taylor, A. A. R., McNicholas, L. D., Popescu, A., and Marion-Poll, F. (2010).** Parallel Reinforcement Pathways for Conditioned Food Aversions in the Honeybee. *Curr. Biol*, 20(24), 2234–2240. <https://doi.org/10.1016/j.cub.2010.11.040>
- Wright, G. A., Nicolson, S. W., and Shafir, S. (2018).** Nutritional Physiology and Ecology of Honey Bees. *Annu. Rev. Entomol*, 63(1), 327–344. <https://doi.org/10.1146/annurev-ento-020117-043423>
- Yi, D., Fang, Z., and Yang, L. (2018).** Effects of Bt cabbage pollen on the honeybee *Apis mellifera* L. *Sci. Rep*, 8(1), 1–6. <https://doi.org/10.1038/s41598-017-18883-w>
- Zwaka, H., Münch, D., Manz, G., Menzel, R., and Rybak, J. (2016).** The Circuitry of Olfactory Projection Neurons in the Brain of the Honeybee, *Apis mellifera*. *Front. Neuroanat*, 10. <https://doi.org/10.3389/fnana.2016.00090>

APPENDIX

Appendix

Appendix A:

A) Sensilla recording indicate Acrylic paint is a inert, robust and reversible block:

To check for the blocking capacity of acrylic paint n=5 *A. dorsata* bees were used. The paint coating successfully blocked the antenna's response to stimulus and the response was restored upon uncovering the antenna (Fig 7). The onset of odor was at 2 sec and continued for 1 sec, response was seen as a deflection in the base line curve. Analysis of the recording was done in MATLAB.

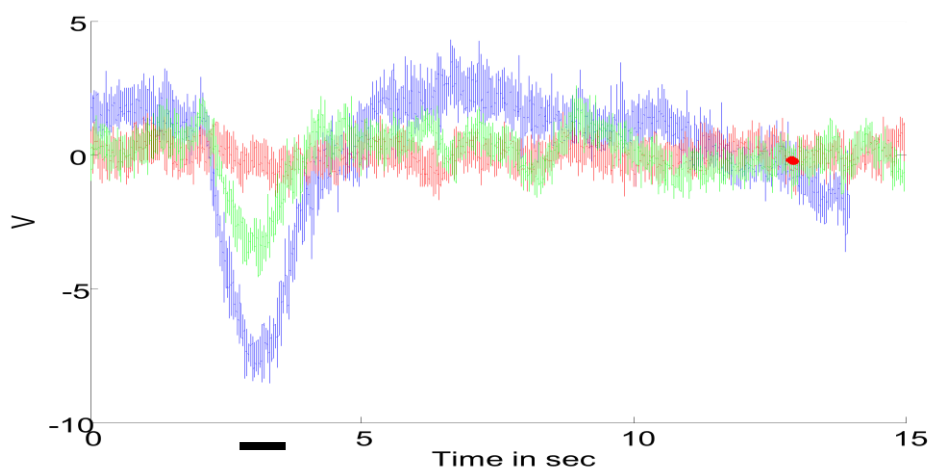


Fig 1: The response to stimulus is seen as the blue deflection. The red line shows the response to the odor was blocked using acrylic paint and response was restored as indicated by the green line. Onset of odor is represented by the black line under the time scale.

Appendix B:B) Recordings from MB show no contralateral input from adjacent olfactory centres:

Recordings were made from the MB while blocking the antenna on the same side, hence checking for any olfactory connections between the two sides of the brain, n=5 *A. dorsata* bees were used for the experiment (Fig 2).

