Visual Learning and Processing in the honeybee, *Apis mellifera*

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Abstract

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The honeybee, *Apis mellifera*, is well-known for her crucial role in crops pollination and for producing an highly-appreciated food, the honey. Indeed, the first historical record of bee-keeping dates from the Mesolithic at least 9000 years ago. The honeybee possesses a miniature brain (approximately one million of neurons) but exhibits incredibly sophisticated behavior. For instance, a forager honeybee can learn abstract concepts such as sameness/difference or above/below, to recognize specific human faces and even count. The neural basis of those behaviors have yet to be elucidated due to the difficulty in linking free-flying behaviors with tethered preparations typically used in *in vivo* neurophysiological recordings. In this dissertation, my goal is to develop a protocol to induce visual learning in a tethered walking honeybee and record from an optic lobe during visually guided behaviors.

In the first chapter, I present an introduction on honeybee learning and memory, how learning protocols were developed, and what we know about the neural bases of these behaviors. I briefly summarize honeybee vision and what is known about internal state and behavioral state modulation of visual processing in insects.

In the second chapter, I present our study on visual learning in tethered walking honeybees. We developed a Virtual Reality Environment (VR) composed of a walking treadmill connected to a computer and surrounded by a screen. Visual stimuli were projected on the screen based on the honeybee motion (closed-loop) or based on predefined inputs (open-loop). We showed that tethered honeybees in our VR could learn to discriminate between visual stimuli, although not all combinations of shapes and colors were learned equally. We hypothesized that optic flow, the motion of the visual panorama on the retina, was critical for learning.

In the third chapter, I present our study on sensory feedback and internal state modulation in an optic lobe of the honeybee brain. In this study, we combined behavioral and neural recordings to explore how internal state and sensorimotor feedback impacted neural activity in the medulla, the second optic lobe of the honeybee. We presented the honeybee with visual stimuli in closed-loop, where the animal had control over the motion of the visual scene (i.e., self-generated optic flow) and subsequently replayed the visual scene motion in open-loop (i.e., externally generated optic flow). We found that neural activity in the medulla is modulated by locomotory state (i.e., walking *versus* non-active locomotion bouts) almost exclusively in the presence of closed-loop behavioral control. Overall, around a third of the neural population recorded was influenced by behavioral control. Honeybees exhibited a surprisingly high ability to adapt to multiple levels of motor gain (i.e., the relationship between her motion and the motion of the visual scene) and this capacity is likely to rely on the release of octopamine, an invertebrate neuromodulator, in the medulla.

In summary, our work provides support to the growing idea that internal and behavioral state are essential for studying how the brain produces behavior. The link between brain and behavior will require an interdisciplinary approach and the devel-

opment of novel methodologies that are spread across disciplines (neurophysiology, behavioral, neurogenetic, psychology, engineering, mathematics, etc.) and species.

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GLOSSARY

COGNITION: ability to acquire, process, store, and act on information

MULTICHANNEL NEURAL RECORDING: multi-channel recording means recording more than two separate channels, synchronized to each other. In the context of neural recordings, a probes composed of multiple recording sites in inserted in the brain. Spike sorting can be applied on the recordings to isolate single units or neurons

OMMATIDIUM: each of the optical units that make up a compound eye, as of an insect. The ommatidium contains photoreceptors, the cells that respond to light. The honeybee had three types of photoreceptors Short-, Medium- and Long-Wavelength Sensitive photoreceptors

OPERANT CONDITIONING: learning procedure in which a certain behavior is associated with a reward or punishment. Also known as instrumental conditioning

OPTIC FLOW: the pattern of apparent motion of objects, surfaces, and edges in a visual scene caused by the relative motion between an observer and a scene. It can be self-generated (motion of the observer) or externally generated (e.g., motion of an object due to the wind)

OPTIC LOBES: brain structures involved in vision. The honeybee brain has three optic lobes: the lamina, the medulla and the lobula

PAVLOVIAN CONDITIONING: learning procedure in which a non-neutral stimulus (e.g. food, electric shock) is associated with a previously neutral stimulus (e.g. a sound, an odor). Also known as classical conditioning

SENSORY FEEDBACK: feedback provided within the sensory systems where information from sensory receptors is returned along the afferent pathways so the brain can monitor the consequence of actions.

SPIKE SORTING: grouping of spikes into clusters based on the similarity of their shapes. Given that, in principle, each neuron tends to fire spikes of a particular shape, the resulting clusters correspond to the activity of different putative neurons. The end result of spike sorting is the determination of which spike corresponds to which of these neurons.

ACKNOWLEDGMENTS

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Thank you to my family and friends, close or distant. I am so lucky to be surrounded by amazing people, the list would be too long to write here (and I am way too afraid to forget to include some). Special thanks are due to my parents, obviously I would not be here without them. I have to say here that my mom is an incredible role model, and among many many other things, she showed me and my siblings that women can have a career and a family. Diego, no hay nadie como tu.

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DEDICATION

To my dad,

may we hijack many more family conversations and talk about science

Chapter 1

INTRODUCTION

> "It is certain that there may be extraordinary activity with an extremely small absolute mass of nervous matter; thus the wonderfully diversified instincts, mental powers, and affections of ants are notorious, yet their cerebral ganglia are not so large as the quarter of a small pin's head. Under this point of view, the brain of an ant is one of the most marvelous atoms of matter in the world, perhaps more so than the brain of man."
> Charles Darwin 1871.

Cognition is the ability to acquire, process, store, and act on information (Simons and Tibbetts, 2019). Even now, in the 20th century, there is this common view of cognition in the animal kingdom with the human species on top, being the most "intelligent" of all. This point of view has created the impression that the smaller animals, with smaller brains, were little robots, composed only of instincts, and not capable of "true" cognition. However, an impressive amount of information about cognition in insects has been generated over the last several decades, and researchers have argued that the origin of complex behaviors, such as the one relying on subjective experience, can be explored by studying these relatively simple organisms (Barron and Klein, 2016). Leaf-cutter ants, ambrosia beetles and termites use fungus-based agriculture (Mueller and Gerardo, 2002). *Polistes fuscatus*, a paper wasp, can identify nest-mates individually using facial and abdominal markings (Tibbetts, 2002). An insect that has inspired researchers with its richness of beha-

vior, social organization, and communication, is the honeybee.

1.1 THE HONEYBEE, A MODEL TO STUDY LEARNING AND MEMORY

In 1927, Karl Von Frisch described the waggle dance in his book *Aus dem Leben der Bienen* (translated into English as *The Dancing Bees*). 50 years later, in 1973, he received the Nobel Prize for his pioneer work on insect communication along with Konrad Lonrenz and Nikolaas Tinbergen. Since then, the honeybee has become a model species for the cellular and molecular study of olfactory learning and memory (Giurfa and Sandoz, 2012), thanks to the abilities of researchers to translate learning paradigms from the field to the laboratory using the olfactory Pavlovian conditioning of proboscis extension reflex. In a now famous experiment, Ivan Pavlov trained dogs to salivate at the sound of a buzzer. Dogs naturally begin to salivate when smelling food odor. Pavlov first associated the sound of the buzzer to food by systematically playing it while feeding the animals. After some repetition of this association, the dogs learned that the sound predicted food and their body reacted by increasing salivation. By the end of the training, the sound by itself, who was neutral before the experiment, triggered the salivation reflex. This classical conditioning as been translated in many species (e.g., primate: Takakuwa et al., 2018; mice: ten Brinke et al., 2017, fruit fly: Shyu et al., 2017) including the honeybee (Bitterman, 1983).

When foraging, a honeybee exhibit a tendency to exclusively visit the same flower species or morphology (Grant, 1950). This floral constancy presuppose that the individual has the ability to remember and have an internal template of the flower type to visit. The contact of the antennae with a sweet solution trigger a reflex extension of the proboscis, a mouth part, to feed from it. Researchers used this reflex

to train honeybee to extend their proboscis in response to odors and this behavioral paradigm has led to an extensive effort of characterization of the honeybee's olfactory pathways and by extension of its learning and memory centers (Fig. 1). The pathways of the olfactory processing from the olfactory sensory neurons in the antennae to the Mushroom Bodies in the brain is well described, as well as the gustatory one, and recent initiatives have been made to integrate this information, along with data from other sensory modalities and brain centers, into a Virtual Atlas of the Honeybee brain (Brandt et al., 2005; Rybak et al., 2010). In addition, several studies in honeybees have highlighted cognitive abilities that were previously thought to exist only in vertebrates (Giurfa, 2003). For example, free-flying honeybees can perform delayed matching-to-sample task (Giurfa, 2001). In this task, honeybees are presented a stimulus that they will be required to remember, known as the 'sample'. They are then required to identify from a subsequent set of stimuli one that 'matches' the sample, therefore showing that they learn the rule rather than the stimulus itself.

The astonishing learning abilities of insects, particularly eusocial insects, ask very interesting question regarding the evolution of cognition. The most studied hypotheses are the 'social intelligence' and the 'ecological intelligence' (Simons and Tibbetts, 2019). The 'social intelligence hypothesis', or 'social brain hypothesis', proposes that enhanced cognition evolved primarily to survive in large social groups (Dunbar, 1998). The 'ecological intelligence hypothesis' proposes that enhanced cognition evolved primarily from the need to find food and other ecological constraints (e.g, DeCasien et al., 2017). While experimental evidences have provided support for both hypothesis, recent studies have proposed that both social and ecological factors work together to shape cognitive evolution. Studies on the evolution of cognition have historically focus on vertebrate, if not mammals. Invertebrate provides a unique opportunity for the study of cognition and its evolution with their large

range of sociality and ecological niche.

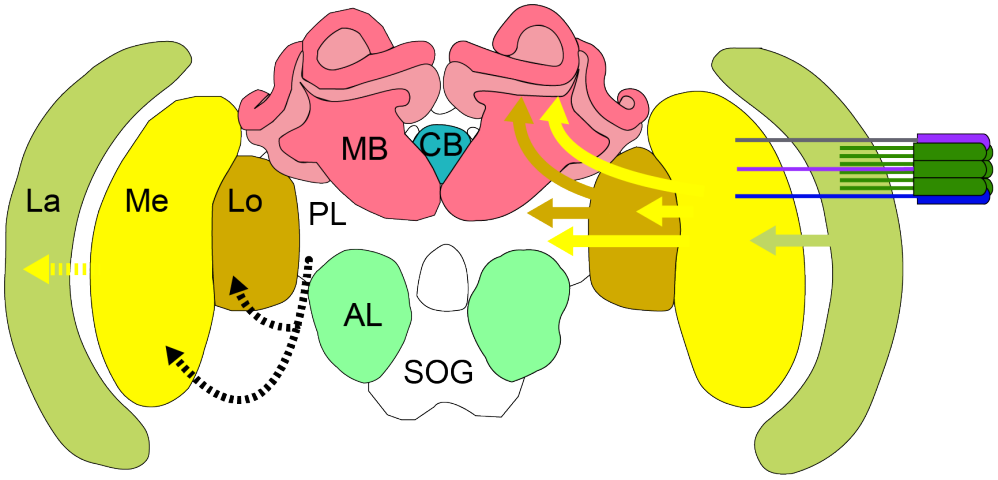

Fig. 1. Honeybee brain and visual processing (Top) The brain of honeybee is composed of multiple compartments. The Antennal Lobe (AL) is involved in olfactory processing and the optic lobes: the Lamina (La), the Medulla (Me) and the Lobula (Lo) are involved in visual processing. The Mushroom bodies (MB) are known to be the center of memory and integrate multimodal sensory information. Visual processing starts in the retina when photoreceptors are exited in the ommatidium. Here is represented a type 1 ommatidia containing 6 LWS, 1 SWS and 1MWS photoreceptors as well as the basal ninth photoreceptor sensitive to UV. The LWS photoreceptors project to the La, and the SWS and MWS photoreceptors directly to the Me. The Me project to the Lo and both send projections to the MB and other parts of the central brain (Protocerebral lobes (PL) and Central Body or complex (CB). The dashed arrows represent the presumed connection from the medulla to the lobula and the putative inputs for sensory and motor feedback. The inputs from the controlateral optic lobes are not represented in this figure

1.2 HONEYBEE VISION

It was using the honeybee that researchers scientifically proved the existence of colour vision in a non-human animal for the first time (Lubbock, 1882). The visual system of the honeybee is composed of two compound eyes and three dorsal ocelli

and each eye contains around 5000 ommatidia. Each ommatidium contains a biconvex cuticle lens and a crystalline cone and receives around 2.5deg of the visual field with an overlap of half a degree with the adjacent ommatidia (Laughlin and Horridge, 1971; Seidl and Kaiser, 1981). Early work have identified that the highest resolution in the honeybee eye is in the frontal part of the bee eye (Seidl, 1982 as reviewed in Horridge, 2009) and that honeybees can discriminate coloured area with a resolution around 10° (Gould, 1985).

Like most insect, honeybees have three retinal cells, or photoreceptors, sensitive to different part of the light spectrum (Menzel et al., 1986). The short-wavelength sensitive photoreceptor (SWS) peaks at 344 nm in the UV, the medium-wavelength sensitive photoreceptor (MWS) at 436 nm in the blue and the long-wavelength sensitive photoreceptor (LWS) at 544 nm in the green part of the spectrum (Fig. 2). The sensitivity tuning of the S and M photoreceptor is relatively narrow whereas the L photoreceptor is sensitive to blue and UV as well. All ommatidia contains 8 large photoreceptors and a small, basal ninth whose sensitivity is still unclear (Menzel and Snyder, 1974; Wakakuwa, Kurasawa et al., 2005). Each ommatidium can be categorized as one of three possible types. In addition to 6 LWS photoreceptors, type 1 ommatidia (44% of the ommatidia) contain one SWS and one MWS photoreceptors, type 2 (46%) contain two SWS photoreceptors and type 3 (10%) contain two MWS photoreceptors.

From the retina, visual information is processed in three successive brain areas: the lamina, the medulla and the lobula (Fig. 1). In the lamina, the axons of the nine photoreceptors of each ommatidia are confined in single cartridge. In each cartridge, the LWS photoreceptors connect to three distinct monopolar cells. A fourth monopolar cell is present in around 25% of the cartridge and provide inhibitory synaptic connection to adjacent cartridge (Ribi, 1975). There are several other neuron types

in the lamina: local amacrine cells, presumed efferent neurons from the medulla and presumed neuroendocrine neurons (Horridge, 2009). Most of the processing taking place in the lamina concern the detection of spatial contrast.

The monopolar cells in the lamina connect to the medulla alongside the axon of the MWS and SWS photoreceptors and form the outer chiasma, keeping the columnar structure and retinotopic organization. In the fruit fly, *Drosophila melanogaster*, a single medulla column contains around 900 neurons, over 2500 presynaptic sites and more than 300000 postsynaptic sites (Takemura, Xu et al., 2015). This impressive numbers suggest that a massive amount of neuronal processing is being done through the medulla. The first step of color vision happens in the medulla, with color sensitive neurons with an ON/OFF responses in the inner layers and colour opponent responses in the outer layers (Paulk et al., 2008; Paulk et al., 2009). The medulla is responsible for mapping the local colour, spatial and temporal contrast as well as detecting the coincidences between feature detector responses (Horridge, 2009). Transmedulla neurons extends their dendrites outside the medulla to the lobula, protocerebrum and mushroom bodies.

The inner chiasma forms the connection between the medulla and the third optic lobe, the lobula. In the lobula, the retinotopic organization and columnar structure is only preserved in the outer part (Hertel et al., 1987). In the lobula, the third optic lobe, more color opponent responses can be found alongside spatial opponent neurons (Hertel et al., 1987) and direction and edge orientation sensitive neurons. Lobula neurons project the protocerebrum and mushroom bodies.

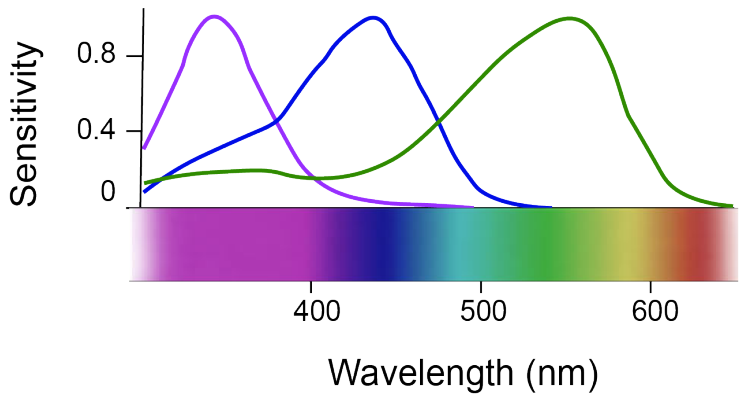

Fig. 2. Sensitivity of the three photoreceptors in the retina of the honeybee. The SWS photoreceptors are sensitive to UV light, with a peak at 344 nm. The MWS photoreceptors are more sensitive to the blue part of the spectrum with a peak at 436 nm. The LWS photoreceptors are more sensitive to the green part of the spectrum with a peak at 544 nm. The LWS photoreceptors presents the wider sensitivity curve.

1.3 BEHAVIORAL AND INTERNAL STATE MODULATION OF SENSORY PROCESSING

In a ever changing environment, it is critical for survival to process sensory information as efficiently as possible. For instance, in many species, the sense of smell is closely linked to how and what to eat. In human, sleep-deprivation increase food intake likely via modulation of olfactory processing (Bhutani et al., 2019). In drosophila, food deprivation modulates olfactory-guided behavior via modulation of the activity of specific glomerulus of the antennal lobes (Ko et al., 2015). Similarly, visual processing is highly modulated by behavioral and internal state.

An obvious sensory feedback on vision concern self-motion. Indeed, motion inform vision, a moving animal generating motion of the visual scene on its retina. Similarly, vision inform motion, it is indeed near impossible to keep a straight path when blinded. Multiple recent studies in insects have explored the impact of locomotion state on visual processing in the optic lobes. Using Virtual Environment combined

with neural recording, researchers have shown that neural activity in the optic lobes in the optic lobes is modulated by locomotion state. The strongest evidence comes from recordings of lobula plate tangential cells (LPTCs) in the fruit fly, Drosophila melanogaster. LPTCs encode visual motion that correspond to self-movement (e.g., roll, pitch, yaw), by integrating locally oriented, directionally selective inputs over a large receptive field. Several LPTCs have been shown to increase their gain or sensitivity during walking (Chiappe et al., 2010) and flight (Jung et al., 2011, Maimon et al., 2010, Suver et al., 2012). A recent study in the fruit fly lamina described wide field neurons that increase their gain and shift their frequency tuning when the animal is flying (Tuthill et al., 2014). In the medulla, several columnar neurons respond to spontaneous leg motion as well as air puffed on the leg (Strother et al., 2018). Taken together, those studies suggest that modulation of visual processing happen in all three optic lobes. However, it is still unclear how and where behavioral state information is integrated in the pathways. Octopamine, an invertebrate neuromodulator, has been identified as the main actor of modulation of visual processing by locomotion-state as octopamine release mimics the increase in gain and shift in frequency tuning triggerred by active behavior (Longden and Krapp, 2009; Jung et al., 2011; Suvert et al, 2012; Tuthill et al., 2014; Strother et al., 2018).
The honeybee brain contains roughly 7 times more neurons than the fruit fly brain and the lobula is a single neuropil in honeybee (i.e., there are no LPTCs). It is thus possible that visual processing in those two species rely on different strategies, with increased modulation in the medulla of the honeybee.

