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Towards classical and operant approaches to learning, memory and navigation in the wood ant, *Formica rufa*

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This thesis is submitted to the University of Sussex in application for the degree of Doctor of Philosophy

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Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration with others except where specifically indicated in the text. No part of this dissertation has been submitted to any other university in application for a higher degree.

Signature:

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30th October, 2019

Summary

In the past century, insects have become models for studying associative learning and memory formation. Learning paradigms have been developed for several insect species and modalities, but they comprise only two main categories: classical and operant conditioning. While the same cues can be learnt in both paradigms, the process by which learning occurs differs on the extent to which animals need to interact with their environment to form a memory. Classical conditioned individuals obtain information about a cue and a reward/punishment passively while operant conditioned animals need perform an action to be rewarded/avoid punishment. Thus, to truly understand how memories are formed and stored in the brain, it becomes essential to identify to which extent self-action within the environment influences learning.

Considering this, I have investigated memory formation in wood ants, *Formica rufa*, through classical conditioning and found that they are able to passively learn the association between a visual cue and a sugar reward. I have explored this paradigm to investigate particularities of this type of learning and showed that these memories can be lateralised, with short- and medium-term visual memories being formed after training with a reinforcement of the right antenna and long-term memories with a reinforcement on the left antenna.

Additionally, I have developed a paradigm in which wood ants can walk on an air supported ball in open- or closed-loop with a virtual world. To demonstrate this paradigm is functional, I have investigated the use of self-generated optic flow on the integration of distance, speed and time of walking ants. I found that ants display repeatable walking behaviour that does not require but can be changed by variations of self-induced or external optic flow. This paradigm allows for a fully controlled comparison and analysis

of active and passive interactions with a virtual environment on a tethered animal from which neural circuits could be accessible.

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Publications

Fernandes, A. S. D., Buckley, C. L., Niven, J. E. (2018). Visual associative learning in wood ants. *Journal of Experimental Biology*, **221**(3), jeb173260.

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Fernandes, A. S. D., Niven, J. E. Lateralisation of short-, medium- and long-term visual memories in an insect. *Proceedings of Royal Society B* (in review)

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General Introduction

Memories are our way into the past and guide our present and future selves. What they are and how they are formed have puzzled humans for thousands of years, with ancient mythology and philosophy already attempting to explain the mysteries of memory (Eliade, 1963; Radvansky, 2017). Today, shaped by crucial findings on cellular biology and genetics, as well as by our relatively recent understanding of evolution, memory formation is one of the most researched topics within the life sciences. Numerous fields are concerned with the study of memory including psychology, psychiatry, neuroscience and animal behaviour, but despite the efforts made to unravel the brain's ability to learn and store information, we are only just starting to grasp its true complexity.

For a memory to be formed, the information about a certain event needs to be acquired, processed and encoded within our brain. A memory can be stored for a short, medium or long period of time and it can then be retrieved and restabilized, disrupted or updated into a new one (Nadel *et al.*, 2012). Each part of the process involves series of electrical and chemical events that induce modifications in the properties of few or many neurons and/or the connections (synapses) between them, which can be dependent upon a series of molecular cascades and epigenetic mechanisms common in many other biological events (Levenson and Sweatt, 2005). Nowadays, rather than only hypothesizing about the ways by which memory formation occurs, we can also observe the neural changes that arise during learning and memory retrieval. Recent technology has allowed us to record blood flow and oxygenation in different brain regions, both an indirect measure of brain activity, as well as directly record neural electrical activity through sensors placed on or underneath the scalp and the magnetic fields produced by electrical activity within the brain (Boly *et al.*, 2016).

However, recordings of the electrical changes that neurons undergo when receiving synaptic inputs and how they excite or inhibit other neurons with which they share synaptic connections was only possible through research using non-human animal models. Several optical imaging and electrophysiological techniques have been developed with the aim of recording neuronal activity while animals are processing information, storing and retrieving a memory (Wickenden, 2000). Furthermore, the development of specific behavioural set-ups transferable between animal models has given us a broad understanding of general as well as species specific learning capabilities, particularly regarding associative memories (Quillfeldt, 2016).