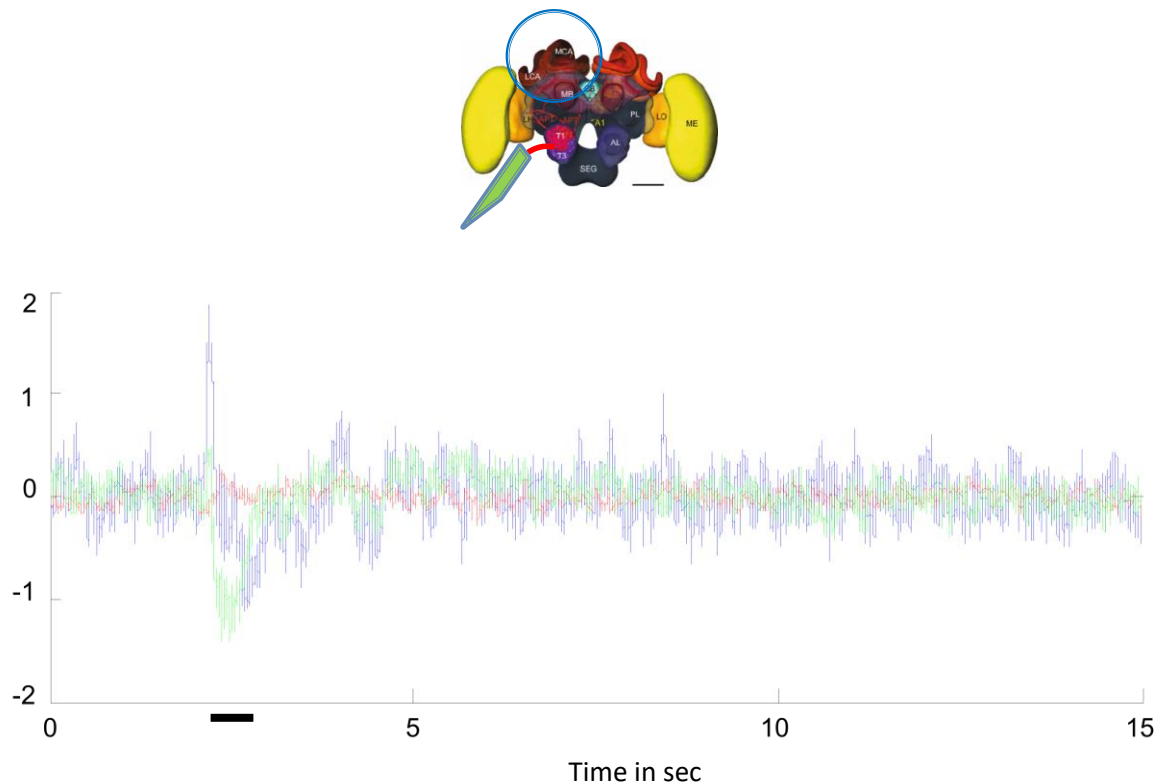


Fig 2: Response to odor by the MB was recorded as represented by the blue deflection. The response was blocked upon applying acrylic paint as seen by the red deflection and the response was restored upon uncovering the antenna represented by the blue deflection. A pictorial representation of the bee brain on the top right panel indicating where the electrode was placed (blue circle around MB) and the antenna on the same side shown with the acrylic block.

Our results suggest that the MB does not receive contralateral input from the olfactory centers of the adjacent brain lobe which would have been evident had there been a response seen in the red line despite acrylic paint being a good odor block. We however observe that the response by the MB neurons upon odor stimulation is shut off by the paint cover on the antenna of same side as the recording electrode.

Appendix C

C) Simultaneous LFP recordings from the α -lobes reveal no olfactory cross-connections between them:

Recordings were taken from both the α -lobes showed there were no cross connections at the olfactory level between the two anatomic structures. n=7 *A. dorsata* bees were used for the experiment. First both the antennae of the bee were left open and response to 1-hexanol was seen, this was followed by blocking one antenna which revealed response only on the open side, then blocking both the antennae shut off response by both the α -lobes and once the cover was reopened the response was restored on both sides (Fig 3). From this experiment we conclude that there seem to be no immediate direct connections at the olfactory level between the α -lobes.