Our sensory systems receive information from the environment continuously and we are aware of only a portion of this information. One way of ensuring that only the most relevant signals are extracted while dedicating as little computational resources as possible can be categorized under the general phenomenon of "attention" (Logan, 1992). Attention allows animals to respond selectively, enabling some

stimuli to evoke behavioral response while others are ignored. The neural basis of visual attention has been extensively studied in primates (see Wolfe, 2000, for review). The visual cortex of the primate brain, the area that receive and processed visual information, is divided in five regions: V1 to V5. Attention modulate neural activity as early as V1, the first region to receive inputs from the retina (Watanabe et al., 1998). The firing rate of visual neurons as well as their receptive field, or portion of the visual world they respond to, is modulated by attention-like processes in all the visual cortex (see McAdams and Maunsell, 1999; Treue, 2001, for review). In the V4 region, attention triggers change in the synchrony between neuronal population. Synchronization increases between neurons that respond to the visual stimuli and decreases with neurons that did not respond (Fries et al., 2001). Little is known about attention in insect, although recent studies have highlighted evidence of attention-like processes in the brain (for review, see De Bivort and van Swinderen, 2016). Recent studies in fruit flies have showed that visual salience increase neural activity at specific frequency and may correlated to behavioral selection and suppression (van Swinderen and Greenspan, 2003; van Swinderen, 2007; Tang and Juusola, 2010; van Swinderen, 2012). In the medulla of the honeybee, responses selectivity preceded behavioral choice, suggesting that modulation of early visual processing centers precedes eventual behavioral decisions (Paulk et al., 2014). Moreover, synchronization of neural oscillation in the medulla and lobula is increased when the animal is in control of its environment (Paulk et al., 2015). In the lobula of the mosquito, *Aedes aegypti*, visual attention is modulated by olfactory input (van Breugel et al., 2015). Female mosquitoes are behaviorally attracted to visual objects only in the presence of CO2 and around 20% of the lobula neurons are modulated by CO2 (Vinauger et al., 2019). Learning and attention impact each other: without attention, learning is impaired, and learning can increase attention to particular cues. If most studies on the neural basis of learning and memory in insect has focus on central brain area, mainly the Mushroom Bodies, recent stud-

ies have described neuronal change in the periphery during and after learning. For example, neurons in the Antennal Lobes of the honeybee change after olfactory learning (Rath et al., 2011). Interestingly, octopamine is known to play a major role in the convergence of reward/punishment information and sensory information in learning protocols. Indeed, local injection of octopamine in the Mushroom Bodies or Antennal Lobes paired with odor delivery is sufficient to trigger learning (Hammer and Menzel, 1998).

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Chapter 2

HONEYBEES IN A VIRTUAL REALITY ENVIRONMENT LEARN UNIQUE COMBINATIONS OF COLOUR AND SHAPE

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SUMMARY STATEMENT

Here, using a novel virtual reality environment and paradigm for visual training in walking honeybees, we show that bees learn certain visual components over others (colour over shape), and interaction between components is critical for visual learning in walking bees.

ABSTRACT

Honeybees are well-known models for the study of visual learning and memory. Whereas most of our knowledge of learned responses comes from experiments using free-flying bees, a tethered preparation would allow fine-scale control of the visual stimuli as well as accurate characterization of the learned responses. Unfortunately,

conditioning procedures using visual stimuli in tethered bees have been limited in their efficacy. Here in this study, using a novel virtual reality environment and a differential training protocol in tethered walking bees, we show that the majority of honeybees learn visual stimuli, and need only six paired training trials to learn the stimulus. We found that bees readily learn visual stimuli that differ in both shape and colour. However, bees learn certain components over others (colour versus shape), and visual stimuli are learned in a non-additive manner with the interaction of specific colour and shape combinations being critical for learned responses. To better understand which components of the visual stimuli the bees learned, the shape-colour association of the stimuli were reversed either during or after training. Results showed that maintaining the visual stimuli in training and testing phases was necessary to elicit visual learning, suggesting that bees learn multiple components of the visual stimuli. Together, our results demonstrate a protocol for visual learning in restrained bees that provides a powerful tool for understanding how components of a visual stimulus elicits learned responses as well as elucidating how visual information is processed in the honeybee brain.

2.1 INTRODUCTION

Many pollinators exhibit astonishing abilities to navigate and locate flowers, their source of nutrients, and their ability to learn floral traits (visual, scent, morphology) has been shown to be critical for maintaining plant-pollinator relationships (for review, Chittka et al., 1999). While seeking out flowers of a particular species while ignoring others, the amount of information perceived by their peripheral system exceeds several orders of magnitudes their brain processing capacity. Attention provides an animal with the ability to attend to one stimulus while filtering out non-relevant ones (Miller et al., 2012). By locking their attention on a subset of physical cues and filtering out environmental noise, they can use specific salient features of their resource. These attended features, for example floral traits (e.g., color, scent, morphology), can then be learned by pollinators.

Among insect pollinators, honeybees (Apis mellifera) were an early model for studying visual preferences and learning and memory due to the honeybee's ability to rapidly learn and retain new associations (Lubbock, 1882; Von Frisch, 1914). Since these early studies, researchers have extensively characterized honeybee's vision (including colour vision and shape detection)(e.g., Srinivasan and Lehrer, 1988; de Ibarra and Giurfa, 2003; Niggebrügge and de Ibarra, 2003; Srinivasan, 2006) and the associated photoreceptors and neural pathways (e.g., Menzel, 1979; Meyer, 1984; Backhaus, 1991; Peitsch et al., 1992; Vorobyev et al., 2001; Wakakuwa et al., 2005; for review see de Ibarra et al., 2014), making bees one of the most studied organisms for vision after primates (Web of Science). Concurrent with these neurophysiological and behavioural studies, visual learning experiments have shown that honeybees exhibit a wide range of visual cognitive abilities, from a classical association of colour, pattern or orientation with a reward (e.g., Srinivasan et al., 1994; Giurfa et al., 1996a; Giurfa et al., 1996b; Giurfa et al., 1997; de Ibarra et al., 2001)

to complex high-order cognitive tasks such as the Delay-Matching-To-Sample task (e.g., Giurfa et al., 2001; Avarguès-Weber et al., 2011; Avarguès-Weber et al., 2012), an ability that was for long believed to exist only in vertebrates. Moreover, bees are great examples of the importance of attentional processes during navigation and learning. They can detect coloured targets using serial visual search (Spaethe et al., 2006), increase the accuracy of their decision when errors are punished (Chittka et al., 2003) and adapt their decision latency to the difficulty of the task (Dyer and Chittka, 2004).

Despite the abundant work on visual preferences, only a handful of studies have been able to demonstrate visual learning in tethered bees, and those learned responses are extremely weak (usually $<$15% responding). However, learned responses can improve, but only after antennae are removed (Hori et al., 2006), the head is not immobilized (Dobrin and Fahrbach, 2012), or the number of trials is dramatically increased (Kuwabara, 1957; Masuhr and Menzel, 1972; Hori et al., 2007; Letzkus et al., 2008; Mota et al., 2011). Learned responses by tethered bees that were comparable to olfactory Proboscis Extension Response (PER) conditioning were only reached when odours were used in combination with colour (Gerber and Smith, 1998), in a context dependent manner with odours (Mota et al., 2011) or when motion was added to the visual stimuli (Balamurali et al., 2015). Furthermore, Niggebrügge and coworkers observed similar level of performance using a differential conditioning procedure in bees with impaired antennae but with poor colour discrimination during the memory test (Niggebrügge et al., 2009). Interestingly, successful visual PER was possible using polarized light (Sakura et al., 2011) or with Africanized honeybees (Jernigan et al., 2014). Most of the visual PER protocols consisted of the illumination of the entire experimental setup with a light of specific wavelength, thus restricting the use of configurational features (e.g., relative position of two stimuli such as above/below or right/left). On the other hand, using

tethered walking bees in a virtual environment would provide the opportunity to maintain the bees in a behavioural context where they are actively sensing their visual environment. Furthermore, such an approach would enable fine-scale quantitative, qualitative and temporal control of the visual stimuli experienced by the bees, as well as allow testing of simple and complex learning tasks in behavioural open- and closed-loop visual contexts. Would bees' learning performances be more comparable to free-flying insects when they are actively behaving? Do they use the attributes of a visual object (e.g. shape, colour) independently or do they merge them into an "object token" (sensu Kanwisher and Driver, 1992) that is the target of their visual attention? To shed more light on these open questions, we developed a new paradigm for visual conditioning in tethered walking honeybees based on their locomotor response and fixation behaviour. Importantly, the system provides feed-back to the behaving animal – similar to the operant control experienced by free-flying bees – that is critical for the learned responses. In the present work, we used a differential conditioning procedure with pairs of stimuli differing in either shape or colour or both, to test: 1) whether bees readily learn visual object-like stimuli; 2) if learning performances are function of the stimulus characteristics; and 3) whether bees learn single or multiple components of a visual stimulus, and if certain combinations are more salient than others.

2.2 MATERIALS AND METHODS:

2.2.1 Honeybee collection and preparation

Approximately 600 forager honeybees from four different hives were used in visual learning experiments; a total 87 bees were discarded due to absence of response to sucrose prior to the experiment, low level of fixation to the visual stimuli (<1 s), or low walking speeds (<5 mm/s) over the course of the pre-test and test phase. Forager honeybees were collected on artificial sucrose feeders (50% w/w, Sucrose

4097, J.T.Baker) placed near or at the entrance of two indoor hives (winter 2015) and two outdoor hives (spring through fall 2016). In order to ensure that the honeybees were behaving similarly between rearing conditions and collecting sites, we compared the distance walked during pre-test (20 sec.) and the level of performance of trained honeybees (i.e., the proportion of bees that changed preference) when presented blue square and green circle (described hereafter). There was no significant difference between indoor and outdoor hives for both the walked distance (indoor hive: 94.1 ± 11.6 mm; outdoor hive: 89.3 ± 10.5 mm; mean ± sem; t-test, indoor vs. outdoor, t= 0.30, df = 31.98, p = 0.76, sample size: 19, 15) and learned preferences of the trained bees (z-score test, indoor vs. outdoor, 0.63 vs. 0.67, p=0.83, sample size: 19, 15). Indoor hives consisted of small colonies (around 2000 workers and a queen) obtained at the beginning of Fall (September 15 onward). Hives were connected to boxes (90 X 90 X 90cm or 90 X 60 X 60cm) filled with plants known to be pollinated by bees and present in Seattle, WA USA. Feeders filled with 50% sucrose solution (w/w, Sucrose 4097, J.T.Baker) were placed at different locations in the box. A homemade pollen mixture was inserted into the hives every two weeks and consisted of 5 doses of fresh pollen (Brushy Mountain Bee Farm) for 2 doses of 50% sucrose solution (w/w). Hives were kept under a 16h/8h Light/Dark cycle by two lamps (one in top of each box, Galaxyhydro 300w LED Grow Light Full Spectrum and Zoo Med ReptiSun T5 HO Terrarium Hood), at 28±2°C and 70±10% humidity. The humidity and temperature were following the light cycle (increase at dawn/decrease by dusk). Forager honeybees were collected the day before the experiments and kept at 29±1°C and under 80±1% humidity in containers (dimensions: 8 X 4 X 5 cm or 11 X 7.5 X 8.5 cm), with a 30% (w/w) sucrose solution (Sigma Aldrich St Louis, USA) available ad libitum and under a 16h/8h Light/Dark cycle. Before experiments, bees were anaesthetized on ice and tethered by the thorax to a metal wire using UV-activated glue (3:1 mix of Loctite 3104 Light Cure Adhesive, Loctite, Düsseldorf, Germany and UV Glue

GL114, JewelrySupply.com, USA). After tethering, bees were fed with up to 30 μL (indoor hives) or 15 μL (outdoor hives) of 30% (w/w) sucrose solution and allowed to recover for at least one hour in a dark, warm and humid environment (25±1°C, 55±5% relative humidity).

2.2.2 Experimental Setup

Locomotion compensator

The locomotion compensator is an omnidirectional treadmill used to measure the planar trajectories (translations and rotation on the plane) of walking bees (Fig. 1A). The compensator comprises: a light plastic ball (W9989 Jumbo Table Tennis Balls 55mm), a custom designed 3D printed (Stratasys, uPrint SE) support structure adapted from (Moore et al., 2014), an aquarium pump (Danner, Air pump, AP-4), and two optic laser sensors (ADNS-9800 Laser Motion Sensor). The laser motion sensors are sampled at 50 Hz using a microcontroller (Red Board, Sparkfun Electronics). The support structure is a cylindrical chamber with a concave recess at the top; air pumped into the chamber flows out through small perforations into the recess. The ball floats in this cup, suspended on a cushion of air, allowing low friction rotation about any axis. Two optical sensors are positioned at the equator at the rear of the ball, 90° apart. These sensors measure the translational optic flow (u and v) of the rotating ball. A bee, tethered to a rigid post on the dorsal side of the thorax, is suspended above the floating ball such that its tarsae engage the surface, rotating the ball while walking. The incremental forward and lateral translations (Δx and Δy, respectively) and yaw rotation ($\Delta\theta$) of the walking bee

are calculated as:

$$\Delta x = \frac{(v_1 + v_2)}{\sqrt{2}} \tag{2.1}$$

$$\Delta y = \frac{(v_1 - v_2)}{\sqrt{2}} \tag{2.2}$$

$$\Delta \theta = \frac{(u_1 + u_2)}{d} \tag{2.3}$$

Where u_i and v_i are the optical motion measurements of the ith sensor (scaled to units of mm) and d is the diameter of the ball (Fig. S1A). The current heading, $\theta(t)$, is calculated as the cumulative sum of angle increments:

$$\theta_n = \sum_{i=1}^{n} \Delta\theta_i \tag{2.4}$$

Visual display

The locomotion compensator sits at the center of a cylindrical visual arena (frosted mylar, 20 cm diameter, 30.5 cm high), subtending 330 with a 30° opening in the rear to allow access to the bee and the compensator (Fig. 1A). A video projector (Acer K132 WXGA DLP LED Projector, 600 Lumens) positioned above the arena projects the visual stimuli downwards onto the mylar screen; the image transformation to project planar patterns onto the cylindrical surface are calculated using the Panda3D package (Carnegie Mellon Entertainment Technology Center) for Python 2.7. The visual stimuli are either presented stationary or rotated in closed-loop as a function of the calculated bee heading (i.e., the visual scene moves laterally with the rotation of the ball). Presentation of visual stimuli in closed-loop (e.g. during the testing phases, see below) provides feed-back and behavioural responses similar to the operant control experienced by free-flying bees, whereas presentation of static visual stimuli in open-loop (e.g. during training phases) ensures that bees are not learning information about the position of the visual objects but only their features.

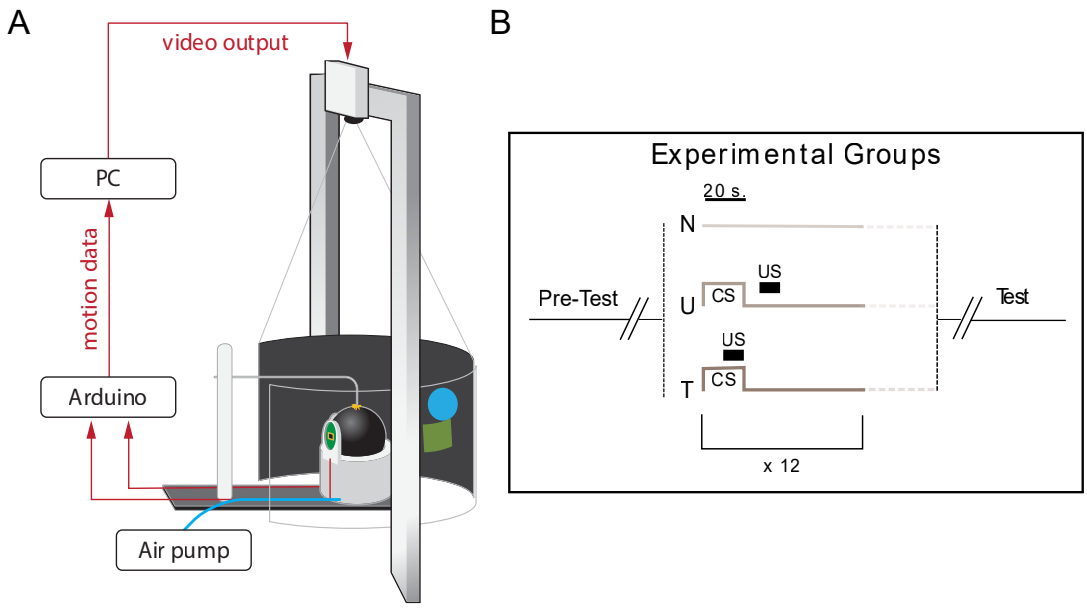

Fig. 1. Arena, visual stimuli and experimental groups. (A) The arena is composed of a locomotion compensator, a screen and a projector. Data from the locomotion compensator is collected via arduino and sent to a computer to control the visual stimuli from the projector. (B) Three experimental groups were tested: naive, unpaired and trained. All groups were exposed to the same visual stimuli during the pre-test and test phases; unpaired and trained groups were exposed to visual stimuli during training. One trial comprised the projection of a stimulus for 20 seconds temporally paired (trained) or not (unpaired) with a reward or a punishment (sucrose or quinine) for 10 seconds and the projection of the ITI pattern during 60 seconds.

Visual stimuli

Visual stimuli – here abbreviated by Blue [B], Green [G], Square [S], and Circle [C] – were composed of the following combinations of colours and shapes: blue circle (BC), blue square (BS), green circle (GC) and green square (GS). The stimuli were similar in brightness (blue stimulus: peak at 451 nm, 18 lux; green stimulus: peak at 537 nm, 21 lux; Fig. S1B) and surface area (circle: 20 cm2; square: 25 cm^2). All experiments were conducted under red light (Bulbrite light bulb, 130V, 10W, red; covered by a red filter: Rosco Roscolux Medium Red, Lighting Filter). Stimuli were positioned on the horizon of the screen, aligned with the top of the locomotion compensator corresponding to a visual angle of 28°. The visual angles of both stimuli were above threshold for discrimination against background and chromatic perception (Giurfa and Vorobyev, 1997). Visual stimuli were displayed on a black background, as this elicited the strongest phototactic responses compared to other backgrounds (Fig. S2). In addition to the blue and green stimuli, during periods between training sessions (described below), a closed-loop pattern of multiple human-grey bars (peaks at 630 [maxima], 451 and 537 nm, 2 cm wide, evenly spaced by 3 cm; 28 3 lux) were used. The pattern of grey bars was only used during inter-trial intervals (ITI). A single bar of the grey pattern and the space between two bars corresponded both to a visual angle of approximately 11°. Spectral characteristics of the visual stimuli were obtained by measuring the relative irradiance of each stimulus (Ocean Optics, USB2000+ Spectrometer, Ocean view software, calibration light HL-2000).

2.2.3 Experimental procedure

After recovering from the cold-induced immobilization and tethering procedure, responsive bees were identified using the proboscis extension reflex (PER) by touching the antennae with a 50% (w/w) sucrose solution. Bees were allowed to accli-

mate in the arena for 2 min, after which they experienced the visual stimuli. An assay consisted of three sequential phases of stimuli presentation: pre-test, training, and testing. During pre-test and testing phases, all groups were exposed to two visual stimuli (e.g., GC - BS) under closed-loop conditions (20 seconds each; Fig. 1B). At the beginning of those phases, the stimuli were located at +40° and -40° (0° being in front of the bee) and their order (e.g. square - circle versus circle - square) was randomized between bees. In addition, trained and unpaired bees were presented combinations of stimuli, rewarded and punished, as described in the experimental groups below. Between stimuli presentations, the Inter-Trial Interval (ITI), bees were presented with a pattern of multiple grey bars in closed-loop. Experimental groups were as follows:

Naive group:

Between the pre-test and test phases, naïve bees were exposed, under closed-loop conditions, to the same pattern of multiple grey bars as displayed during ITIs (see Unpaired and Trained groups). The time elapsed between both phases was equal the total duration of the training phase (1140 sec). This treatment controlled for exposure to the experimental setup and the duration of the experiment.