Associative learning

Associative learning is the ability of an animal to relate independent stimuli, events and/or actions to one another and is involved in most forms of memory formation, from the most complex to the simplest. Thus, by associating the neural representation of one stimulus to another, the perceived value of that stimulus changes and so does the animal's behaviour in response to it (Mackintosh, 1983). Associative learning in animals has been studied using two main behavioural paradigms, classical and operant conditioning. These two mechanisms for studying memory formation differ mainly in the active or passive contributions of the trained individual, i.e., if action is required for the learning outcome.

Classical conditioning was the first well established learning paradigm and was originally observed in dogs by Pavlov, in 1897. Pavlov, who investigated the role of digestive glands in dogs, accidentally witnessed that repeatedly pairing the sound of a bell, to which animals did not respond initially, with the presence of food that elicited salivation, would prompt salivation to the sound of the bell even when food was absent. He concluded that dogs were able to form an associative memory between the bell's sound, termed the conditional stimulus (CS), and the presence of a food reward, termed the unconditional stimulus (US). Thus, the reward value of the US became paired with the CS, activating in the same manner the motor output salivation, termed the unconditional response (UR) (Pavlov, 1897). The nature of the CS and US can vary, as well as the UR elicited by the

US. The conditioned stimulus can be a sound, an odour, a touch or a visual stimulus, as long as it does not initially prompt a similar response to the unconditioned stimulus. In turn, the US can be appetitive, such as a food reward, or aversive, such as an electric shock or air puff, thus prompting a specific UR depending on its nature (reviewed in Mackintosh, 1983). Because both CS and US are controlled by the experimenter and the UR is a reflex type of response, the animal does not have to perform a specific behaviour to form a memory of the association between the two stimuli.

Conversely, in operant conditioning animals need to perform a specific behaviour to acquire a reward or avoid a punishment. If performing a behaviour has positive consequences it is more likely to be repeated, whereas if the consequences are negative the probability of repeating that behaviour is lower (Mackintosh, 1983). Although this was theorized in the late ninetieth century by Thorndike (1898), who also designed some experimental paradigms to study operant conditioning, the most well established paradigm was only published in 1948, by Skinner. He designed an experimental paradigm, termed 'Skinner's box', in which the behaviour of the animal controls the presence of a reward or punishment (Skinner, 1948). Because animals need to engage with their environment and integrate their own actions with the positive or negative change in the environment for operant conditioning to occur, this constitutes an active form of learning, distinguishable from the passive memories formed through classical conditioning.

Since the establishment of these paradigms, several adaptations have been developed to investigate memory formation in multiple species including high order mammals such as primates, as well as birds and, most typically, rats and mice (Shettleworth, 2001). Although great contributions to this field have been made through research with these animal models, the complexity of the neural structures of these species and their relatively recent evolutionary origin poses difficulties in investigating the true basis of memory formation. To understand the basic characteristics underlying memory formation in all living beings with such capabilities, one ought to investigate simple forms of learning within small and simple brain models compared to vertebrates. This became evident with the boom of researchers focusing on learning and memory in invertebrate models. In particular, social

insects such as bees and ants have been widely investigated due to their extraordinary capabilities of forming memories while foraging for food to bring back to their colonies, despite their relatively small brain and low number of neuronal connections compared to mammals (Menzel, 2012). Research in these species of insects and other invertebrates have made incredible contributions on the cellular and molecular mechanisms of learning and memory formation and have shed a light on numerous related aspects, such as the evolution of the nervous system, perception, information encoding and processing, energy efficiency and compartmentalization of these events within the brain, amongst many others (reviewed in Chittka and Niven, 2009; Burns *et al.*, 2010; Yan *et al.*, 2014).

Associative learning in insects

Classical conditioning approaches

Although classical conditioning paradigms had been developed for several species of mammals since its discovery in 1927 (Pavlov), the first attempt to demonstrate learning using this methodology on an insect species was only made in 1961, by Takeda (Takeda, 1961). His research could not clearly demonstrate that the changes in behaviour he observed in honey bees, *Apis melifera*, were a consequence of learning but subsequent research conducted by Bitterman *et al.* (1983) did show that classical conditioning could induce learning in the same insect species (reviewed in Alloway, 1972). This paradigm consists in a set of simple appetitive learning experiments in which the US, typically sugar, evokes a UR, the proboscis extension reflex (PER) and can be paired with a CS, usually an odour. Honey bees learn to associate the CS and US and perform PER to the presence of the CS alone, after just a few training trials.