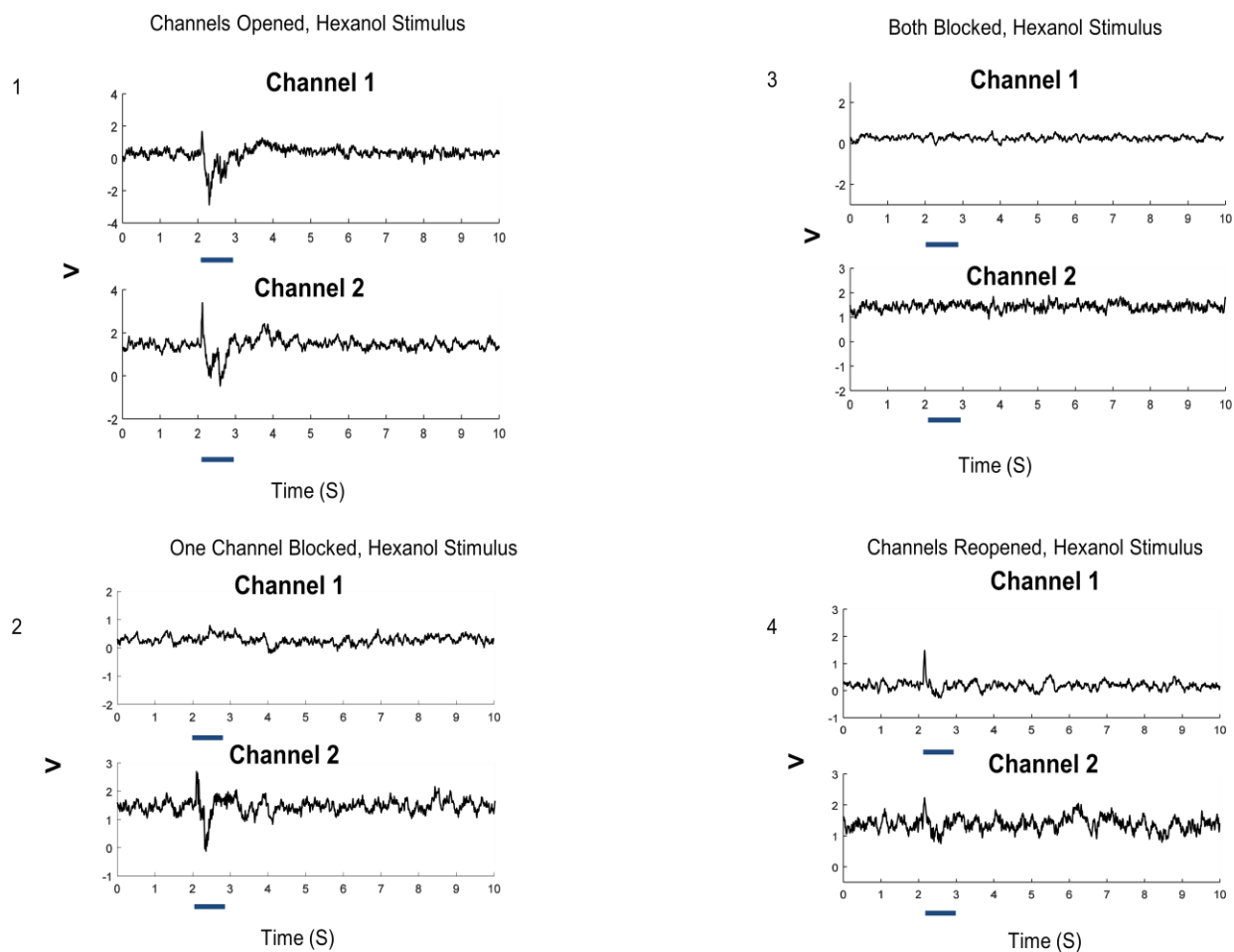


Fig 3: Each panel represents the treatment type for that recording. Channel 1, 2 represent the recordings from the two electrodes placed in the α -lobes. The response to odor is seen as a deflection in the base line as seen in channel 1,2 in the 1st panel and channel 2 in the second panel, this response is blocked by the acrylic paint cover, as seen in channel 1 in panel 2, and channel 1,2 in panel 3. The response is restored as shown by the response to odour in channel 1 and 2 in panel 4.