Trained group:

During training, 50% w/w sucrose was used as a reward (positive unconditioned stimulus; US+) and 60 mM quinine was used as a punishment (negative unconditioned stimulus; US-). One of the two visual stimuli was rewarded (conditioned stimulus, CS+) and the other one punished (CS-). During a trial, one of the two conditioned stimuli was presented individually in open-loop for 20 seconds and centered on the screen (i.e., 0° on the azimuth). This centred stationary presentation during training ensured that the bees learn the stimuli components and not its position. Ten seconds after the onset of the CS, the US was delivered to both

antennae for 10 seconds (Fig. 1B). If the bee exhibited a PER, she was allowed to drink a drop of the solution. Bees were exposed to 1 minute of ITI in closed-loop between trials and 2 minutes of the ITI pattern at the end of the training session. The training phase consisted of 6 presentations of each stimulus in a randomized order. To control for innate visual preferences at the individual level, the stimulus that was preferred during the pre-test phase was systematically punished and the non-preferred stimulus systematically rewarded during training.

Unpaired group:

For the unpaired group, rewards and punishments were delivered in a pseudo-random order, as described in the trained group, but without temporal contingency with the CS (i.e., a reward or punishment was delivered during the ITI, also in a randomized manner, and for 10 seconds; Fig. 1B).

For all groups, the time spent walking towards each visual stimulus was used to determine the individual preference for the stimulus, with the preferred stimulus corresponding to the longest fixation time. Fixation was defined as the insect maintaining a stimulus within the interval of [-20, 20°] (i.e., directly in front of the bee). All bees walking less than 10 mm, or those that did not fixate ($<$ 1 sec) were discarded from the analysis ($<$12% of bees).

2.2.4 Experimental series

A series of experiments were conducted to assess: (a) whether bees can learn to discriminate between two stimuli that differed in their shape and colour; (b) whether bees can learn to discriminate stimuli that differed in only one component (either shape or colour); and (c) whether single or multiple components of the visual stimuli are learned by the bees (Table 1). Experimental treatments are described below:

- To test whether bees learn to discriminate between two stimuli that differ

in their shape and colours (Table 1a). In this first experimental series, bees were trained with visual stimuli that differ in both shape and colour (i.e., GC versus BS) over the course of 12 trials (6CS+/6CS-). The stimuli were different in both shape and colour, thus potentially enhancing training success. To ensure that learned responses were not a function of a specific combination of colour and shape, we also trained a group of bees to GS versus BC. Control groups of naive and unpaired bees were also tested, and groups were run in parallel. We also examined the acquisition rate of the learned responses, where bees were exposed to the same protocol as for the trained group but with either 4 trials (2CS+/2CS-) or 8 trials (4CS+/4CS-) between the pre-test and test phases.

- To test whether bees discriminatively learn when trained with visual stimuli that differ in colour but not shape (i.e., BS versus GS; or BC versus GC) or in shape but not colour (i.e., GS versus GC; or BS versus BC)(Table 1b), bees were trained with visual stimuli over the course of 12 trials (6CS+/6CS-). For each set of stimuli, a control group of naive bees were also pre-tested and tested in parallel.

- In the last series of experiments, the shape-colour association of the stimuli was reversed either after or before training (i.e. after the pre-test phase)(Table 1c). The aim of this experimental series was to examine if bees learned single or multiple components of the visual stimuli over the course of training by decoupling the relation between shape and colour. To examine this, two different sets of stimuli were used in two experimental groups: BS versus GC and BC versus GS. In the first set of experiments, bees were pre-tested and trained with GC versus BS, and tested with BC versus GS; this experiment allowed testing of whether bees learned only one component of the stimulus, because if bees learned one component during their training phase then they

should respond to that component in the test phase regardless of the presentation of the other component (e.g. if bees learn the colour blue when trained to BS, then they should also respond to BC in the test). In this experiment, a control group of naïve bees was tested in parallel.

Experimental series	Pre-test	Training	Test
A			
BS versus GC	BS versus GC	BS versus GC	BS versus GC
BC versus GS	BC versus GS	BC versus GS	BC versus GS
B			
Shape only: B	BS versus BC	BS versus BC	BS versus BC
Shape only: G	GS versus GC	GS versus GC	GS versus GC
Colour only: S	BS versus GS	BS versus GS	BS versus GS
Colour only: C	BC versus GC	BC versus GC	BC versus GC
C			
Constancy pre-test and training	BS versus GC	BS versus GC	GS versus BC
Constancy training and test	BS versus GC	BC versus GS	BC versus GS

Table 1. Visual stimuli presented to honeybees during pre-test, training and test for all the experimental series.

In the second set of experiments, bees were pre-tested with GC versus BS, and then trained and tested with GS versus BC; this experiment allowed testing of whether bees learned the shape-colour combination, because if bees learned the combination then changing the stimuli between training and test would negatively impact their learning performance, while their performance should not be affected by changing the stimuli between pre-test and training. The assignment of the rewarded and punished CS was randomized and learning performance was assessed by comparing

bees' performances with an unpaired group. The unpaired group was used as a control because quantifying learning based on a change of bees' preference would have introduced confounding factors (i.e. the training is applied on stimuli that differ from the pre-tested set of stimuli). In the unpaired group, the rewarded and punished CS were randomized.

2.2.5 Statistical Analyses

All statistical analyses were conducted using R (R Core Team, 2016) and the package 'Plotly' (Sievert et al., 2016) for the creation of 2D histograms. The standard error of a proportion was calculated as (Le, 2003):

$$se = \frac{\sqrt{p * (1 - p)}}{n} \tag{2.5}$$

. A binomial test was used to compare proportion of change to a random choice (p = 0.5) and a multiple-sample proportion test (e.g., z-score test) to compare two or more proportions. Two-sample t-tests were used to compare between walking distances or fixation times after verification of normality, and Wilcoxon tests were performed for non-normal data. For the second experiment in experimental series (c), we used two different approaches to calculate the proportion of "correct choice" (defined as the proportion of bees responding to the rewarded stimulus) in the control group: (1) determining the proportion of correct choices for the unpaired group when experiencing the same sequence of CS+/CS- stimuli as the trained group; and (2) conducting a permutation test by resampling the choices from the trained sequence 1000 times and comparing those results to the proportion of correct choices from the unpaired group using a binomial exact test.

2.3 RESULTS

2.3.1 Visual training changes the innate preference of honeybees

During the first 20 s of the pre-test to the visual stimuli (a green circle and a blue square), honeybees fixated one or both visual stimuli for 8.8 ± 3.56 sec. (mean ± sem)(Fig. S3). Innate preference did not influence the amount of time fixating the stimuli (t-test, blue square vs. green circle, t = 1.62, df = 76.25, p = 0.11) (Fig. S3). Similar levels of fixation were observed during the test phase as well (t-test, t = 1.47, df = 98.11, p = 0.14) and across experiments (i.e., with different sets of stimuli). When first exposed to those stimuli in closed-loop, the majority of bees innately preferred the green circle ($P_{GC} = 0.65$, binomial exact test, $p<0.001$). In the absence of conditioning, naïve bees remained constant in their visual preferences between the pre-test and the test phase ($P_{GC,\ pre\text{-}test} = 0.70$ and $P_{GC,\ test} = 0.73$, z-score test p=0.8; Fig. 2A). By contrast, trained honeybees were significantly more prone to switch their preferences between pre-test and test phases ($P_{switch,\ trained} = 0.65$ and $P_{switch,\ naïve} = 0.33$, z-score test, $p<0.01$; Fig. 2A). An independent group of bees were presented "unpaired" visual stimuli and reward, the temporal relation between the cognate CS and US was disrupted. This group did not differ from the naïve group in terms of its change of preference ($P_{switch,\ unpaired} = 0.4$ and $P_{switch,\ naive} = 0.33$, z-score test, p=0.52; Fig. 2A). And similar to naïve bees, the unpaired group was also significantly less prone to change preference than the trained group ($P_{switch,\ trained} = 0.65$ and $P_{switch,\ unpaired} = 0.4$, z-score test, p=0.04; Fig. 2A), demonstrating the associative nature of their learning.

To also test whether bees could learn the blue circle (BC) versus the green square (GS), honeybees were trained with those stimuli. When first exposed to this set of stimuli, naïve honeybees did not have an overall preference as a group for either stimulus ($P_{GS} = 0.63$, binomial exact test, p=0.20, sample size = 30), and were constant in their choice (P_{GS}, pre-test = 0.63 and P_{GS}, test= 0.67, z-score test, p=0.79)

but trained honeybees were significantly more prone to switch preferences between the pre-test and the test phase ($P_{switch,\ naive}$ = 0.30 and $P_{switch,\ trained}$ =0.60, z-score test, p=0.04, sample size = 30, 20; Fig. S4). We next examined the influence of the number of training trials for the acquisition of visual memory. As training trials increased from 4 to 12, the change in visual preference by trained honeybees correspondingly increased ($P_{switch,\ trained}$, 4t = 0.4 and $P_{switch,\ trained}$, 12t = 0.65, z-score test, p=0.04; Fig. 2B).

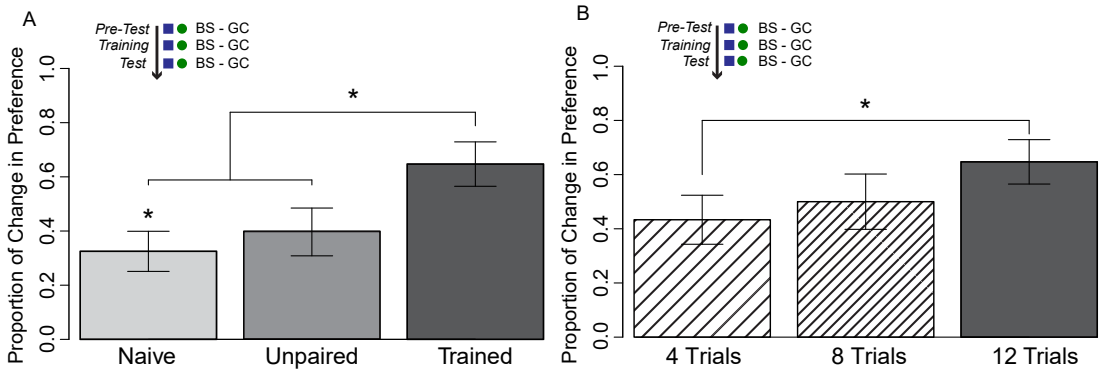

Fig. 2. Learned responses of honeybees exposed to a green circle and a blue square. (A) Proportion of bees that changed their stimulus preference between the pre-test and test phases for naive, unpaired and trained bees. Asterisks denote p<0.05; sample sizes were 40, 30, and 34 for naive, unpaired and trained groups, respectively. (B) Proportion of change in stimulus preference between pre-test and test phases for bees trained with 2, 4 or 6 paired trials. Learning performance increased with the number of trials, with 6 trials being significantly different from 2 trials (asterisk denotes z-score test: p<0.05; sample sizes were 31, 24 and 34 bees for the 2, 4 and 6 training trial groups, respectively). Insets for (A) and (B) are the set of stimuli used for this experiment.

The bee's visual preferences and learned responses were confirmed by quantitative analysis of the bee's fixation time to the visual stimuli (sensu Paulk et al., 2014)(Fig. 3). Naïve bees spend significantly more time in front of their innately

preferred stimulus during both pre-test and test phases (Fig. 3A; Wilcoxon test, pre-test: V = 820, p <0.001; test: V = 571, p=0.03; sample size: 40). Unpaired bees did not spend more time fixating the innately preferred stimulus during the test phases (Fig. 3B; Wilcoxon test, pre-test: V = 465, p<0.01; test: V = 258, p = 0.61). By contrast, learned bees significantly shifted their fixation time: during the pre-test they spent more time in front of the CS- than the CS+, but during the test phase they spent more time in front of the CS+ than the CS- (Fig. 3C; Wilcoxon test, pre-test: V = 253, p<0.001; test: V = 0, p<0.001). Examining the temporal dynamics of the locomotor response, we observed that the distribution of cumulative angles was similar between the pre-test and test phases for all groups except for the trained bees that changed their stimulus preference, as these bees walked more in the direction of the rewarded stimulus during the test phase (Fig. 3). Finally, similar level of walking speed was found over the course of the experiment and across experimental treatment (i.e., naïve, unpaired and trained groups; Figs. S5, S6). Together, these results show that both the fixation time and the bee preference during the test phase captured the significant visual learned responses in the trained group (Figs. 2,3).

2.3.2 The effect of shape or colour alone on the learned responses by honeybees

To better understand the contribution of different visual features in honeybee learning, bees were trained to shape or colour alone (e.g., BC versus BS, or BC versus GC). Across all treatment groups (naïve and trained), when honeybees were pretested with stimuli that differed in their shape alone, they innately preferred the square form when both stimuli were green ($P_{\text{square | green}} = 0.65$, binomial exact test, p=0.04) but did not show any shape preference when both stimuli were blue ($P_{\text{square | blue}} = 0.53$, binomial exact test, p=0.77). Nonetheless, there was an interaction between the treatment group and the components of the visual stimuli. Naïve honeybees were constant in their shape preference when the shapes

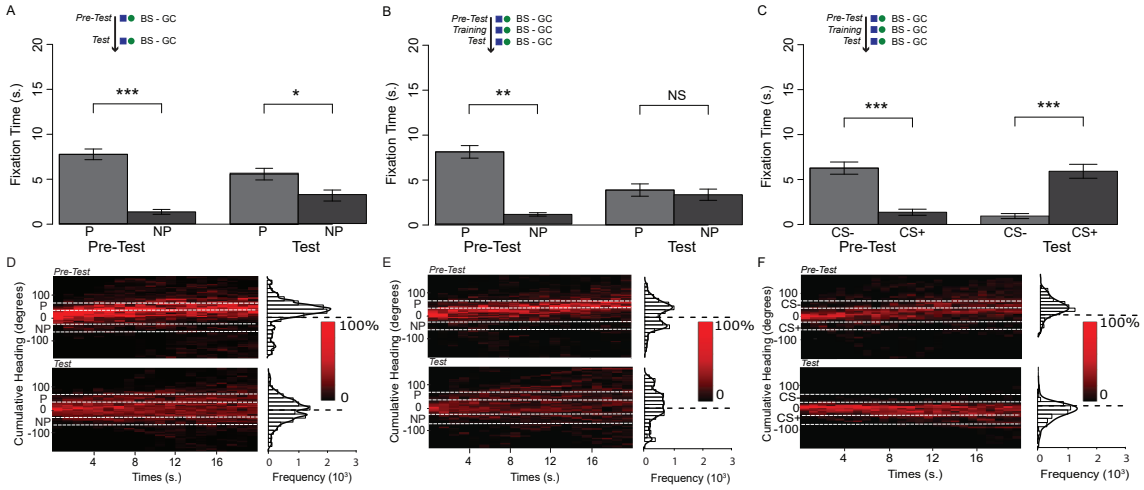

Fig. 3. Fixation time and temporal dynamic of locomotion responses for the naive (A, D), unpaired (B, E) and learners of the trained group (C, F) during the pre-test and test phases. (A, B, C) Time spent fixating the preferred stimulus (P) or CS- and the non-preferred stimulus (NP) or CS+ during pre-test and test phases for naïve (A), unpaired (B) and trained bees (learners, C). Naïve bees spend significantly more time in front of their innately preferred stimulus during both pre-test and test phases (Wilcoxon test, pre-test: V = 820, p <0.001; test: V = 571, p=0.03; sample size: 40). Unpaired bees did not spend more time fixating the innately preferred stimulus during the test phases (Wilcoxon test, pre-test: V = 465, p<0.01; test: V = 258, p = 0.61) whereas trained bees spend more time in front of the CS- than in front the CS+ during pre-test but more time in front of the CS+ than in front of the CS- during the test phase (Wilcoxon test, pre-test: V = 253, p<0.001; test: V = 0, p<0.001). (D, E, F) Temporal dynamics of the locomotor response during the pre-test and test phases for naïve (D), unpaired (E) and trained (learners, F) groups. Trained bees walked and oriented in the direction of the rewarded stimulus during the test phase compared to the pre-test phase. Histograms to the right of the surface plots represent orientations (cumulative angles) for the 5 last seconds of the response. Trained bees shifted their orientation toward the CS+ in the test phase. Insets for (A), (B) and (C) are the set of stimuli used for this experiment.

were green ($P_{\text{circle, pre-test}} = 0.28$ and $P_{\text{circle, test}} = 0.40$, z-score test, p=0.37; Fig. 4A), whereas, when the shapes were blue, naïve bees did not maintain their preference over the course of the experiment ($P_{\text{switch | green}} = 0.36$ and $P_{\text{switch | blue}} = 0.60$, z-score test, p=0.02; Fig. 4A). Trained honeybees did not learn shape stimuli, regardless of colour, exhibiting responses not significantly different from naïve bees ($P_{\text{switch, naïve | green}} = 0.36$ and $P_{\text{switch, trained | green}} = 0.42$, z-score test, p=0.68; $P_{\text{switch, naïve | blue}} = 0.6$ and $P_{\text{switch, trained | blue}} = 0.5$, z-score test, p=0.48; Fig. 4A). When bees were first exposed to stimuli that differ only in their colour and not shape, bees significantly fixated on the green stimulus ($P_{\text{green, pre-test}} = 0.73$, binomial exact test, $p<0.001$) regardless of shape. For naïve honeybees, this green preference was constant between the pre-test and test phases ($P_{\text{green, pre-test}} = 0.73$ and $P_{\text{green, test}} = 0.65$, z-score test, p=0.20; Fig. 4B). When trained with blue versus green squares, honeybees did not learn the predictive value of the colour of the stimuli ($P_{\text{switch, naive}} = 0.32$ and $P_{\text{switch, trained}} = 0.41$, z-score test, p=0.53; Fig. 4B). However, circles elicited a different learned response: when bees were trained with blue versus green circles, bees significantly changed their preference compared to naïve honeybees ($P_{\text{switch, naive}} = 0.33$ and $P_{\text{switch, trained}} = 0.64$, z-score test, p=0.03; Fig. 4B).

2.3.3 Bees learn unique combinations of visual stimuli

In the previous experiments, bees could learn to correctly choose between a green circle and a blue square, a blue circle and a green square, or between two coloured circles, but it was unclear whether one or both components (i.e., shape or colour; shape and colour) of the stimulus were learned. To answer this question and further explore how keeping the visual stimuli constant over the course of the experiment impacts visual learning, we exposed honeybees to two different experimental series. The first experimental series swapped the colour-shape combination between the pre-test and training/testing phases of the experiment, and the second experi-

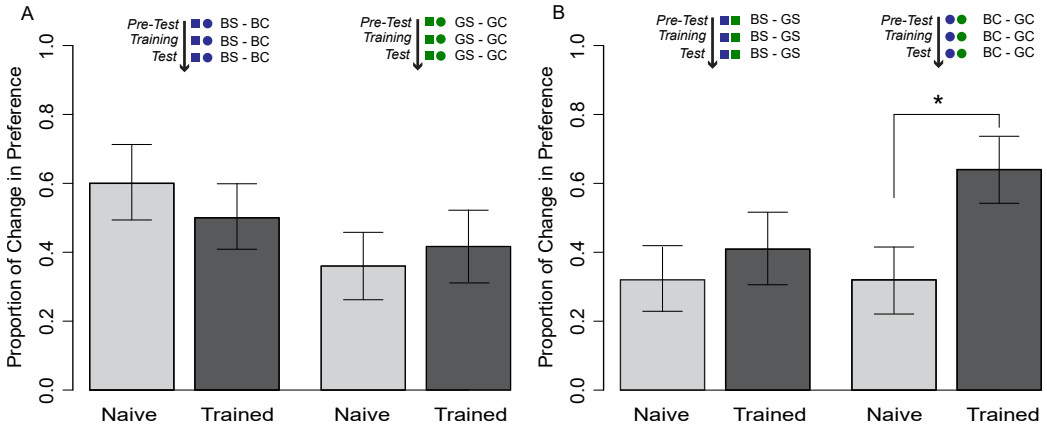

Fig. 4. Learned responses of honeybees exposed to stimuli of similar shapes or colours. (A) Proportion of change in stimulus preference between pre-test and test phase for shape alone (blue square versus blue circle and green square versus green circle) for the naive and trained bees (sample size: n=25, 24, 25, 24). (B) Proportion of change in stimulus preference between pre-test and test phases for blue square versus green square and blue circle versus green circle for naive and trained bees. Bees could learn to fixate the rewarded stimuli for the set of circles (asterisk denotes z-score test, $p<0.05$) but not for the set of squares (sample sizes: n=25, 22, 25, 25).

mental series swapped the colour-shape combination between the pre-test/training and testing phases. Our reasoning is the following: if a single component of the visual stimuli is learned, the lack of maintaining the visual stimuli between training and test should not impact learning performance. For instance, if bees were trained on and learned a colour, then the bees should respond to the learned colour irrespective of its shape. Conversely, if bees learn the combination of shape and colour, we expect changing the stimuli between training and test to impact their learning performance, while their performance should not be affected by changing the stimuli between pre-test and training. This latter was tested in a second experimental series. In the first experimental series, when control (naïve) honeybees were exposed to a green circle and a blue square during pre-test, and then to a green square and blue circle during the test phase, without any training in between, the bees exhibited a random preference to both visual stimuli ($P_{green} = 0.64$, binomial exact test, p=0.18). Similarly, trained honeybees that were exposed to a green circle and a blue square during pre-test and training, and then to a green square and blue circle during the test phase, did not show a preference during the test ($P_{green} = 0.65$, binomial exact test, p=0.26). Moreover, during the test phase, trained bees did not show a preference for the colour of the rewarded stimulus, here denoted as the "correct choice", and control bees showed similar responses for the stimuli they initially rejected ($P_{\text{correct choice, naive}} = 0.54$, z-score test, p=0.85; $P_{\text{correct choice, trained}} = 0.50$, p=0.81; Fig. 5A). For example, among the bees that preferred the green circle in pre-test, half preferred the blue circle (shape preference) and the other half the green square (colour preference) during the test (Fig. 5A). In the second experimental series, when bees were pre-tested with a green circle and a blue square but trained and tested with a green square and a blue circle, bees tended to choose the rewarded ("correct") stimulus, although responses were not statistically significant ($P_{\text{correct choice}} = 0.65$, binomial exact test, p=0.18). As opposed to the previous experiments where innate preferences were negatively reinforced,

here for each individual bee the rewarded and punished stimuli were randomly assigned. The rationale was to avoid confounding factors originating from quantifying learning performances based on changes in preference where stimuli differed in both shape and colour between the pre-test and test phases. As a control, we used an unpaired group where the CS+ and CS- were also randomly assigned similar to the trained group – thereby allowing explicit control of the experienced stimuli during the pre-test versus training and test phases, something that is not possible in the naïve group. When comparing the trained and control groups, trained bees made more correct "choices" than control bees, albeit these differences were not statistically significant ($P_{\text{correct choice, trained}} = 0.64$ and $P_{\text{correct choice, unpaired}} = 0.45$, z-score test, p=0.18; Fig. 5B). To ensure that our results were not biased by the arbitrary assignment of reward sequence in the unpaired group, we conducted a permutation test by randomly resampling the choices from the reward sequence 1000 times and found no significant difference between the two methods (binomial exact test, p=0.82). Together, these results suggest that bees learn multiple components of the visual stimuli.

2.4 DISCUSSION

Our results demonstrate that bees can achieve visual learning while tethered on a locomotion compensator, and that the visual stimuli used as the CS play a critical role in the observed learning performance. Stimuli that differed in both colour and shape were consistently learned. Similarly, learning was achieved with stimuli that differed in their colour if they were circles, which may be linked to bees' natural attraction to flower-like patterns (Lehrer et al., 1995; Dafni et al., 1997). However, squares that differ in their colour and stimuli that differed only in their shape were not learned, at least in the configurations tested here. Nonetheless, we are confident that bees can distinguish between the circles and squares given their initial preferences during the pre-test phase when exposed to two green shapes. Although bees

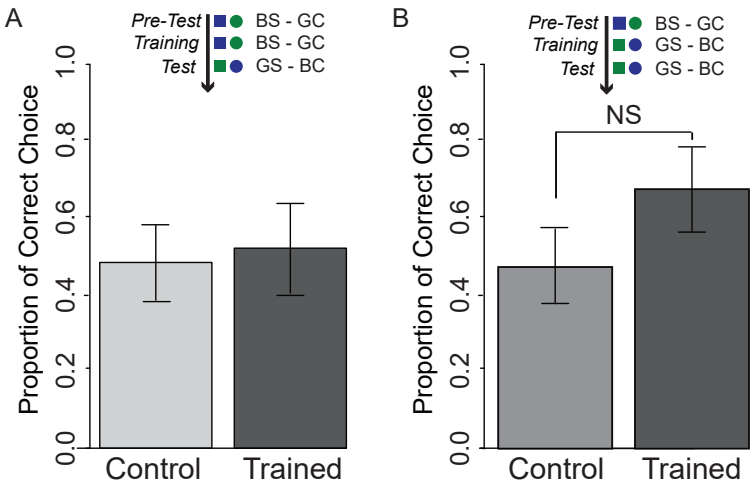

Fig. 5. Learned responses of honeybees exposed to different set of stimuli over the different phases of the experiment. (A) Proportion of bees responding to the "correct" stimulus, here defined as the stimulus that shared the same colour as the rewarded stimulus (trained group), or the stimulus they initially rejected (control and trained group). Bees were pre-tested and trained with a green circle and a blue square and tested with a green square and a blue circle. Trained honeybees did not behave differently than naïve (sample sizes were 28 and 20 for control and trained groups, respectively). (B) Proportion of bees that fixated the rewarded stimulus (i.e., correct choice) when pre-tested to a blue square and a green circle and trained and test with a blue circle and a green square. Trained honeybees fixated on the rewarded stimulus, but these responses were not significantly different from the unpaired group (z-score test, p=0.18; sample size were 20 and 28 for the control and trained groups, respectively).

are known to prefer blue over other colours (Giurfa et al., 1995), in our experiments, bees fixated preferentially the green stimulus when given the choice between green and blue objects of the same shape or between green circle and blue square. As the green stimuli have a higher intensity than the blue stimuli, this preference could be linked to a phototactic response. However, when presented a green square and a blue circle, bees did not fixate preferentially one stimulus, showing that innate preference may result from a complex interaction between shapes and colours.

Previous studies have shown that honeybees are able to learn visual stimuli on the basis of one or multiple features or components (e.g., size, contrast, orientation, bilateral symmetry)(e.g., Srinivasan et al., 1994; Giurfa et al., 1996a; de Ibarra and Giurfa, 2003; Niggebrügge and de Ibarra, 2003). Moreover, novel visual stimuli that share one or several components of the previously learned pattern could evoke subsets of the same neural representation sufficient enough to drive the learned behaviour (e.g., Ronacher, 1992; Horridge and Zhang, 1995; Horridge, 1997; Stach et al., 2004). In our experiments, bees may learn to choose between a green circle and a blue square based only on the colour or shape component of the stimuli, or, alternatively, one of the components may be predominant in the bee's perception of the stimulus. By training bees with the green circle and a blue square and then testing them with the reversed association of shape and colour, we showed that trained and naïve bees responded similarly to the novel stimuli. Both components of the stimuli may thus be implicated in the decision-making of bees when presented this ambiguous choice. It is also possible that half of the bees learned only the colours while other learned only the shapes, leading to similar results as the one we observed. Further studies could explore the relative importance of these features and their generalization in our paradigm by testing bees with stimuli of intermediate wavelength or different shapes (e.g., diamonds or stars).

Finally, we explored the importance of the pre-test phase by presenting a different set of stimuli during this phase and during the training and test phases. Analogous protocols have been used to modulate innate visual or spatial responses in diverse animals, from insects to mammals, suggesting the utility of our protocol (Tang and Guo, 2001; Bolin et al., 2012; Kent et al., 2013). As bees exhibited a strong innate preference in most of our experiments, the pre-test phase provided insight in the individual preferences and allowed us to train the bees against those innate preferences. Otherwise, the randomized assignment of the reward to one stimulus might have biased the performance level, as the experiment shown in Figure 5B suggests.

Why then, when using the PER in honeybees, do we observe such low performances in visual learning compared to the high performances of olfactory learning? Olfactory conditioning using PER may be especially relevant in the context of feeding behaviours, where taste and olfaction are coupled. By contrast, visual learning by PER may not be contextually relevant given that bees are using their vision for a wide variety of behaviours, including locomotor responses in flight, navigation and landing. This is supported by studies showing that bee performances in visual PER conditioning increased in presence of motion cues (Balamurali et al., 2015). In addition, visually oriented insects depend on the optic flow, the image shift on the retina during self-motion, as their main source of information about their 3D surrounding environment. The optic flow can be separated into translational and rotational components that can be segregate by a saccadic flight structure strategy (Collett and Land, 1975). This strategy allows the animal to enhance depth perception by actively shaping the visual inputs (e.g., Boeddeker and Egelhaaf, 2005) and is explicitly coupled to the flight and foraging behaviours of bees (e.g., Srinivasan et al., 1991; Boeddeker and Hemmi, 2010). Such active behavioural states play profound roles in modulating visual responses and processing in bees and flies (Paulk

et al., 2014; Weir et al., 2014).

Thus, the locomotion compensator may provide similar sensorimotor pathways and responses as those used by naturally behaving bees. Moreover, the treadmill and virtual environment enable a fine control of stimuli, an important feature for neuronal recordings. The experiments presented here have allowed us, using simplistic stimuli (circle and square) to demonstrate and characterize associative visual learning in tethered bees. However, virtual environments can be used to display more naturalistic stimuli. For example, it is possible to record the trajectory of a walking animal, reconstruct the panorama experienced by the animal, and replay it in the virtual environment. It is also possible to include more sensory modalities in the environment (e.g., odors) and explore, for example, multimodal learning. Implementation of depth perception can be used to explore navigation and its neural basis. Overall, locomotion compensators and virtual environment are a good intermediate between a complete tethered and unrestrained behaving animal, allowing us to explore and characterize behaviour and its related neural basis in a controlled environment and a behaving animal. The higher learning performances observed here, in comparison to most studies of visual PER conditioning, may also be linked to the presentation of discrete stimuli. In many visual PER conditioning studies (Kuwabara, 1957; Masuhr and Menzel, 1972; Hori et al., 2006; Hori et al., 2007; Mota et al., 2011), the conditioned stimulus consisted of the ambient illumination of the entire experimental area by a specific wavelength. However, it has been shown in bumblebees that a colour stimulus with strong contrast to the background is necessary for behaviour (Lunau et al., 1996; Spaethe et al., 2001). In addition to potential increase in detection and discrimination, discrete stimuli allow the experimenters to place the bees in an operant behavioural context, where bees have closed-loop control of the position of the stimuli. Behaving bees thus receive both motor and visual feedback. Discrimination performances in free-flying learning ex-

periments have been hypothesized to be dependent on attention level (Giurfa, 2004) and studies have highlighted the importance of attention-like processes for visual discrimination and fixating behaviour (e.g., Paulk et al., 2014, Van De Poll et al., 2015; de Bivort and van Swinderen, 2016). Moreover, temporal coordination in the insect brain was promoted by closed-loop behavioural control in a virtual environment (Paulk et al., 2015). It is thus possible that honeybees need visual and motor feedback to actively sense their visual environment and maintain their attention on a visual stimulus.

In summary, the methods and results described here provide a framework to examine the components of visual learning in tethered bees. In addition to fine-scale environmental control and feedback, the system provides motivation for experiments characterizing the neural bases of visual learning and memory. Major advances in our understanding of the neural basis of sensory processing have been recently made through the use of virtual reality environments, either in walking (Guo and Ritzmann, 2013; Paulk et al., 2013; Paulk et al., 2015) or flying simulators (Suver et al., 2012; Tuthill et al., 2013; Weir and Dickinson, 2015). We believe that such virtual reality environments are an important tool for characterizing learning and visually-guided behaviours and identifying the neural processes that underlie these behaviours.

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2.6 SUPPLEMENTARY INFORMATION

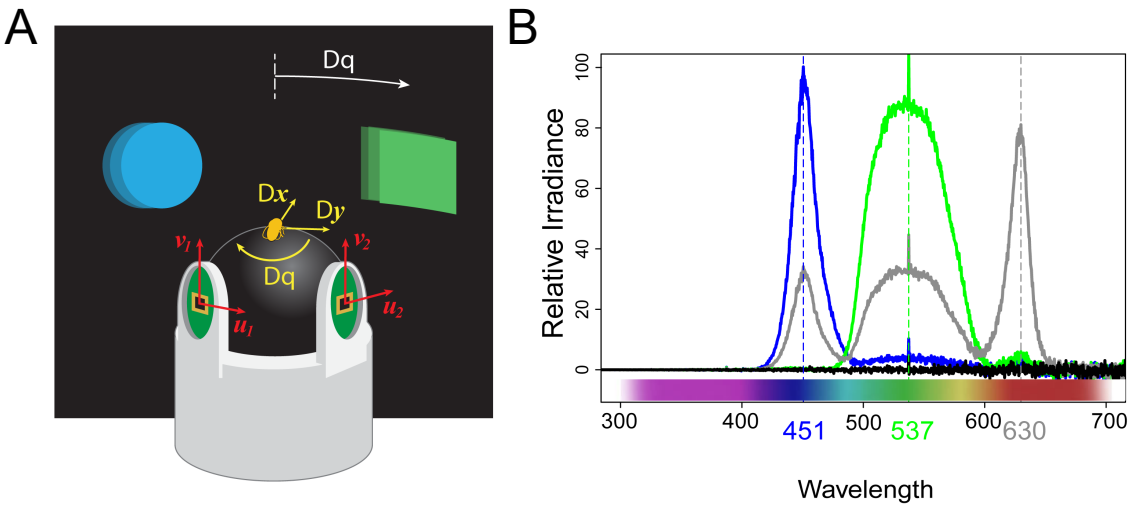

Fig. S1. Arena and visual stimuli. (A) Recording of bee motion and translation to stimuli. Rotation of the ball is recorded by optic laser sensors and optical motion measurements (ui, vi) are used to calculate the lateral translation (x, y) and yaw rotation (q) of the walking bee. Closed-loop control of the stimuli are based on this last measurement. (B) Relative irradiance of visual stimuli. Blue stimuli present a peak at 451 nm and green stimuli at 537 nm. The gray bars pattern presents peaks in the blue, green and red spectrum with similar intensity in the blue and green and a higher peak in the red spectrum (=630 nm).

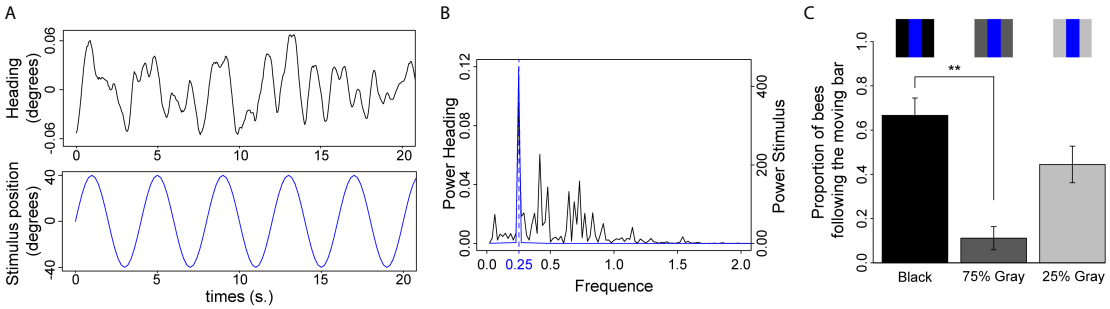

Fig. S2. Response to a moving projected stimulus. A blue bar moving as a sinusoid of 0.25 Hz was projected during 45 sec. (A) Example of the instantaneous heading (top) and stimulus position (bottom) during the experiment. (B) Associated periodogram for the visual stimuli (blue) and the bee's heading (black). (C) Percentage of bees following the stimulus for the three different backgrounds (black, level of grey 75the stimulus were projected on a black background (Friedman post-hoc test, $p < 0.01$, sample size: 9).

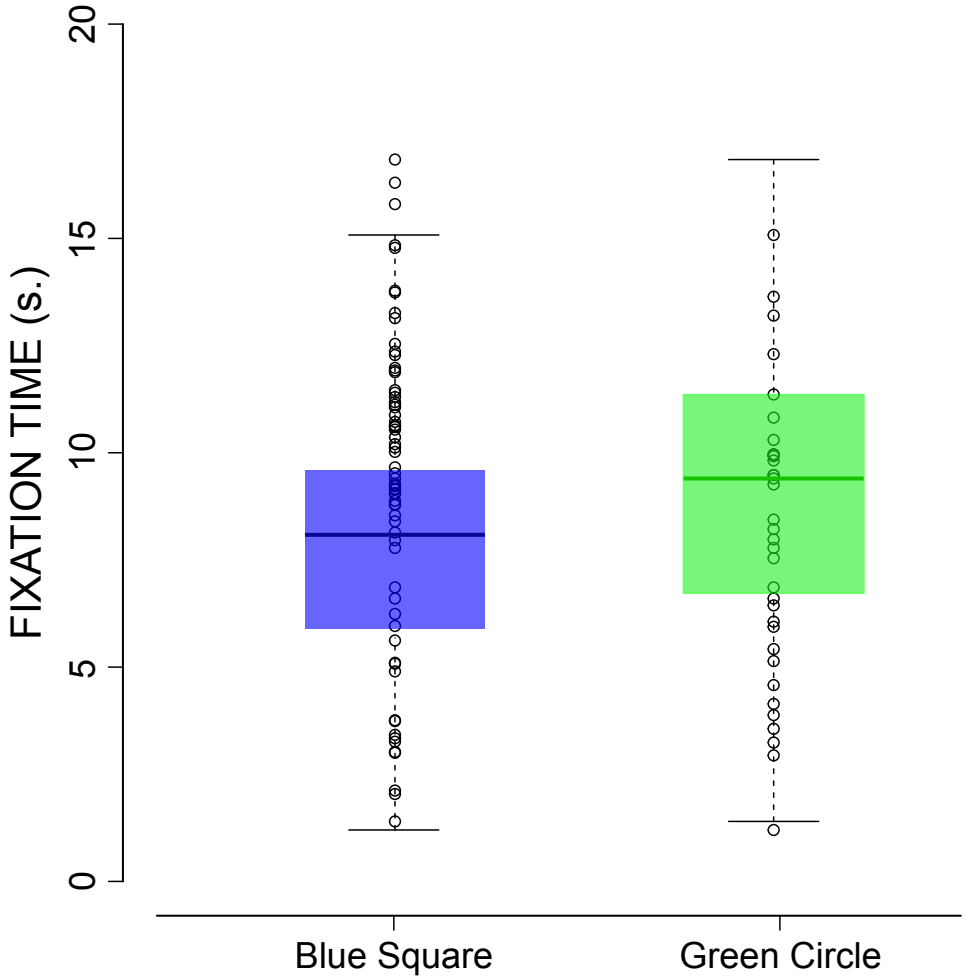

Fig. S3. Time spent fixating the stimuli during pre-test according to the innate preference for the naive, unpaired and trained bees. Bees spend similar amount of time fixating stimuli regardless of their innate preference (t-test, p=0.1095, sample size = 34, 70).

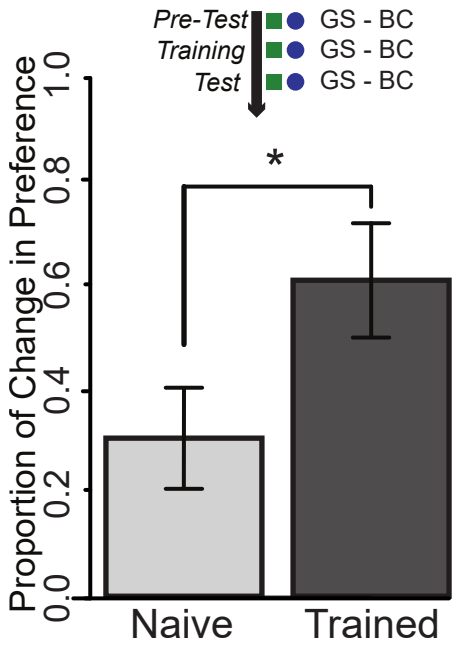

Fig. S4. Learned response of honeybees exposed to a green square and a blue circle. Proportion of bees that changed their stimulus preference between the pre-test and test phases for naïve and trained bees. Asterisks denote $p<0.05$ (z-score); sample sizes were 30 and 20 respectively.