Appendix DD) Electroretinogram revealed seizure like behavior in foragers allowed to free fly:

The responses of three groups of foragers to light stimulus was recorded, foragers caught and placed in the chamber immediately, foragers cooled for ten minutes and placed in the chamber and foragers caught and harnessed. Recordings were made 30 minutes after the captured and immediately boxed and captured cooled and boxed foragers were allowed to free fly. The response to stimulus did not vary in the three groups and the waveform was the same for all the three groups with the typical on and off response. The amplitude of the on an off response was between 2-4 mV. The activity of foragers captured and allowed to free fly (n=7) exhibited maximum intensity in deflections from the base line compared to the cooled foragers (n=16) and the harnessed foragers (n=9) . The power spectrum of the individual bees and the mean of the responses showed the deflections were not significantly different for the bees captured and put in the chamber compared to the two other groups (Kruskal Wallis one way analysis of variance). These deflections however seemed similar to the disrupted pattern and bursting activity seen in seizure like conditions. Our results indicate that free flying foragers when captured exhibit seizure like activity before the onset of death, which usually occurs within the first hour of capture.

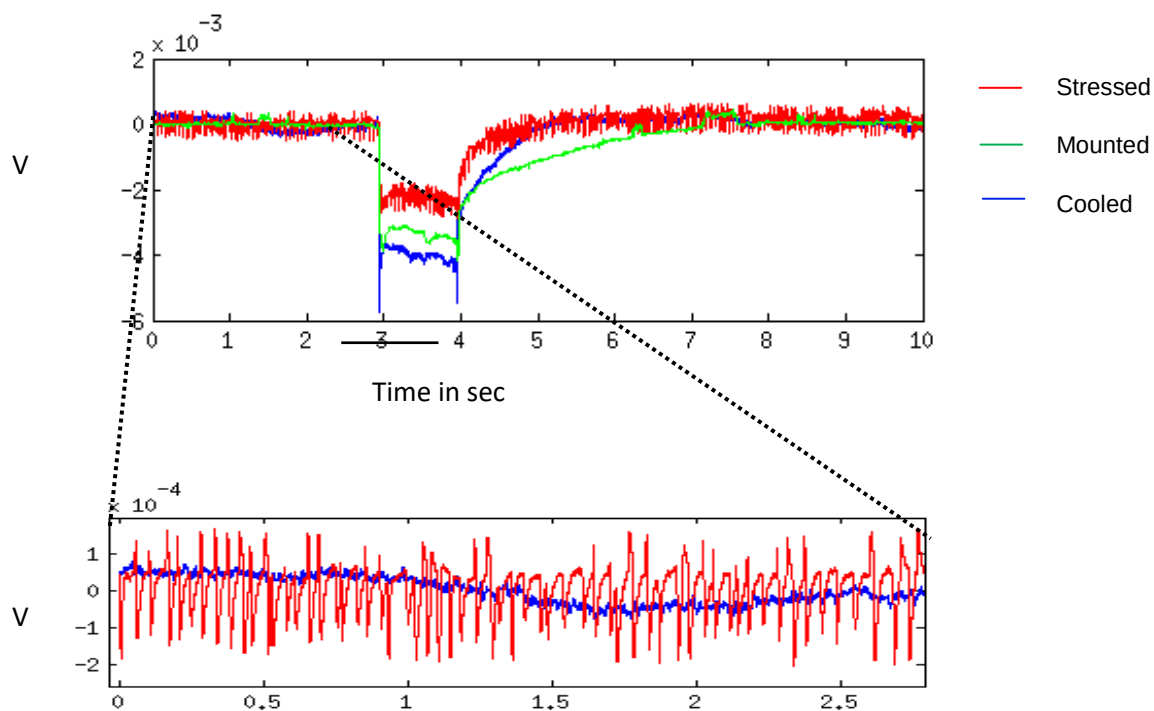


Fig 4: The electroretinogram recording of foragers allowed to fly freely immediately upon capture (red line), foragers mounted upon capture (green line) and foragers cooled then allowed to free fly (blue line). The response to stimulus is seen as a deflection (2-4 mV) at 3 seconds, the time of stimulus is represented by the black bar between 3-4 seconds. The lower panel represents the magnification of the base line activity of a stressed bee and a forager cooled and allowed to free fly. The amplitude of the fluctuations of the stressed forager is seen but was not found to be significantly different from the two other forager groups.