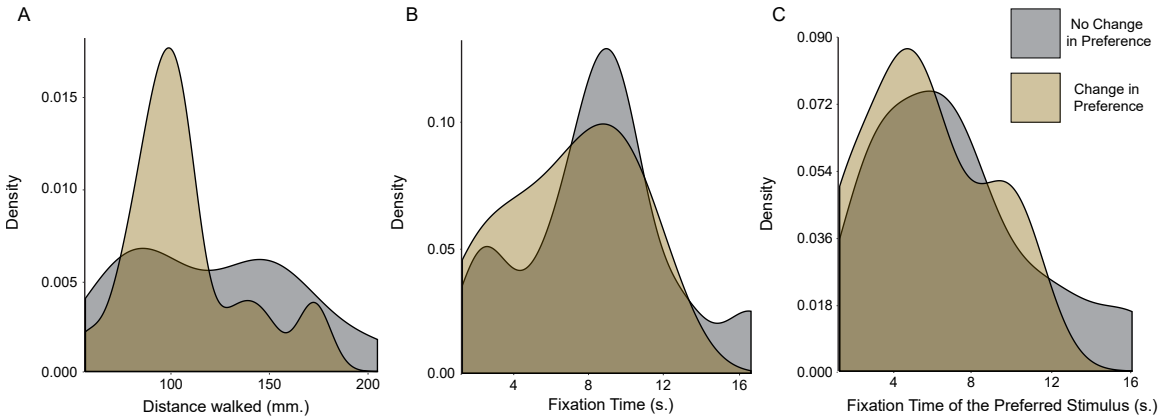

Fig. S5. Density histograms of distance walked and fixation times during the pre-test for the trained group exposed to a green circle and a blue square as a function of the change in preference between pre-test and test phases. There was no difference in the distribution of (A) distance walked (t-test, t = -0.99, df = 16.72, p = 0.34), (B) total fixation time (t-test, t = -1.03, df = 19.78, p = 0.32) and (C) fixation time of the preferred stimuli (t-test, t = 1.03, df = 17.95, p = 0.32) between bees that change preference and bees that did not (sample size: no change in preference = 12, change in preference = 22).

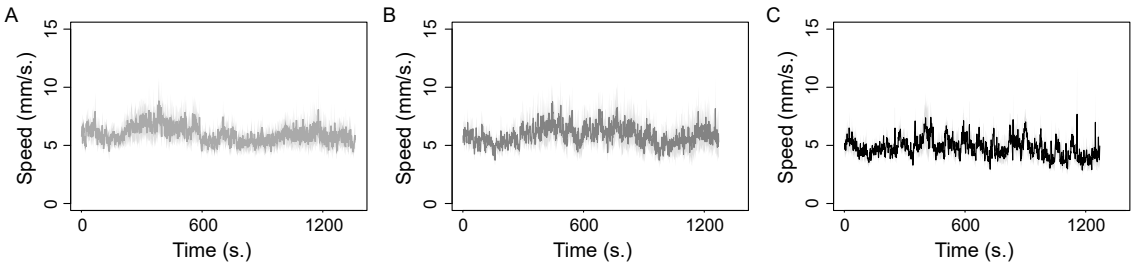

Fig S6. Average speed and associated sem of naïve (A), unpaired (B) and trained (C) groups across an experiment (pre-test, training and test with blue square and green circle). Bees walked with similar speed level during all the experiment and across treatments.

Chapter 3

BEHAVIORAL STATE-DEPENDENT MODULATION OF VISUAL PROCESSING IN THE MEDULLA OF THE HONEYBEE

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3.1 ABSTRACT

Behavioral and internal state modulation of sensory processing has been described in several organisms. In insects, visual neurons in the optic lobe are modulated by locomotion, but the degree to which visual-motor feedback modulates these neurons remains unclear. Moreover, it also remains unknown whether self-generated and externally generated visual motion are processed differently. Here, we implemented a virtual reality system that allowed fine-scale control over visual stimulation in relation to animal motion, in combination with multi-channel recording of neural activity in the medulla of a honeybee (*Apis mellifera*). We found that this activity was modulated by locomotion; however, in most cases, only when the bee had behavioral control over the visual stimulus (i.e., in a closed-loop system). Moreover, closed-loop control modulated a third of the recorded neurons, and application of octopamine (OA) recapitulated the changes in neural responses observed in a closed-loop. Additionally, in a subset of modulated neurons, fixation on a visual stimulus was preceded by an increase in firing rate. To further explore the relation-

ship between neuromodulation and adaptive control of the bee's visual environment, we modified the sensitivity of motor gain while locally injecting an OA receptor antagonist into the medulla. Whereas honeybees were tuned to a motor gain of -2 to 2 (between the heading of the bee and its visual feedback), local disruption of the OA pathway in the medulla abolished this tuning, resulting in similar, low levels of response across levels of motor gain. Taken together, our results show that behavioral control modulates neural activity in the medulla, and ultimately impacts behavior.

3.2 SIGNIFICANCE

When moving, an animal generates motion of the visual scene over its retina. We asked whether self-generated and externally generated optic flow are processed differently in the insect medulla. Our results show that closed-loop control of the visual stimulus modulates neural activity as early as the medulla, and ultimately impacts behavior. Moreover, octopaminergic modulation allows the sharpening of object tracking and neural responses. Our results suggest that the medulla is an important site for context-dependent processing of visual information, and that placing the animal in a closed-loop environment may be essential to understand its visual cognition and processing.

3.3 INTRODUCTION

An animal's response to a stimulus often depends upon the context under which the stimulus is processed. This context may depend upon physiological state, or other conditions associated with the sensory environment. For example, the influence of internal state on vision has been extensively studied in the visual cortex of vertebrates, including primates (Kastner and Buschman, 2017) and mice (Khan and Hofer, 2018; Koay et al., 2019), where both body motion and features of the visual environment impact neural responses. In insects, the locomotion state (e.g., walking, running, or flying) also modulates visual processing in multiple brain regions, including the central complex and lobula region of the optic lobe (Paulk et al., 2015; De Bivort and Van Swinderen, 2016; Green et al., 2017). The degree to which self-motion modulates upstream visual brain loci, however, remains less well understood (Tuthill et al., 2014).

The small brains of insects are particularly well-suited for studying the links between sensorimotor context, neural processing, and behavior. With these tractable and comparatively simple brains, insects nevertheless exhibit diverse behaviors that are integrally linked to sensorimotor state. For instance, free-flying honeybees (Apis mellifera) can learn the concept of sameness and difference (Giurfa et al., 2001), and can discriminate different human faces (Dyer et al., 2005). However, visual learning abilities are dramatically reduced when bees are tethered (Hori et al., 2006; Hori et al., 2007; Sakura et al., 2012), or when head motion is restrained (Niggebrugge et al., 2009; Mota et al., 2011). Recent studies have demonstrated that allowing a bee to walk or move its head while placed in a virtual reality environment increases learning success (Rusch et al., 2017; Buatois et al., 2017). These results suggest that sensorimotor feedback is critical for visual learning in honeybees.

The spatial and temporal stimuli an insect experiences as it navigates will depend on features of its surroundings and the dynamics of its locomotion. In interactions with environment, the ability to modify behavioral output to generate the expected consequences requires closed-loop control of behavior, and is critical for survival. This closed-loop control of behavior has important implications for neural processing of sensory stimuli, and an insect's sensitivity to feedback control. For instance, there is growing evidence that flying a straight path is primarily an operant behavior in the fruit fly Drosophila melanogaster (see Brembs, 2009 for review). Recently, researchers have trained bumblebees (Bombus spp.) to adjust their motor actions to perform complex learning tasks, such as successfully pulling strings or rolling balls to obtain a reward (Alem et al., 2016; Loukola et al., 2017). Such studies demonstrate that the insect sensorimotor system continually updates behavior to achieve goal-oriented tasks. Although feedback sensitivity to visual stimuli has been well characterized (Wolf and Heisenberg, 1991; Wolf et al., 1992; Heisenberg et al., 2001; Bahl et al., 2013), the interplay between motor gain and neural processing is less clear (Wolf Heisenberg, 1986; Fank et al., 2014; Paulk et al., 2015).

Octopamine (OA), a well-known neuromodulator in many insect species, is integrally linked to locomotion state, and presumably facilitates the processing of images at increased rates during locomotion (Roeder, 1999; Chiappe et al., 2010; Maimon et al., 2010; Suver et al., 2012; Tuthill et al., 2014). For example, in flies, the release of OA during flight increases gain in lamina and lobula plate neurons (Suver et al., 2012; Tuthill et al., 2014, Strother et al., 2017). However, whether self-generated versus externally generated motion modulates neural activity in the medulla is still unclear, as is the interaction between OA and motor gain in tuning visually-evoked responses. In this study, we ask 1) how optic flow impacts neural processing in the honeybee medulla; 2) how behavior is impacted by the relation between honey-

bee motion and visual input; and 3) if OA is involved in the observed modulation. Together, our results show that neural responses in the medulla are strongly modulated by sensorimotor feedback, and that OA is critical for the sensitivity of motor gain at both the behavioral and neural levels.

3.4 MATERIALS AND METHODS

3.4.1 Honeybees collection and preparation

Sixty-eight forager honeybees were used in electrophysiological and behavioral experiments between 2018 and 2020 (11 bees for the neuronal recording experiments, 57 for the behavioral experiments including 21 for the local-injection pharmacology experiment). Honeybees were either collected from the University of Washington campus (Seattle WA, USA) or were shipped from Arizona State University (Tempe, AZ USA). Based on the behavioral experiments, there were no significant differences between bees from these locations (2-group Mann-Whitney U Test, distance walked: Seattle vs. Tempe, W = 7796, P = 0.437, n = 20 and 16 respectively). Bees were kept at 251Celsius and under 50±5% relative humidity in containers (11 7.5 8.5 cm), with a 30% (w/w) sucrose solution (Sigma-Aldrich, St Louis, MO, USA) available ad libitum. Before experiments, bees were anesthetized on ice and tethered by the thorax and head to a custom 3D printed holder using UV-activated glue (3:1 mix of Loctite 3104 Light Cure Adhesive, Loctite, Düsseldorf, Germany, and UV Glue GL114, JewelrySupply.com, USA) and dentist wax (Patterson Dental Supply, Saint Paul, USA)(Fig. 1A). The custom holder allowed us to precisely cut a small window in the cuticle (from the ocelli to the antennae and from one eye to the other) to access the brain in the electrophysiological or pharmacological experiments. After tethering, bees were fed with up to 30µl of 50% (w/w) sucrose solution to check for motivation and allowed to recover for at least 20 min to an hour in a dark, warm and humid environment (25±1°Celsius, 55±5% relative humidity).

Around 7% of bees were discarded due to the general lack of locomotion activity after recovering and placed on the locomotion compensator (forward velocity < 5 mm per second over the experiment).

3.4.2 Experimental setup

The virtual environment is composed of a locomotion compensator, a screen, and a projector (Fig. 1A). The virtual environment has been described in Rusch et al., 2017. Briefly, the locomotion compensator is an omnidirectional treadmill used to measure the planar trajectories (translation and rotation on the plane) of walking bees. The treadmill consists of a light plastic ball (W9989 Jumbo Table Tennis Balls 55 mm, SS Worldwide), a custom-designed 3D printed support structure (adapted from Moore et al., 2014), an aquarium pump (air pump AP-4, Danner Manufacturing, Islandia, NY, USA) and two optic laser sensors (ADNS-9800 Laser Motion Sensor, Jack Enterprises, Cookeville, TN, USA), sampled at 50 Hz using a microcontroller (RedBoard, SparkFun Electronics, Niwot, CO, USA). The locomotion compensator sits at the center of a cylindrical visual arena (frosted mylar, 20 cm diameter, 30.5 cm high), subtending 250° with a 110° opening in the rear to allow access to the bee and electrode insertion. A video projector (Acer K132 WXGA DLP LED Projector, 600 lm) positioned above the arena projects the visual stimuli downwards onto the mylar screen. The visual stimuli are either presented stationary, rotated in closed-loop as a function of the calculated bee heading (i.e., the visual scene moves laterally with the rotation of the ball) or rotated in open-loop based on predefined inputs.

3.4.3 Visual stimuli

All experiments were conducted in a dark room. Visual stimuli on the screen were single green or blue bars (5 cm width, or a visual angle of 28 degrees) projected on a black background. The stimuli were similar in brightness (blue stimulus: peak at

451 nm, 18 lux, 0.02 W/m^2; green stimulus: peak at 537 nm, 21 lux, 0.03 W/m^2) and surface area (152.5 cm^2). All experiments were conducted under red light (Bulbrite light bulb, 130V, 10W, red; covered by a red filter: Rosco Roscolux Medium Red, Lighting Filter). The visual angles of both stimuli were above the threshold for discrimination against the background and chromatic perception (Giurfa and Vorobyev, 1997). Spectral characteristics of the visual stimuli were obtained by measuring the relative irradiance of each stimulus (Ocean Optics, USB2000+ Spectrometer, Oceanview software, calibration light HL-2000). Visual stimuli were displayed on a black background, as this elicited the strongest phototactic responses compared with other backgrounds (Rusch et al., 2017). In addition to the blue and green stimuli, a closed-loop pattern of multiple human-grey bars was presented between trials.

3.4.4 Behavioral experiments

Three experimental series were performed in the virtual environment: simultaneous behavioral and electrophysiological recording experiments (n = 11 bees, 90 total units; recording length of approximately 7200 seconds), behavioral experiments alone (n = 36 bees, 1800 seconds), and behavioral experiments coupled with pharmacological manipulation of OA in the bee optic lobe (n = 21 bees, 1200 seconds). The first series was performed to explore the impact of closed-loop control on neural activity in the medulla, whereas the second series examined the impact of gain on the behavioral responses. The last series served to explore further the involvement of OA in the observed behavioral adaptation to levels of motor gain. All experiments were performed in a dark room and started with a 120 seconds acclimation period with a black screen. For all experiments, the forward and angular velocities, the animal heading, and the stimulus position were recorded at 20 Hz. We defined fixation when the stimulus was maintained within ±20° in front of the honeybee for at least one second while it walked towards the visual stimulus. For the analysis of

the impact of fixation bouts on neural activity, only bees that fixated more than 5-times throughout the closed-loop presentations were used in the analyses (n = 9 bees). Bees fixated, on average, 20 bouts throughout the experiment (20 ± 4.14, mean ± s.e.m.) and for 2.33 seconds per bout.

Open-loop, Closed-loop, and Replay experiments:

In the first series of experiments, we presented the honeybee with either a single green or blue bar that moved across the screen in the yaw direction at 100 degrees per second. We next presented the bar in closed-loop for a 20-second duration, followed by a replay trial where the motion of the closed-loop visual stimulus was repeated to the honeybee in open-loop (Fig. 1B). In between presentations, a black screen was presented for 20 seconds. In all trials, the initial position of the visual stimulus was -40°, on the left side of the screen, corresponding to the side of the recorded medulla (see below). The number of closed-loop and replay presentations ranged from 16 to 50 per preparation.

Gain experiments:

In the second experimental series, we manipulated the gain between the animal motion and the motion of the visual scene to explore further the sensitivity and impact of closed-loop control on visual processing (Fig. 1B). A gain of -1 is the default in our virtual environment and is defined as 1° change in the animal heading corresponding to a 1° motion of the visual stimulus in the other direction. Bees were presented a green or blue bar in closed-loop at 12 different levels of motor gain, [-5, -3, -1.5, -1, 0.5, -0.1, +0.1, +0.5, +1, +1.5, +3, +5]. Each presentation lasted for 20 seconds, and the levels of motor gain were randomly assigned and presented at least four times each. To maintain similar levels of behavioral responses throughout the experiment, between each trial, a pattern of gray bars was presented in closed-loop for 10 seconds at a gain of -1.

3.4.5 Multi-channel recordings

A hole was cut in the cuticle of the head capsule, as described above, to expose the medulla on both sides of the brain. The brain was superfused continuously with physiological saline solution (130 mM NaCl, 6 mM KCl, 4 mM MgCl2, 5 mM CaCl2, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, pH 6.7) at room temperature. The multi-channel electrode was positioned in the left medulla under visual control using a microscope and micromanipulator. Recordings were made with 16-channel silicon multielectrode (A2x2 3 mm, 150-150-121 and A4x4 3mm, 50-125-177, Neuronexus Technologies, Ann Arbor, MI). Electrophysiological signals were amplified 10,000X and filtered (lowpass: 6kHz; highpass: 300Hz), recorded and digitized using Tucker-Davis acquisition software (Tucker-Davis Technologies, FL, USA). Spike data after thresholding was subsequently manually clustered using Offline Sorter (Plexon, Dallas, TX, USA). Only well-clustered units that separated in a three-dimensional space (PC1-3) after statistical verification (multivariate Anova, $P < 0.05$), were used in the analysis. Unit responses were initially examined in Neuroexplorer (Nex Technologies, Winston-Salem, NC, USA) before being analyzed through custom-written and open-source code in Matlab and the Statistics Toolbox (release 2019a, The MathWorks, Inc., Natick, MA, USA). Firing rate response were estimated using bins of 50 ms and used to classify units based on their responses to visual stimulation. In total, 90 units from 11 individuals were recorded simultaneously with behavior (mean = 8 units per preparation). From those 90 units, 44 responded to the presentation of the blue or green bars, and 18 responded significantly to visual motion (Fig. 1E, F). Only 55 units were used to explore the impact of the closed-loop control as their spontaneous activity, and visual-evoked responses were stable over time (Fig. 2B).

3.4.6 Pharmacological experiments

The OA antagonist (Epinastine hydrochloride, Sigma Aldrich, St Louis, MO, USA) and agonist (OA hydrochloride, Sigma Aldrich, St Louis, MO, USA) were dissolved in saline at a concentration of 4.10^{-3}M and 10^{-4}M respectively. Epinastine is a highly specific octopamine receptor antagonist in honeybees, with a much lower binding affinity (104-times) for other bioamine receptors (Roeder et al., 1998). Our Epinastine concentrations were similar to those of previous studies in honeybees (Rein et al., 2013; Tedjakumala et al., 2014). Drugs or saline alone (control) were injected either into the brain as bath application (Epinastine, 10x20 nl) or directly inside each medulla (OA, 2x2nl). For the local injection, volumes injected were calibrated before and after injection using a Malassez cell, and injections were performed 15 minutes before the experiment started.

3.4.7 Immunohistochemistry

Microelectrode insertion:

To image the insertion of the microelectrodes in the medulla, the tips of the silicon electrodes were coated with a solution of 2% Texas Red (Thermo-Fisher Scientific, Waltham, MA, USA) mixed in saline. After the experiment, heads were removed into cold (4° Celsius) fixative containing 4% paraformaldehyde in phosphate buffered saline, pH 7.4 (PBS, Sigma-Aldrich, St Louis, MO, USA) and left overnight at 4° Celsius. The following day, the heads were washed two times over 20 minutes in PBS, and the brains dissected and then embedded in agarose. The resulting embedded tissue was cut into 60 µm serial sections using a Vibratome. Sections were washed in PBS containing 0.5% PBS-Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) two times over 20 minutes. Then 50 µL normal serum was added to each well containing 1 mL PBS-TX. After 1 hour, the primary antibody was added to each well, and the well plate was left on a shaker overnight at room temperature.

The next day, sections were washed six times over 20 minutes, and 2.5 μL of Cys3 Donkey alpha-mouse (Alexa Fluor 488) was added to 1 ml of PBS-TX in each well and the well plate was again left on the shaker overnight. Tissue sections were then washed in PBS six times over 3 hours and embedded on glass slides in Vectashield (Vector Laboratories, Burlingame, CA, USA). After at least 24 hours at 4° Celsius, the brain tissue was imaged using a Leica SP5 laser scanning confocal microscope, and the resulting image stacks were processed using ImageJ (National Institutes of Health)

Characterization of pharmacological microinjections:

To allow us to both visualize the insertion site of capillaries and the spread of the solution after microinjection, we mixed the saline or OA antagonist solution with 2% Texas Red (Thermo-Fisher Scientific, Waltham, MA, USA). After the experiment, the head of the honeybee was removed and placed in 4% PFA and left overnight at 4° Celsius. The following day, the heads were washed 6 times in PBS for 20 minutes each before the brains were removed from the head capsule. Brains were dehydrated in a series of increasing ethanol solutions followed by clearing in methyl salicylate (1:1 with 100% ethanol, then 100% methyl salicylate). After at least 24 hours at 4° Celsius, the brain tissue was imaged using a Leica SP5 laser scanning confocal microscope. The images were processed using ImageJ (National Institutes of Health).

3.4.8 Data Analysis

Normalized firing rate:

To compare firing rate between closed-loop and replay trials, we calculated a normalized difference

$$\Delta(FiringRate) = \frac{MeanFiringRate_{\text{Closed-Loop}} - MeanFiringRate_{\text{Replay}}}{MeanFiringRate_{\text{Closed-Loop}}} \quad (3.1)$$

where mean firing rate responses for closed-loop or replay stimulus presentations were calculated over a 16-second window 2 seconds after the onset of the stimulus and averaged over 16-50 trials for each unit. To statistically analyze closed-loop and replay responses, for each unit we assessed the difference in ΔFiring rate (hereafter: ΔFR) by comparing the closed-loop and replay datasets to a null distribution of 100 bootstrapped pairwise differences randomly drawn from the combined closed-loop and replay visual stimulus datasets.

Generalized linear model:

For each responsive unit, we applied the following Generalized linear regression model assuming a Poisson distribution:

$$log(Spikecount) = 1 + S.P. + F.V. + H. + B.C. \quad (3.2)$$

where the Stimulus Position (S.P.) variable corresponded to the position of the bar on the screen, the Forward Velocity (F.V.) and Heading (H.) to the instantaneous forward velocity and heading of the animal at the same instant and the Behavioral Control (B.C.) variable corresponded to the level of motor gain during the trial, either open-loop (replay) or closed-loop. All variables were recorded at 20 Hz, and the model was trained on 80% of the dataset and the 20% left were used to predict firing rate using the trained model. The random sampling of the dataset for training and testing was balanced across behavioral control labels (closed-loop and replay trials). For each unit, the model was run 100 times, and the mean of the P-values against the null model and of predictors was extracted to assess if the model was different from a null model and if the behavioral control was a significant

predictor of spiking activity. The resulting AIC values were used to evaluate the different variables (with and without Behavioral Control) on the model results.

Singular value decomposition:

To reduce the dimensions in our dataset, we applied singular value decomposition (SVD) where the mean-centered matrix A of normalized firing rate:

$$A = U\Sigma V^{*} \tag{3.3}$$

The U and V matrices are orthogonal, unitary, and their columns correspond to the eigenvectors of AAT and ATA. The U matrix corresponds to the modes of the decomposition and the weight associated with each mode is given by their corresponding singular values (diagonal of the matrix). The SVD was applied either to a population of neurons or a single unit. When applied to a population of neurons, the mean-centered matrix A corresponded to the normalized mean across trials for each unit. When applied to a single unit, the mean-centered matrix A corresponded to the firing rate under different conditions (e.g., fixation bouts and randomly selected bouts). We then compared the energy contained in each mode across the different conditions.

k-nearest neighbor classification

To build a model that would classify and predict behavioral control based on the firing rate of the population, we separated the matrix into two subsets, one containing 80% of the data for training our model and the other 20% for testing. We then applied the SVD to the training matrix to extract the most important features. The testing matrix was projected on the SVD space obtained with the training matrix to obtain the Vtest matrix:

$$V_{\text{test}} = U' A_{\text{test}} \tag{3.4}$$

A k-nearest neighbor classifier algorithm using the three closest neighbors was trained with the matrix V and tested with the matrix Vtest. The training-testing steps were repeated 100 times to assess the mean accuracy in both training and testing.

Gain experiment behavioral data analysis:

The data collected (stimulus position, animal heading, and velocity) in the gain experiment were analyzed, and all statistical tests were computed using R software (R Development Core Team, 2016) and the circular package. A random walk model was developed to ensure the difference in duration of fixation bouts observed was not only due to the difference in the animal velocity. In the model, a fictive animal can take a step in one of three different directions (left, right, forward) with the same probability at every timestamp, and the "amplitude" of the step was randomly drawn from the angular velocity distribution of the epinastine- and saline-injected bees.

3.5 RESULTS

3.5.1 Locomotion state and turning behavior impact neuronal activity in the medulla

To explore the impact of locomotion on neuronal responses in the optic lobe, we placed tethered, walking bees in a virtual reality arena, while performing multi-channel recording in the bee medulla (Fig. 1A). Visual stimuli included static and moving vertical gratings or single bars (size 28°, velocity 100°/sec) that were presented in closed-loop trials and subsequently replayed in open-loop trials (Fig. 1B). Bees exhibited robust fixation responses to all visual stimuli, and during closed-loop

trials they maintained the visual stimulus in the frontal region of the eyes (Fig 1C, D). Based on the single unit responses to these visual stimuli, approximately 69% of neural units were sensitive to color, or to color and motion (Fig. 1E, F).

While walking speed is known to modulate neural activity in insect optic lobes (e.g., Chiappe et al., 2010; Paulk et al., 2015), it is unclear how closed-loop control impacts early brain loci such as the medulla. We examined bee walking and turning behaviors with respect to position of a visual stimulus, and classified the behavior into active and non-active bouts based on our observations and recordings. We considered the honeybee to be walking if its forward velocity was at least 2.5 mm/sec. (Fig. 2A), or turning when its absolute angular velocity was greater than 20°/sec (Fig. 2A). During closed-loop trials, bees walked 66.25 ± 8.27% of the time (mean ± standard error), and 66.56 ± 8.49% during replay trials. The average forward velocity of walking bouts was 9.48 ± 1.55 mm/sec (mean ± s.e.m.) during closed-loop trials, and 1.14 ± 0.09 mm/sec during bouts defined as non-walking.

We found no difference in walking or turning velocities between closed-loop and replay trials, across all bees and all trials (Fig. 2B, Wilcoxon rank-sum test for closed-loop versus replay trials, forward velocity: z = -1.761, P = 0.07; angular velocity: z = 0.56, P = 0.57). However, the locomotion state did impact single unit responses (Fig. 2D). A significantly greater number of units were modulated during closed-loop turning responses (19 units, 35% of all units), compared to only 4 units modulated by angular velocity in replay trials (two-way ANOVA, firing rate angular velocity * behavioral control, $P_{angular\ velocity} < 0.01$; $P_{behavioral\ control} < 0.01$). Similar results occurred when comparing neural responses during closed-loop versus no-visual stimulus (black screen, ITI) trials (two-way ANOVA, firing rate angular velocity * behavioral control, $P_{angular\ velocity} < 0.01$; $P_{behavioral\ control} < 0.01$). Walking activity also impacted neural responses. During closed-loop trials,

firing rate responses were significantly altered for 8 units (15% of units), compared to only 2 units modulated in replay trials or during presentation of the black screen during ITI (Kruskal Wallis test, walking versus not walking, $P < 0.01$; two-way ANOVA, firing rate forward velocity * visual stimulus condition, Pforward velocity $<$ 0.01; Fig. 2D).

3.5.2 Characterizing and modeling the impact of behavioral control on single unit and ensemble responses

To further characterize the effects of behavioral control on single unit and ensemble responses, we first compared firing rate responses between closed-loop and replay trials during the interval from 2 to 18 sec (Fig. 2C, E). This time window was chosen to capture the dynamics of closed-loop control, while excluding any ON response to the onset of the visual stimulus. In 17 units, firing rate responses differed significantly between the two conditions (Fig. 2F, Wilcoxon rank sum test, ΔFR versus 0, $P < 0.05$), and this difference could not be explained by variability in firing rate activity, independent of behavioral control (ΔFR versus bootstrapped ΔFR, $P < 0.05$). To further explore the impact of behavioral control on dynamic responses of medulla neurons, we fitted a generalized linear model to the responses of each unit for all trials (16-50 trials per condition), and compared the model with and without the behavioral control variable (defined as a two-level categorical variable: closed-loop or replay) (Fig. 3A). For 25 units, behavioral control (BC) was a significant predictor of spiking activity, and the model differed significantly from a null model fit to the same data ($P < 0.05$). For each of those 25 units, we compared the Akaike Information Criterion (AIC) of the models with or without the control variable. We found that for 14 units, inclusion of the behavioral control variable in the model significantly lowered the AIC values (one-tailed Wilcoxon rank sum test, AICwithBC versus AICwithoutBC, $P < 0.05$, Fig. 3D) and increased the R-squared values (one-tailed Wilcoxon rank sum test, adjusted R-squaredwithBC versus ad-

justed R-squaredwithoutBC, $P < 0.05$, Fig. 3B). The predicted firing rate based on the models for those 14 units captured the observed single unit responses, and predictions were improved by inclusion of the behavioral control variable in the model (Fig. 3D). In contrast, for the other 11 units, predicted firing rates did not match the measurements, regardless of w the behavioral control variable was included (Fig. 3C, D).

We next used dimension reduction techniques (singular value decomposition, SVD) to explore the impact of behavioral control at the neural population level. The SVD was applied to a matrix containing the normalized mean firing rate under three conditions: closed-loop trials, replay trials, and trials with no visual stimulus presented (Fig. 3E). At the onset of visual stimulus, firing rate responses were both higher and less variable in the closed-loop condition compared to the replay stimulus, which exhibited greater firing rate response variability in the preceding 16 sec window. To exclude ON responses, the SVD was applied to the interval from 2 to 18 sec in the stimulus trials (Fig. 3E, blue dots). The ensemble responses showed distinct clustering in multivariate space. In the first mode of the SVD , closed-loop trials were separate from the replay trials and the unstimulated (ITI) condition, whereas in the second mode, closed-loop and replay trials were separate from responses to unstimulated condition (Fig. 3F). We next trained a k-nearest neighbors classification algorithm to sort the data in closed-loop, replay, and no visual stimulus trials using the first 20 modes, since these singular values were higher than for a noise model based on a shuffled dataset (Fig. 3G). Interestingly, the algorithm performed better at correctly assigning the closed-loop trials than the replay or unstimulated (ITI) trials (Fig. 3H). Classification by the algorithm was better than random chance for both training and testing (Fig. 3I).

3.5.3 OA application increased similarity between closed-loop and replay trials

Neuroamines play key roles in regulating neural activity during different locomotion states, and OA is well known to be involved in modulation of neural activity during insect flight (Maimon et al., 2010; Suver et al., 2012; Tuthill et al., 2014). To explore potential participation of OA in the modulation we observed during closed-loop trials, we superfused the brain with OA (10-6 M) while recording neural and behavioral activity. Both forward and angular velocities of honeybees increased after OA application (Kruskal Wallis test for saline versus OA, forward velocity: degrees of freedom [df] = 1, Chi-squared = 8509.53, $P < 0.001$; angular velocity:F = 181.01, P <0.001, Fig. 4A, B). Neural responses also reflected OA modulation, as follows. Prior to OA application, 6 of the 14 units recorded showed significant differences in firing rate between closed-loop and replay trials (Wilcoxon rank sum test, ΔFR versus 0, $P < 0.05$ and ΔFR versus bootstrapped ΔFR, $P < 0.05$). After OA application, firing rate responses did not differ significantly between the two stimulus conditions (Fig. 4C). In 12 of 14 units, OA evoked an increase in firing rate during both closed-loop and replay trials (Wilcoxon signed rank test, baseline firing rate with OA versus saline, $P < 0.05$). However, application of OA during replay did not completely recapitulate the ensemble response dynamics observed in closed-loop (Fig. 4D). Nonetheless, SVD applied to the firing rate matrices showed separation between the groups in the first modes (Fig. 4E), and was reflected in the Euclidean distance in the first 20 modes (i.e., the modes with singular values above the noise model of all experimental trials), in which OA increased the similarity between replay and closed-loop (Wilcoxon signed rank test, $P = 0.02$, Fig. 4F).

3.5.4 Fixation behavior is dependent on the visual stimulus and is preceded by an increase in firing rate in medulla neurons

The differences in neural responses between closed loop and replay motivated us to examine how the response dynamics change as the honeybee first begins to fixate on the visual stimulus. During closed-loop trials, 9 of 11 bees (82%) fixated for 2 to 4 sec (Fig. 5A). In certain units, we observed an increase in firing rate preceding fixation (Fig. 5B-E). This increase could not be explained solely by the position of the visual stimulus on the screen (Fig. 5B-D). To further explore the impact of fixation on those neurons, we extracted the spiking activity during the first second of all fixation bouts in the closed-loop trials, as well as during the preceding second. Using these data, we built a "fixation-triggered average" matrix of firing rate responses, as well as a matrix of similar size containing firing rate responses during random time windows of non-fixation within closed-loop trials. The resulting covariance matrices had higher eigenvalues in the first mode for the fixation matrix than the corresponding random one, for all neurons exhibiting the prefixation increase in firing rate. To explore the impact of fixation on the population, we averaged the firing rate across fixation and random bouts for all units, including units that showed no difference between fixation and random bouts (Fig. 5E). After SVD analysis, the two populations separated significantly across the first mode (Kruskal-Wallis test, Vclosed-loop versus Vreplay, df = 1, Chi-squared = 33.69, $P < 0.001$; Fig. 5F-H).

3.5.5 OA in the medulla is critical for fixation behavior

To further explore how OA influences the sensitivity of visual responses and feedback during closed-loop, we tested the ability of honeybees to fixate a visual stimulus at different levels of motor gain, and under different pharmacological interventions. Immediately before the experiment, either the OA receptor antagonist Epinastine (410-3 M) or saline control was focally microinjected into both medullas.

For saline-injected bees, the duration of fixation bouts differed significantly among individual bees and with gain, with increased duration of fixation bouts at gains of -2 to 2 (two-way ANOVA, duration of fixation $\sim$ gain * bee ID, gain: $F = 2.49$, $P = 0.006$; bee ID: $F = 3.32$, $P < 0.001$; interaction factor: $F = 1.22$, $P = 0.146$; $n = 36$; Fig. 6A). Saline-injected bees significantly oriented toward and fixated on the visual stimulus; however, bees injected with Epinastine were unable to maintain the visual stimulus in the frontal field of view (Watson's two-sample test of homogeneity, test statistic 318.22, $P < 0.001$; Fig. 6B). Saline-injected bees showed the same pattern as that in uninjected bees, with a significant increase in duration of fixation bouts at gains of 0.1-1.0 (Kruskal-Wallis test, duration of fixation $\sim$ gain, Chi-squared = 18.04, df = 4, $P = 0.001$, Fig. 6C). By contrast, Epinastine-injected bees showed no increase in duration, and fixated for a similar amount of time at all gains (Kruskal-Wallis test: duration of fixation $\sim$ gain, Chi-squared = 7.53, df = 4, $P = 0.11$, Fig. 6C). Overall, injection of Epinastine significantly decreased the duration of fixation bouts (Kruskal-Wallis test, duration of fixation $\sim$ injection type: Chi-squared = 11.13, df = 1, $P < 0.001$, Fig. 6C). Similar to the results for duration of fixation, the proportion of trials with fixation increased in saline-injected bees at gain levels from -5 to -1.5, and decreased thereafter (Fig. 6D). In Epinastine-injected bees, however, the proportion of trials with at least one instance of fixation did not change with gain level, and was lower than in saline-injected bees (Fig. 6D). In addition, Epinastine-injected bees had higher forward velocities, lower angular velocities, and walked longer distances than saline-injected bees (Kruskal-Wallis test, distance walked $\sim$ injection type: Chi-squared = 51.67, df = 1, $P < 0.001$; Fig. 6E, F).

To ensure that the decreased fixation observed in Epinastine-injected bees was not a result of higher walking velocity, we developed a random walk model, and compared the duration of fixation bouts of fictive bees with the collected data. In the

model, fictive bees could turn right, left, or go forward with the same probability and with an angular velocity drawn randomly from the distributions observed in saline-injected or Epinastine-injected honeybees at a motor gain of -1 (Kruskal Wallis test, instantaneous angular velocity injection type, $P < 0.001$). As in actual bees, the modeled duration of fixation of fictive bees was shorter when based on data collected in Epinastine-injected bees than in saline-injected bees (Kruskal Wallis test, duration of fixation type of fictive bees, $P < 0.001$). However, fictive bees based on the Epinastine-injected data fixated on the visual stimulus in 55% of trials, whereas fictive bees based on the saline-injected data fixated in only 47% of trials. This last result is the opposite of what we observed experimentally (Fig. 6D), demonstrating that a decrease of angular velocity associated with greater forward velocity cannot fully explain the impact of OA antagonist on fixation behavior.

3.6 DISCUSSION

The brain is inherently a closed-loop system. The nervous system processes sensory signals from the environment to shape behavior, and behaviors consequently modify the environment. In the present study, we combined behavioral and multi-channel recordings in walking honeybees to explore how behavioral state and sensorimotor feedback modulate visual processing in the honeybee medulla. Our results showed that when honeybees had behavioral control over the horizontal displacement of the visual scene, a subset of spiking neurons exhibited increased responses during the duration of the stimulus and prior to the onset of behavioral fixation, which was in contrast with results during their replay in open-loop.

3.6.1 Behavioral modulation of visual processing

The behavioral context-dependence of the neural response raises the question of mechanism. OA is an important neuromodulator of visual processing in many in-

vertebrates, and in insects has been showed to play an important role during locomotion (Stern et al., 1995; Longden and Krapp, 2010; Jung et al., 2011; Suvert et al., 2012; Tuthill et al., 2014; Strother et al., 2018). In honeybees, OA neurons innervate the three optic lobes with a relatively homogeneous distribution (Bicker, 1999), whereas only the medulla and the lobula are innervated in the fruit fly (Busch et al., 2009). In locusts, OA neurons with dense arborizations in the optic lobes were proposed to participate in mediating dishabituation or arousal of the visual system (Stern et al., 1995). In our honeybee study, application of OA triggered a general increase in gain in the neural population, similar to that observed during closed-loop stimulation, in which the honeybee had behavioral control over the visual scene (Fig. 4F). The speed-tuning of visual processing was consistent with efficient coding, wherein tuning of neurons should match that of the environment—when shifting from non-active behavioral bouts to walking or flying bouts, the optic flow shifts toward higher frequencies.

Our study adds to the ongoing redefinition of insect visual processing as more than a strictly feed-forward processing stream from the lamina to the lobula. Flying insects live in a fast world: a bumblebee, for example, flies at 7.1 m/sec, or approximately 400 body lengths/sec (Riley et al., 1999). Behavioral modulation of visual processing may ensure correct and rapid behavioral responses under different environmental conditions – as image frequencies increase during flight, or decrease during hovering – while reducing neuronal energy consumption by dynamically altering neuron sensitivity according to locomotion state (Niven, 2016). To our knowledge, optic lobe neuronal recordings have not been performed in non-active, walking, and flying locomotion states in the same individual. Such recordings would facilitate better understanding of how neuronal activity is modulated across the different locomotion states. Moreover, different species must rely on different strategies to process visual stimuli. For instance, the extensively studied lobula plate of the fruit fly is absent in the honeybee. Comparative studies are thus critical to achieve a

more thorough understanding of neural modulation.