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RESEARCH ARTICLE

Evidence for absence of bilateral transfer of olfactory learned information in *Apis dorsata* and *Apis mellifera*

Meenakshi Vijaykumar^{1,2}, Sandhya Mogily¹, Aparna Dutta-Gupta² and Joby Joseph^{1*}

ABSTRACT

The capacity and condition under which the lateral transfer of olfactory memory is possible in insects is still debated. Here, we present evidence in two species of honeybees, *Apis mellifera* and *Apis dorsata*, consistent with the lack of ability to transfer olfactory associative memory in a proboscis extension response (PER) associative conditioning paradigm, where the untrained antenna is blocked by an insulating coat. We show that the olfactory system on each side of the bee can learn and retrieve information independently and the retrieval using the antenna on the side contralateral to the trained one is not affected by the training. Using the setup in which the memory on the contralateral side has been reported at 3 h after training, we see that the memory is available on the contralateral side immediately after training. In the same setup, coating the antenna with an insulator on the training side does not prevent learning, pointing to a possible insufficiency of the block of odor stimuli in this setup. Moreover, the behavior of the bee as a whole can be predicted if the sides are assumed to learn and store independently, and the organism as a whole is able to retrieve the memory if either of the sides have the memory.

KEY WORDS: PER conditioning, Mushroom body, Olfactory coding, Insect olfaction, Learning and memory

INTRODUCTION

Lateral transfer of information helps environmental stimuli acquired and learned on one side to become accessible to both lobes of a bilateral brain (Aboitiz and Montiel, 2003; Gazzaniga, 2000). This helps to maximize the computational ability of the brain by allowing each side of the brain to co-opt the other for joint decision-making or to avoid duplicity of storage for efficient use of the substrate (Aboitiz and Montiel, 2003; Gazzaniga, 2000, 2014). Information transfer across the midline has also been theorized to be the basis of unified consciousness (Barron and Klein, 2016) and its importance has been highlighted in split-brain patients (Gazzaniga, 2014). In higher mammals, this crucial function is carried out by the corpus callosum, a tissue present in eutherian mammals alone (Aboitiz and Montiel, 2003; Gazzaniga, 2000, 2014; Suárez et al., 2014). The formation of the corpus callosum has been suggested to be an evolutionary innovation (Mihirshahi, 2006), highlighting the importance of developing and evolving the process of transfer of information as an evolutionarily stable strategy. Does this

evolutionary jump have correlates in invertebrates such as insects? Despite their primitive nature, insects are known to be able to perform complex tasks with their rather simple brains consisting of a few 100,000 neurons. Insects such as ants, wasps and honeybees, belonging to the order Hymenoptera, can perform complex tasks involving locating food sources, nesting sites and foraging back and forth between the food source and nest, which would require coordination of a range of modalities (Hansson and Stensmyr, 2011; Kaupp, 2010; Matsumoto et al., 2012; Roper et al., 2017; Sanes and Zipursky, 2010; Su et al., 2009).

In free-flying bees (Masuhr and Menzel, 1972), it was reported that side-specific olfactory conditioning does not transfer to the contralateral side. It was later reported that in *Apis mellifera*, if the bee is trained in the proboscis extension response (PER) to associate an odor with reward when a stimulus is applied to only one antenna, the odor memory can be retrieved by applying trained odor to the untrained contralateral antenna, 3 h after training (Sandoz and Menzel, 2001). In these experiments, a wall was used to separate the two antennae and deliver the odor in a side-specific manner, arguing that blocking the antenna using a coating influences the context of training and impairs transfer. In their study, 3 h post training, up to 50% of the bees responded by extending their proboscis when the learned odor and not a novel odor was applied only to the contralateral antenna, suggesting the presence of a commissure relaying encoded odor-specific memory between sides. Guo et al. (2016) reported changes on a molecular level in the contralateral side of *A. mellifera* after training even if the contralateral side was isolated by coating the antenna. That study, however, did not show transfer using behavior, compared with controls trained with both sides closed. This group used silicon paste to block one antenna while training the exposed antenna to an odor. Post 24 h transcriptomic analysis was carried out and the results showed an upregulation in memory and learning-related genes on the untrained side of the brain, indicating a possible lateral transfer of this learned information and memory. The above experiments and works pointed to the possible presence of a commissure dedicated to the relaying of olfactory learned information from one brain lobe to the other. If this is true, then recording the activity of the neurons in this commissure would also provide insight into the nature of olfactory code, an exciting prospect.