3.6.2 Adaptive control of visual processing

Using virtual reality, researchers have shown that insects can modify their motor patterns to achieve a goal. Fruit flies placed in a flying arena can modify their leg motion to achieve steering at both positive and negative levels of motor gain (i.e., the relationship between the insect motion and visual scene motion) (Wolf et al., 1992). Such behavior demonstrates a high level of motor pattern flexibility, and the need to combine visual processing with information from locomotion. In our experiment, honeybees showed strong aptitude for adapting their locomotion in order to fixate on visual stimulus (Fig. 6A). We then asked whether OA was important for adapting motion to obtain the expected visual motion. Microinjection of an OA antagonist into the medulla disrupted fixation behavior, with loss of adaptive responses to different levels of motor gain, suggesting that OA is involved in visual response tuning (Fig. 6C, D).

3.6.3 Behavioral control and visual learning

Operant behavior has classically been used in learning protocols. The ability to learn the consequence of a particular action is critical for adaptive behavior and thus for survival. Previous studies have shown that the presence of optic flow is critical for visual learning in tethered honeybees (Rusch et al, 2017; Buatois et al., 2018), and it is possible that behavioral control will prove critical for complex learning protocols, such as the learning of abstract concepts. One means of ensuring extraction of only the most relevant signals during learning, while dedicating the least possible amount of computational resources, can be categorized when an animal "fixates" and attends to a visual object (Logan, 1992). In the presence of competing stimuli, selective fixation allows an animal to respond to specific visual objects, permitting some stimuli to evoke behavioral response while ignoring others.

Visual fixation has been studied extensively in primates (reviewed in Wolfe, 2000). For instance, attention alters the synchrony of a neuronal population (Fries et al., 2001), and can modulate both the firing rate and the receptive field of neurons (for reviews see McAdams and Maunsell, 1999; Treue, 2001). An overall boost in signal gain, comparable to the increase in firing rate we observed in honeybees, has been described in the visual cortex of mice (Niell and Stryker, 2010; Schneider 2020 for reviews). Recent studies in fruit flies have shown that visual salience increases neural activity at a specific frequency, and may be correlated to behavioral selection and suppression (van Swinderen and Greenspan, 2003; van Swinderen, 2007; Tang and Juusola, 2010; van Swinderen, 2012). However, these studies relied on measures of local field potential, which is proposed to reflect the synchronous activity of a population of neurons. While we did not present competing stimuli in our study, we did observe an increase in firing rate that preceded fixation behavior during closed-loop trials (Fig. 5E). This finding is consistent with the local field potential change in the medulla that was observed to precede fixation upon one of two competing stimuli (Paulk et al., 2014). Behavioral experiments that record activity in multiple areas of the brain while presenting the animal with distractors will shed more light on attention-like processes, and their role during learning.

Our results emphasize the importance of behavioral closed-loop control on visual processing in the medulla, and its interplay with behavioral and internal state modulation. The medulla has received less attention than the lobula in studies of behavioral and internal state modulation, even though a single fruit fly medulla column contains approximately 900 neurons, over 2500 presynaptic sites, and more than 300000 postsynaptic sites (Takamura, Xu et al., 2015). The precise location in the visual pathway that integrates feedback from leg or wing position is still unknown. Answering this question will likely require simultaneous recordings in different brain areas, and better knowledge of the connectomics of the visual pathway. Given the

impressive amount of processing taking place in the medulla, it may be a strong candidate for behavioral and internal-state modulation during visual processing.

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3.8 Conflict of interest statement:

The authors declare no competing financial interests.

3.9 Author contributions:

C.R, D.A.S.A and J.R designed research; D.A.S.A designed sofware; C.R performed research, C.R analyzed data, C.R and J.R wrote the paper.

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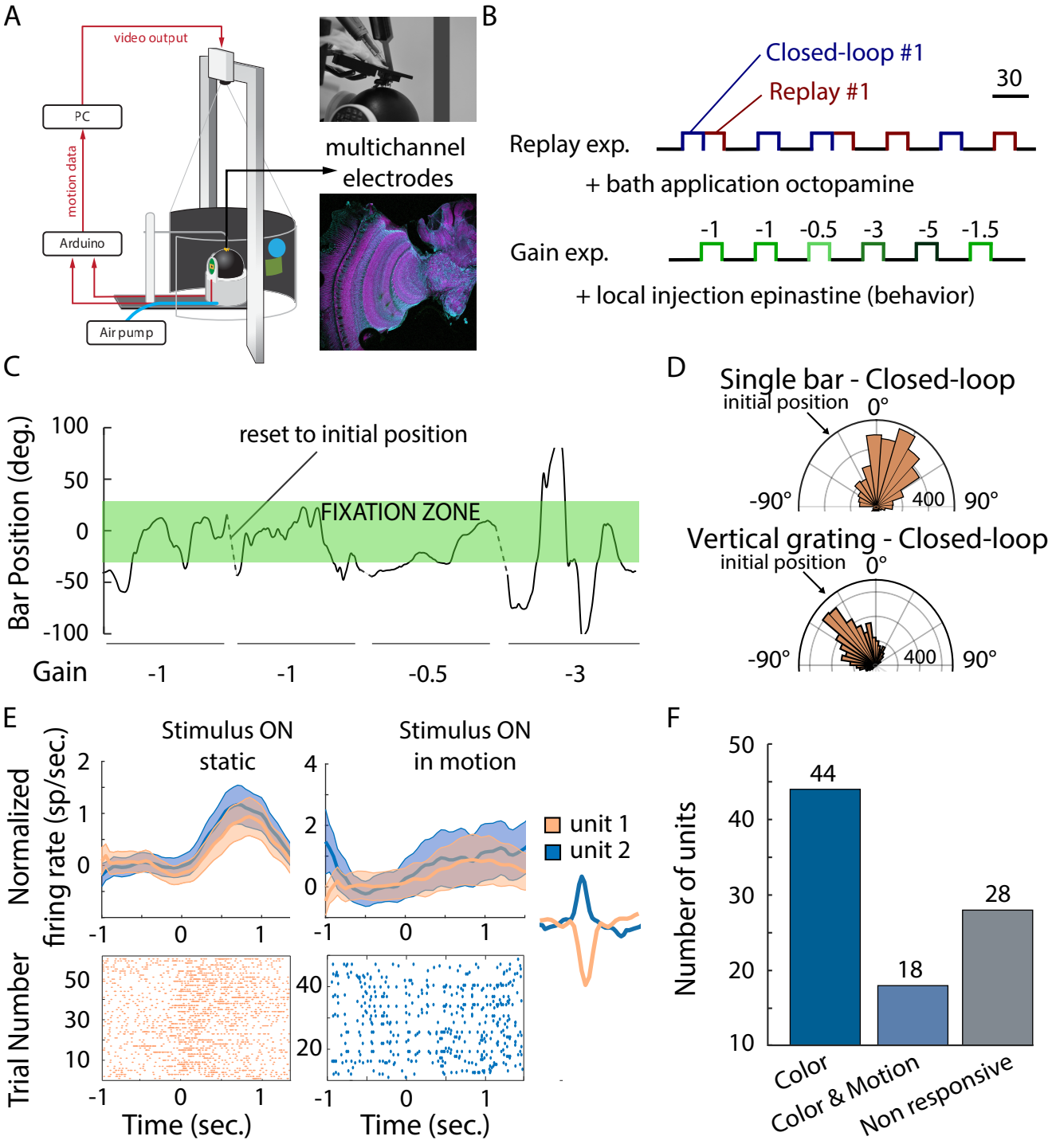
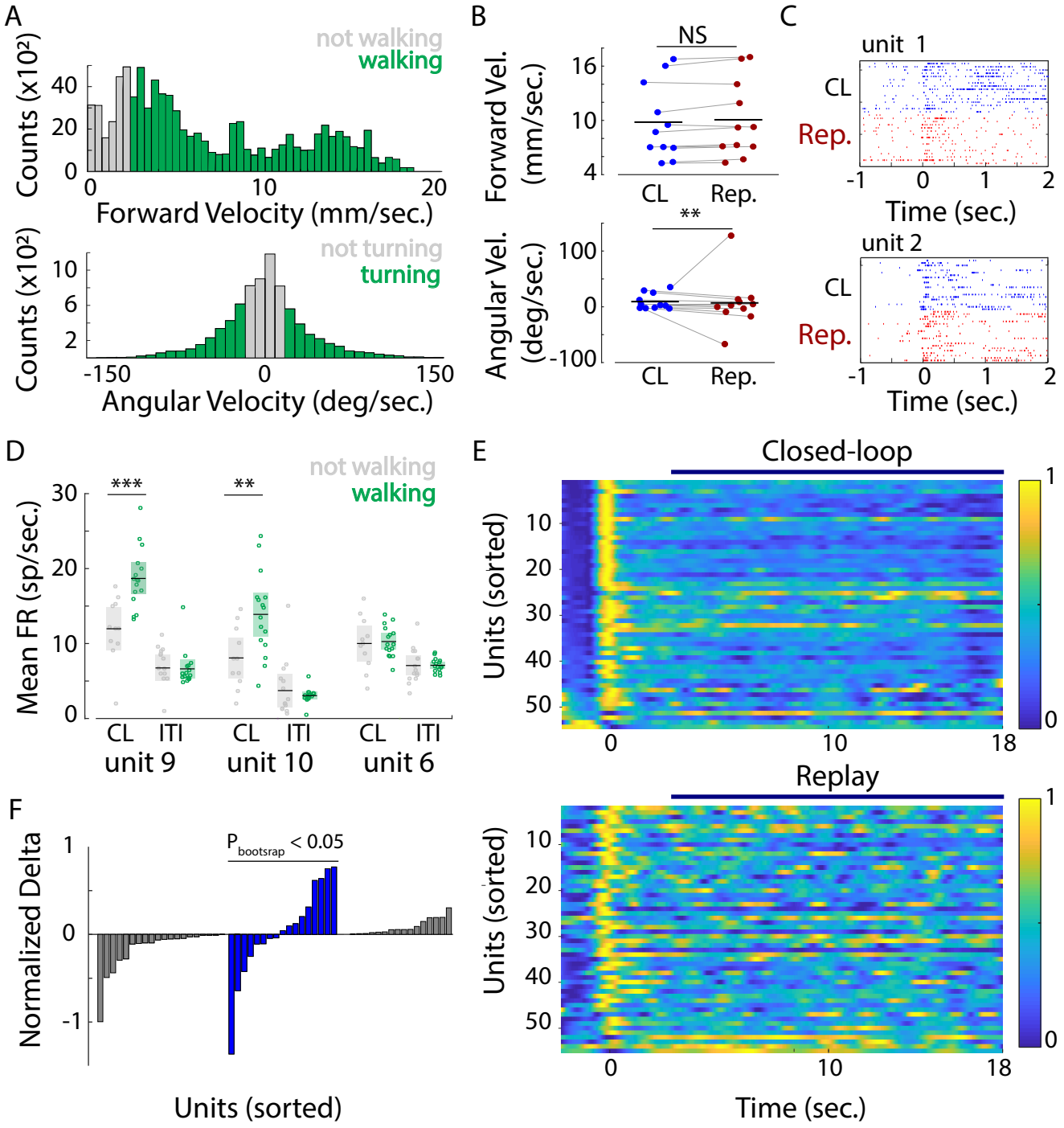
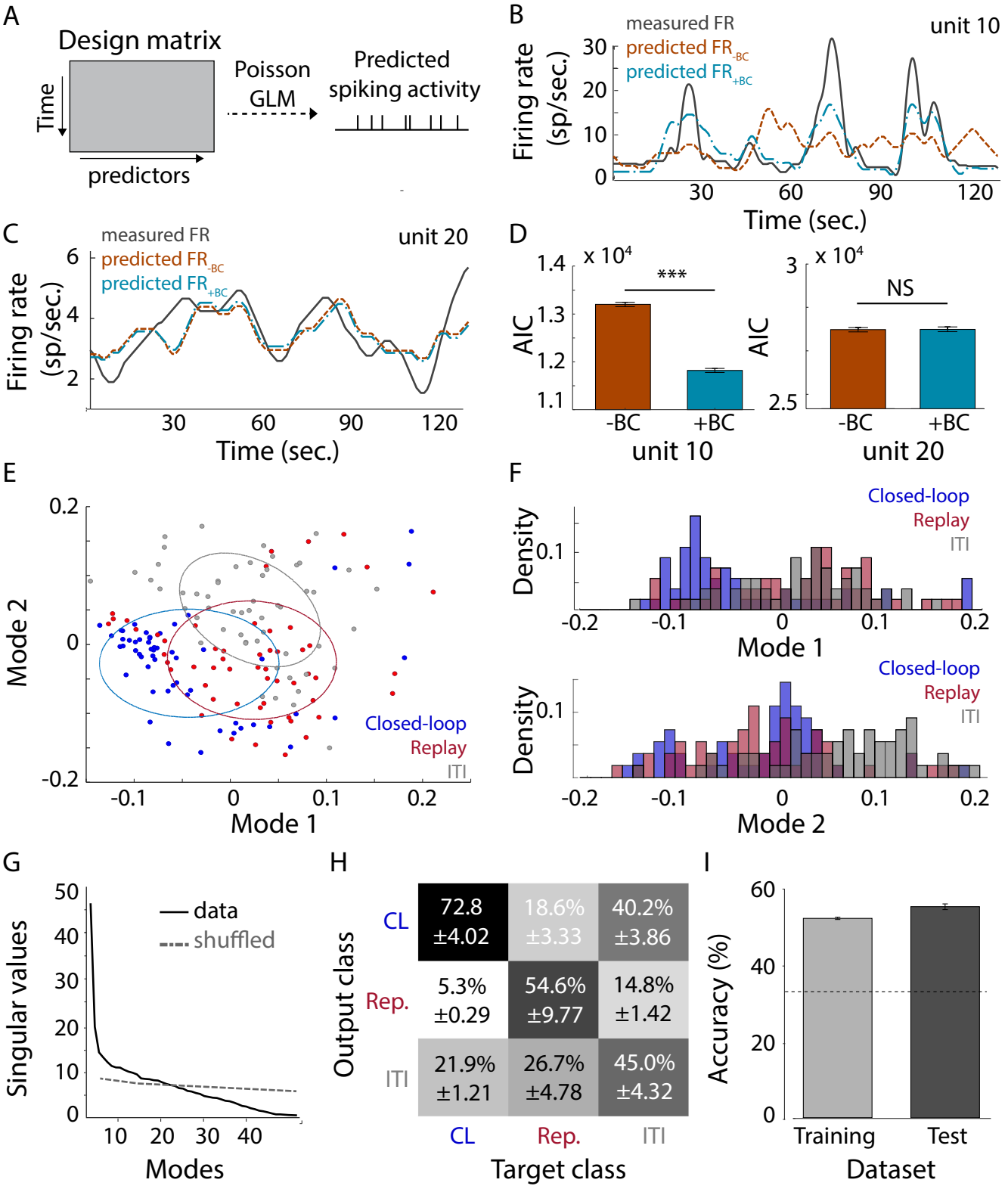

A
video output
PC
motion data
Arduino
Air pump
multichannel
electrodes
B
Closed-loop #1
Replay #1
30
Replay exp.
+ bath application octopamine
-1 -1 -0.5 -3 -5 -1.5
Gain exp.
+ local injection epinastine (behavior)
C
reset to initial position
FIXATION ZONE
Bar Position (deg.)
100
50
0
-50
-100
Gain
-1
-1
-0.5
-3
D
Single bar - Closed-loop
initial position
0°
-90°
90°
400
Vertical grating - Closed-loop
initial position
0°
-90°
90°
400
E
Stimulus ON
static
Stimulus ON
in motion
Normalized
firing rate (sp/sec.)
unit 1
unit 2
Trial Number
Time (sec.)
Time (sec.)
F
Number of units
44
18
28
Color
Color & Motion
Non responsive

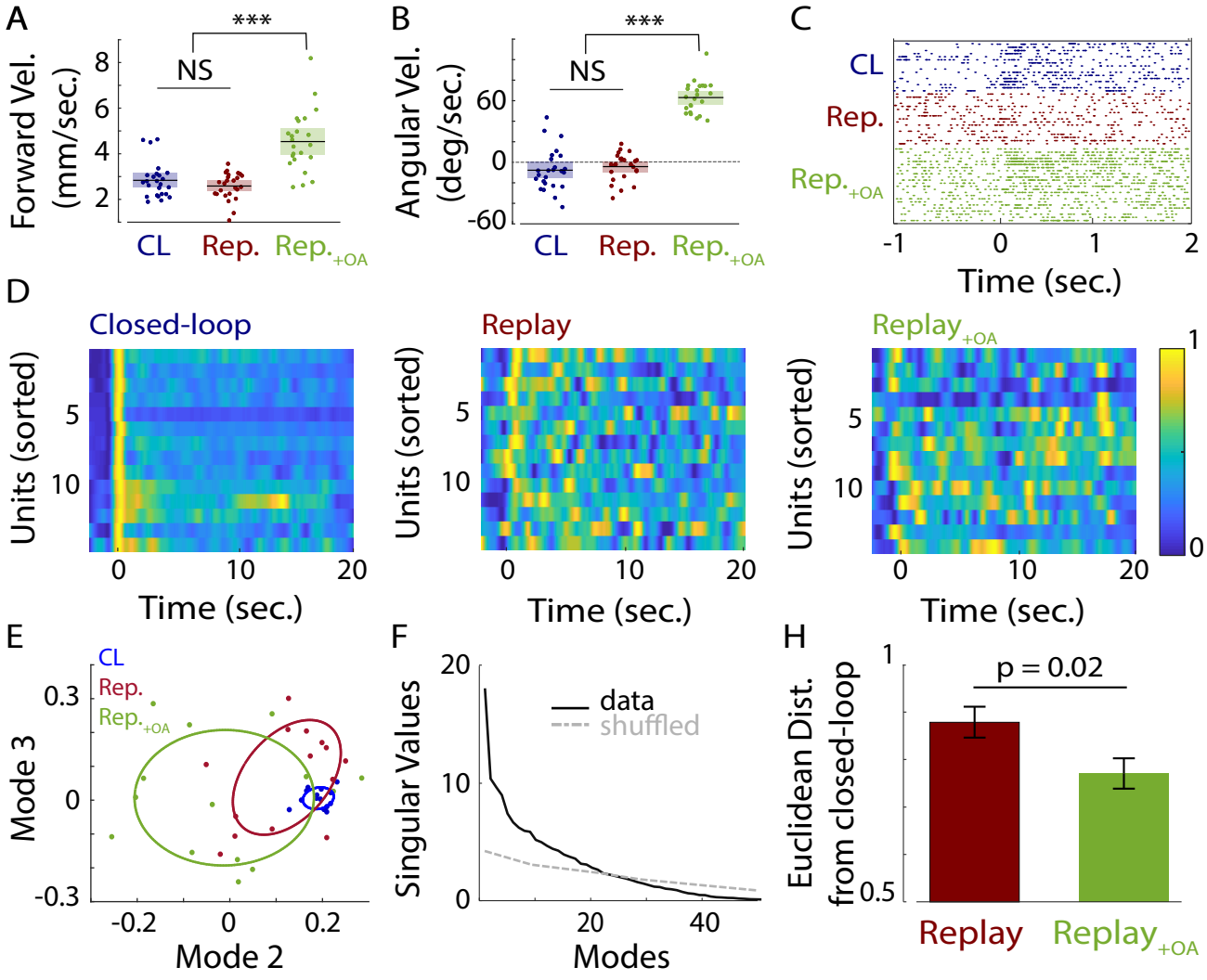
(previous page): Figure 1. A Virtual Reality Environment (adapted from Rusch et al., 2017). A custom-made 3D-printed holder was glued to the head and thorax of the honeybee to prevent head motion, perfuse the brain with saline solution and ensure walking behavior (insert, topright). The multi-channel tetrodes were inserted using a micromanipulator in the medulla of the honeybee and the reference electrode was lowered in the saline bath near the tetrode. Placement of the electrodes was verified after the experiment (insert, bottom left). B Experimental series: The replay experiment consisted in presenting to the honeybee a single bar in closed-loop for 20 seconds and subsequently presenting the replay of the visual stimulus motion in open-loop. A black screen was presented to the honeybee between trials and presentations were randomized so that a replay trial did not necessarily follow a closed-loop trial. This experiment series was paired with multi-channel electrodes recording and bath application of OA agonist. The second experimental series, the gain experiment, consisted in presenting a single bar to the honeybee at different levels of motor gain. This series of experiments was paired with local injection of epinastine, an OA antagonist, into the medulla. C Single-bar position on the screen during closed-loop trials at different levels of motor gains. The honeybee tends to fixate on the visual stimulus (fixation zone, [-20 20] deg, green rectangle) but fails to do so at the highest level of motor gain (-3). D Position on the screen of the single-bar position (top) and of the vertical grading (bottom) during closed-loop trials. When presented with a single visual stimulus, honeybees fixated on it and this behavior was not found when presented a vertical grating. E Neuronal activity in responses to visual stimulus (normalized mean firing rate and sem, top; raster plot, bottom; static stimulus: left; stimulus in motion: right). The insert corresponds to the spike waveform of the corresponding unit. F In total, 90 units were extracted from the neuronal recordings. 44 responded to the presentation of a static visual stimulus, 18 responded to the presentation of a moving visual stimulus. 28 did not respond to any of the visual stimuli presented. From the 62 responsive units, 7 were excluded from the analysis due to inconsistency in activity across the experimental series


A
not walking
walking
Counts (x10²)
Forward Velocity (mm/sec.)
not turning
turning
Angular Velocity (deg/sec.)
B
NS
Forward Vel. (mm/sec.)
**
Angular Vel. (deg/sec.)
CL
Rep.
C
unit 1
unit 2
Time (sec.)
D
not walking
walking
***
**
Mean FR (sp/sec.)
CL
ITI
unit 9
unit 10
unit 6
E
Closed-loop
Replay
Units (sorted)
Time (sec.)
F
Normalized Delta
P bootsrap < 0.05
Units (sorted)

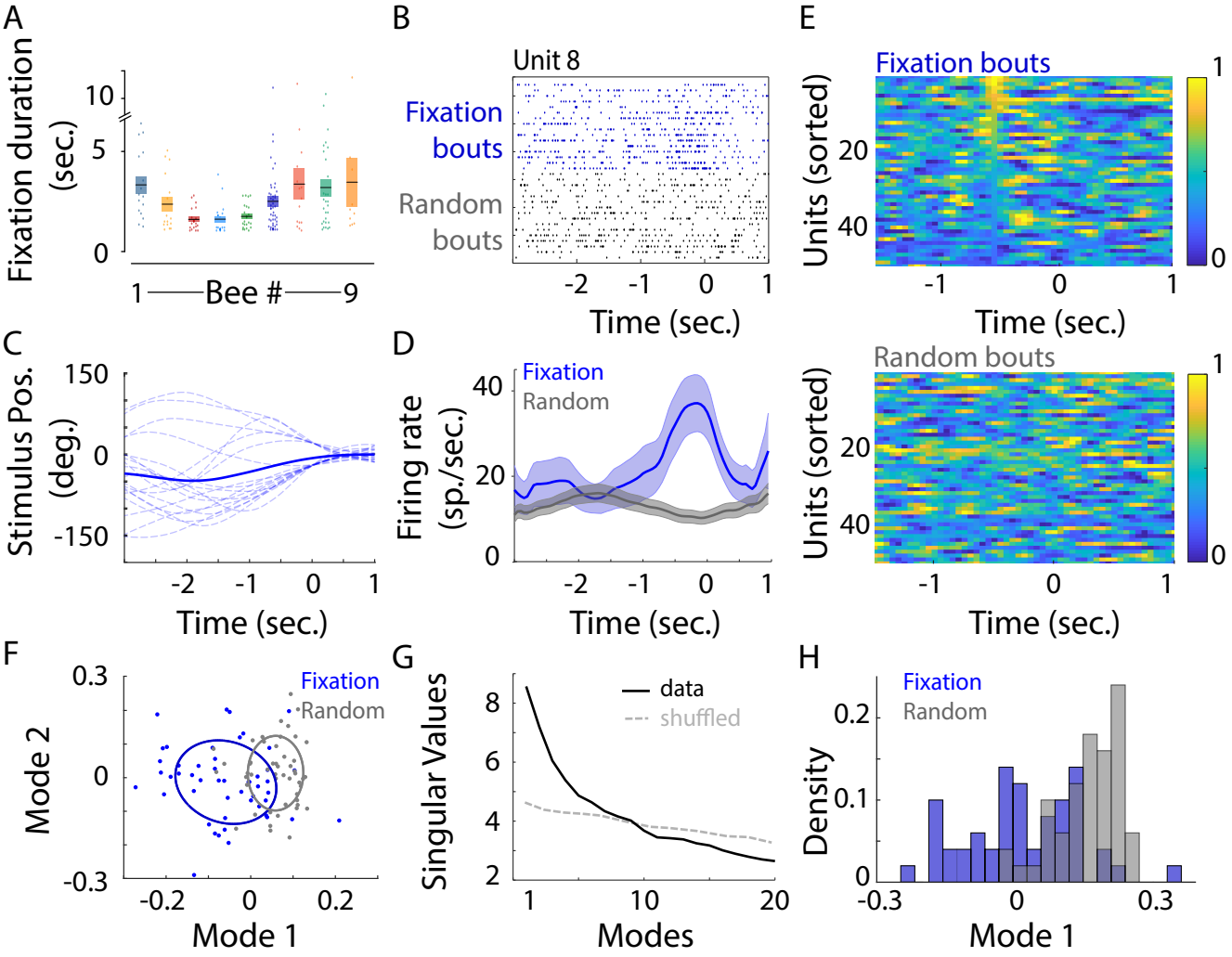
(previous page): Figure 2. A Top: Forward velocity (Forward Vel.) distribution for one honeybee. The data was divided in walking bouts (forward velocity inferior to 2.5 mm/sec, gray bars) and not walking bouts (forward velocity superior to 2.5 mm/sec, green bars). Bottom: Distribution of angular velocity (Angular Vel.) for one honeybee. The data was divided in turning bouts (angular velocity >20°/sec or <-20°/sec, green bars) and not turning bouts (angular velocity between -20° to 20°/sec, gray bars). B Top: Forward velocity averaged across closed-loop or replay trials for each bee (Wilcoxon rank sum test, P = 0.05, each dot = 1 bee). Bottom: Angular velocity averaged across closed-loop or replay trials for each bee (Watson-Williams multi-sample test, F = 6.87, P = 0.02, each dot = 1 bee) C- Neural responses (raster plots) of two units during closed-loop (blue) and replay (red) trials. Unit 1 shows decreased rate of spiking during the replay trials. D For 3 different units, mean firing rate (spikes/sec.) during walking (green) and non-walking (gray) bouts, averaged for each trial of closed-loop (CL) and between trials (ITI: inter-trial interval) where no visual stimulus (black screen) was presented to the honeybee. Shaded rectangles represent the 95% confidence interval. Unit 9 and 10 showed a significant increase in firing rate during walking bouts and in the presence of visual feedback (Kruskal Wallis test, **: P < 0.01, ***: P < 0.001). E Matrix of normalized firing rate of all units averaged across trials for the closed-loop trials (top) and the replay trials (bottom). Units were sorted according to their activity during closed-loop trials at time 0 sec. F Normalized difference in firing rate between closed-loop and replay trials. The colored units showed a significant difference from 0 (Wilcoxon rank sum test, P < 0.05) and from a bootstrap analysis (Wilcoxon rank sum test, P < 0.05).


A
Design matrix
Time
predictors
Poisson
GLM
Predicted
spiking activity
B
measured FR
predicted FR-BC
predicted FR+BC
unit 10
Firing rate
(sp/sec.)
Time (sec.)
C
measured FR
predicted FR-BC
predicted FR+BC
unit 20
Firing rate
(sp/sec.)
Time (sec.)
D
x 10^4
***
AIC
-BC
+BC
unit 10
NS
unit 20
E
Mode 2
Mode 1
Closed-loop
Replay
ITI
F
Density
Mode 1
Mode 2
Closed-loop
Replay
ITI
G
Singular values
data
shuffled
Modes
H
Output class
CL
Rep.
ITI
72.8 ±4.02
18.6% ±3.33
40.2% ±3.86
5.3% ±0.29
54.6% ±9.77
14.8% ±1.42
21.9% ±1.21
26.7% ±4.78
45.0% ±4.32
Target class
I
Accuracy (%)
Training
Test
Dataset

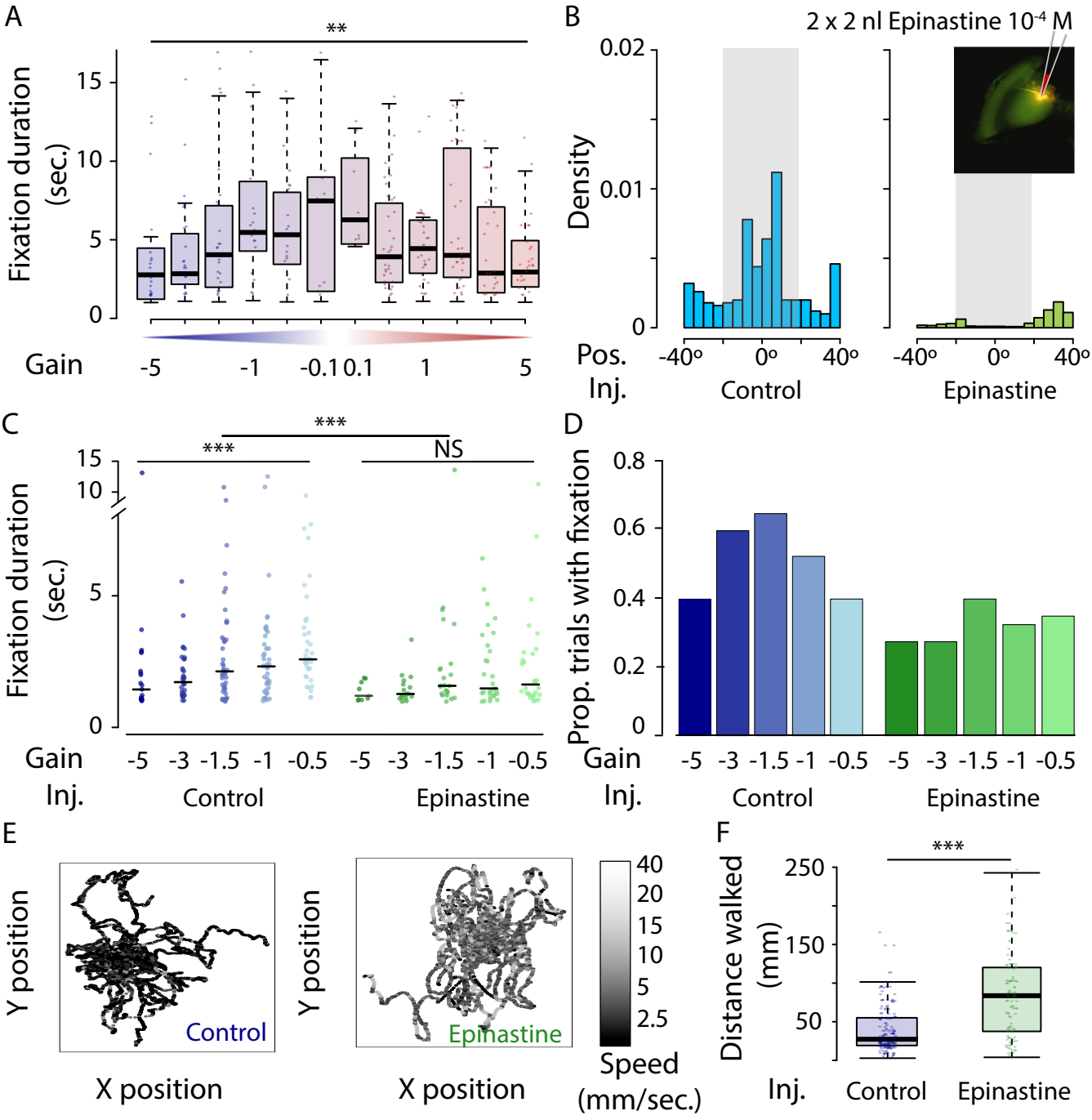
(previous page): Figure 3. A Generalized linear model. The design matrix contains the predictor variable: Stimulus position, Forward Velocity, Heading, Spike history and Behavioral Control (BC). The response variable is the spike count. The model assumed a Poisson distribution of the response variable. B Measured firing rate (gray line) and predicted firing rate without the BC variable (orange line) or with the BC variable (blue) for one unit. C Measured firing rate (gray line) and predicted firing rate without the BC variable (orange line) or with the BC variable (blue) for one unit. D AIC of the model with and without the BC variable for the two units plotted in B and C. E Distribution of the closed-loop (blue) and replay (red) trials, and random inter-trial intervals (e.g., without presentation of visual stimulus, gray) in the two first modes after Singular Value Decomposition. The ellipses correspond to the 50% confidence interval. F Distribution of the closed-loop (blue), replay (red) trials and inter-trial intervals (gray) in the first mode (top) and second mode (bottom). G Singular values along modes for the matrix of firing rates and a shuffled matrix. The first 20 modes of the data matrix contain more information than the ones of the shuffled data. H Confusion matrix of the classification algorithm for the condition closed-loop (CL), replay (Rep.) and inter-trial intervals (ITI). I Percentage of accuracy in training and testing for our k-nearest neighbor classification algorithm. The dashed line corresponds to chance level (0.33).


A
Forward Vel. (mm/sec.)
***
NS
CL
Rep.
Rep.+OA
B
Angular Vel. (deg/sec.)
C
Time (sec.)
D
Closed-loop
Replay
Replay+OA
Units (sorted)
E
Mode 3
Mode 2
F
Singular Values
data
shuffled
Modes
H
Euclidean Dist. from closed-loop
p = 0.02

(previous page): Figure 4. A Forward velocity (mm/sec) during closed-loop trials (blue), replay trials (red) and replay trials with OA bath application (green). The black lines represent the means. The average forward velocity is higher when OA is delivered (Kruskal-Wallis test, forward velocity: saline vs. OA, df = 1, Chi-squared = 8509.53, P $<$ 0.001). B Angular velocity (/sec) closed-loop trials (blue), replay trials (red) and replay trials with OA bath application (green). The black lines correspond to the means. OA triggered a tendency to turn right (Kruskal-Wallis test, angular velocity, saline vs. OA, df = 1, Chi-squared = 3545.86, P $<$ 0.001). C Raster plot of the response of a unit to a visual stimulus in closed-loop trials (blue), replay trials (red) and replay trials with bath application of OA (green). D Matrix of normalized mean firing rate for 14 units during closed-loop trials (left), replay trials (middle), and replay trials with bath application of OA (right). E Projection of the closed-loop trials (blue), replay trials (red) and replay trials with OA bath application (green) on mode 2 and 3 after SVD. The ellipses correspond to the 50% confidence interval. F Distribution of the singular values across mode for the matrix of firing rate (closed-loop, replay and replay with OA data) and its shuffled version (shuffled data). G Euclidean distance from the closed-loop trials to the replays trials with or without OA application. Population responses in replay trials with bath application of OA were more similar to closed-loop than replay trials without OA (Wilcoxon signed rank test, P = 0.02).


A
Fixation duration (sec.)
10
5
0
1
Bee #
9
B
Unit 8
Fixation bouts
Random bouts
-2
-1
0
1
Time (sec.)
E
Fixation bouts
Units (sorted)
20
40
-1
0
1
Time (sec.)
1
0
C
Stimulus Pos. (deg.)
150
0
-150
-2
-1
0
1
Time (sec.)
D
Fixation
Random
Firing rate (sp./sec.)
40
20
0
-2
-1
0
1
Time (sec.)
Random bouts
Units (sorted)
20
40
-1
0
1
Time (sec.)
1
0
F
Mode 2
0.3
0
-0.3
Fixation
Random
-0.2
0
0.2
Mode 1
G
Singular Values
8
6
4
2
data
shuffled
1
10
20
Modes
H
Density
0.2
0.1
0
Fixation
Random
-0.3
0
0.3
Mode 1

(previous page): Figure 5. A Duration of fixation bouts (sec) for each bee. The mean ± s.e.m. sd is represented by a rectangle. B Raster plot of the response of a unit 3 second before to 1 second during fixation (Fix.) or during 4 randomly selected periods (Random bouts). Note the increase in firing rate just before fixation. C Position of the stimulus on the screen °) 3 seconds before to 1 second during Fixation for the same unit as represented in B. D Mean firing rate (spike/sec) and s.e.m. centered around fixation for the same unit as in B and C. E Matrix of normalized mean firing rate for fixation (top) and randomly selected bouts (bottom). F Projection in the first two modes after SVD showed little separation between fixation bouts (blue dots) and randomly selected bouts (gray dots). The ellipses correspond to the 50% confidence interval. G Density distribution in the first mode for the fixation bouts (dark blue) and random samples (gray). F Singular values across mode. The first 10 modes explain more of the variance than a shuffled and label-removed version of the data.


A
**
Fixation duration (sec.)
15
10
5
0
Gain
-5
-1
-0.1
0.1
1
5
B
2 x 2 nl Epinastine 10-4 M
0.02
0.01
0
Density
Pos.
Inj.
-40°
0°
40°
Control
Epinastine
C
***
***
NS
Fixation duration (sec.)
15
10
5
0
Gain
Inj.
-5 -3 -1.5 -1 -0.5 -5 -3 -1.5 -1 -0.5
Control
Epinastine
D
Prop. trials with fixation
0.8
0.6
0.4
0.2
0
Gain
Inj.
-5 -3 -1.5 -1 -0.5 -5 -3 -1.5 -1 -0.5
Control
Epinastine
E
Y position
Control
X position
Y position
Epinastine
X position
40
20
15
10
5
2.5
Speed (mm/sec.)
F
***
Distance walked (mm)
250
150
50
Inj.
Control
Epinastine

(previous page): Figure 6. A Duration of fixation (sec) across different levels of motor gain [-5; -3; -1.5; -1; -0.5; -0.1; 0.1; 0.5; 1; 1.5; 3; 5]. Duration was significantly different across gain and honey-bee (two-way ANOVA, duration of fixation bouts gain * bee ID, gain: $F = 2.49$, $P = 0.006$, bee ID: $F = 3.322$, $P < 0.001$, interaction factor: $F = 1.222$, $P = 0.146$, n=36). B Distribution of bar position (deg. on screen) for all trials for a saline-injected bee (blue) and an epinastine-injected bee (green). The insert represent the injection in the medulla. C Duration of the fixation bouts (sec) across different levels of motor gain [-5; -3; -1.5; -1; -0.5] in saline-injected (blue, n = 11) and Epinastine-injected bees (green, n = 10). The duration of fixation was significantly different across levels of motor gain in saline-injected bees (Kruskal-Wallis test, duration of fixation gain, Chi-squared = 18.048, df = 4, P = 0.001) but not for Epinastine-injected bees (Kruskal-Wallis test: duration of fixation gain, Chi-squared = 7.533, df = 4, P = 0.11). Overall duration of fixation was different between saline-injected and Epinastine-injected bees (Kruskal-Wallis test, duration of fixation injection type: Chi-squared = 11.132, df = 1, $P < 0.001$). D Proportion of trials with at least one fixation event and across levels of motor gain for saline-injected bees (blue) and Epinastine-injected bees (green). E Fictive path of saline-injected (left) and Epinastine injected (right) bees at all levels of motor gain and colored according to their forward velocity (mm/sec). F Overall distance walked during trials for saline-injected (blue) and Epinastine injected (green) bees. Epinastine-injected bees walked larger distances than saline-injected bees (Kruskal-Wallis test, distance walked injection type: Chi-squared = 51.672, df = 1, $P < 0.001$).

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PUBLICATIONS

Rusch C., Roth E., Vinauger C. and Riffell J.A. (2017) Honeybees in a virtual reality environment learn unique combinations of colour and shape. *J. Exp. Biol.*

Rusch C., Broadhead G.T., Raguso R.A. and Riffell J.A. (2016). Olfaction in context – sources of nuance in plant-pollinator communication. *Curr. Opin. Insect. Sci.* **15**, 53-60

AWARDS AND FUNDING

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