Work in our laboratory recently showed the presence of bilateral extrinsic neurons of the mushroom body calyx in a species of grasshopper, *Hieroglyphus banian* (Singh and Joseph, 2018 preprint). In addition, a cluster of lateral horn neurons in *Schistocerca americana* have been shown to have a bilateral innervation (Gupta and Stopfer, 2012). Thus, there are very few possible substrates for lateral transfer of olfactory memory in insects and none reported in Hymenoptera. We attempted to look for the neuronal basis of the phenomenon of bilateral transfer of information in a species of honey bee native to South East Asia, *Apis dorsata*, also referred to as the giant honey bee or the rock bee, which is one of the crucial pollinators in the region. In our

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Characterization of the olfactory system of the giant honey bee, *Apis dorsata*

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Abstract

Apis dorsata is an open-nesting, undomesticated, giant honey bee found in southern Asia. We characterized a number of aspects of olfactory system of *Apis dorsata* and compared it with the well-characterized, western honeybee, *Apis mellifera*, a domesticated, cavity-nesting species. *A. dorsata* differs from *A. mellifera* in nesting behavior, foraging activity, and defense mechanisms. Hence, there can be different demands on its olfactory system. We elucidated the glomerular organization of *A. dorsata* by creating a digital atlas for the antennal lobe and visualized the antennal lobe tracts and localized their innervations. We showed that the neurites of Kenyon cells with cell bodies located in a neighborhood in calyx retain their relative neighborhoods in the pedunculus and the vertical lobe forming a columnar organization in the mushroom body. The vertical lobe and the calyx of the mushroom body were found to be innervated by extrinsic neurons with cell bodies in the lateral protocerebrum. We found that the species was amenable to olfactory conditioning and showed good learning and memory retention at 24 h after training. It was also amenable to massed and spaced conditioning and could distinguish trained odor from an untrained novel odor. We found that all the above mentioned features in *A. dorsata* are very similar to those in *A. mellifera*. We thereby establish *A. dorsata* as a good model system, strikingly similar to *A. mellifera* despite the differences in their nesting and foraging behavior.

Keywords *Apis dorsata* · Olfactory system · Digital atlas · Mushroom body · Olfactory conditioning

Introduction

Honey bees were described as magic well for discoveries in biology by Karl Von Frisch. The Western honey bee, *Apis mellifera*, is well established as a model system to investigate various fundamental scientific questions at the behavioral, neural, and molecular levels. The olfactory conditioning paradigm in bees is extensively used for research in learning and memory (Menzel and Erber 1978; Menzel 1993; Menzel and Muller 1996; Giurfa 2007) as features and mechanisms of learning and memory in bees are found to have similarities

to those in mammals and humans (Squire 1987; Menzel et al. 1996; Menzel 2012).

In *A. mellifera*, the odor molecules are detected by around 60,000 olfactory receptor neurons (ORNs) present in sensilla located on the antennae (Esslen and Kaisling 1976; Kropf et al. 2014). ORNs from each side innervate the ipsilateral antennal lobe (AL), the primary olfactory center, through the T1–4 tracts of the antennal nerve (AN) (Suzuki 1975; Mobbs 1982; Galizia et al. 1999; Abel et al. 2001; Kirschner et al. 2006). In the AL of *A. mellifera*, ORNs synapse on to around 800 projection neurons (PNs) (Bicker et al. 1993; Hammer 1997; Galizia 2008) and approximately 4000 local neurons (LNs) (Witthöft 1967; Sachse and Galizia 2006; Galizia 2008; Galizia and Rossler 2010) in dense spheroidal structures called glomeruli, the morpho-functional unit of the AL (Hildebrand and Shepherd 1997; Anton and Homberg 1999; Hansson and Anton 2000). PNs project to the higher olfactory centers, lateral horn (LH), and the mushroom body (MB) through five antennal lobe tracts (ALTs). In the MB, the PNs synapse on approximately 180,000 Kenyon cells (KCs) (Mobbs 1982; Abel et al. 2001; Muller et al. 2002; Kirschner et al. 2006; Rossler and Brill 2013; Zwaka et al.

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