The original classical conditioning methodology has been widely used since then and adapted to investigate the learning abilities of several other insect species, including other species of bees (Laloi *et al.*, 1999; Mc Cabe *et al.*, 2007), flies (Fukushi, 1976, 1979; Chabaud *et al.*, 2006), locusts (Simões *et al.*, 2011) and ants (Guerrieri and d'Ettorre, 2010). These paradigms are particularly advantageous for investigating memory forma-

tion because they allow full control over the cues being learnt. Indeed, simple modifications of this behavioural set-up have allowed particular aspects of memory formation to be observed in insects, similarly to the learning abilities found in higher order species such as mammals. These include aversive conditioning, in which the US is a punishment and the animals respond displaying aggressive or avoidance reactions (Vergoz *et al.*, 2007), differential conditioning, i.e., being able to discriminate one cue from another and associate each cue to its own reward or punishment value (Giurfa *et al.*, 1999), second order conditioning, in which a first CS is paired with a US, and then a second CS is associated to the first, eliciting the same UR (Hussaini *et al.*, 2007), amongst others.

In classical conditioning paradigms, not only the characteristics of the CS and US are controlled, but also the duration the animal is allowed to perceive them and the time between presentations. This full control over the temporal characteristics of the learning experimental procedure has allowed researchers to evaluate the mechanisms involved in the formation of short-, medium- and long-term memories (Menzel, 1999), which would not be accessible to the same extent if memory acquisition would depend on the animal's own will to perform a particular behaviour. Indeed, classical conditioning experiments with honey bees have unravelled five different memory stages, from early and late short-term memory, to medium-, early and late long-term memory, each involving different molecular processes within the olfactory learning pathway (Menzel, 2001). Furthermore, adaptations of this paradigm to several species of bees have shown that, similarly to mammals, memories seem to be lateralised within the bees' brain, which opened new possibilities in understanding the evolution of memory formation and functional brain lateralisation (Frasnelli *et al.*, 2014).

To have this precise control over the learning process, animals tend to be restrained in classical conditioning paradigms. Harnessing animals, in turn, poses other crucial advantages when investigating the neural basis of such processes. The neural circuits of a live and behaving individual become accessible while perceiving the cues, learning them and recalling that memory at different time points. For instance, olfactory classical conditioning in honey bees has been performed in parallel with artificial excitation of a particular

neuron which replaced the presentation of the unconditioned stimulus, providing direct access to the cellular mechanisms underlying the reinforcing properties of the US pathway (Hammer, 1993). This advantages of classical conditioning paradigms have had a crucial impact in our understanding of the molecular and cellular mechanisms of learning, which would not be observable in freely moving animals.

However, these approaches are very reductionist and do not reflect an animal's natural behaviour while perceiving, interacting and learning about its environment. In a natural situation, an animal is in closed-loop with its environment; not only it perceives and learns its characteristics, but acts and changes it. In turn, self-generated changes in the environment need to be perceived distinctly from passive changes and integrated into learning. Because this is not achieved in classical conditioning paradigms where animals form memories passively, many complex neural mechanisms used for memory formation in natural contexts may be underestimated.

Operant conditioning approaches

The first recorded observations of learning associations in freely moving insects date the early nineties, with cockroaches and bees sharing the attention (reviewed in Alloway, 1972). Aversive learning was demonstrated in cockroaches, which learnt the association between a dark environment and the presence of an aversive stimulus, an electric shock, changing their natural preference for darker places to show avoidance (Szymanski, 1912; Turner, 1913). In addition, appetitive learning was demonstrated in bees that learnt the location of artificial feeders while foraging (Frisch, 1914). Shortly after, associative learning was demonstrated in many other insect species, including beetles (Alloway, 1969), fruit flies (Wustmann *et al.*, 1996), ants (Schneirla, 1933) and more recently locusts (Simões *et al.*, 2011). Social insects deserve particular attention regarding this type of paradigm, because they display complex learning and navigation abilities to be able to find food and return to their nest efficiently (Collett and Collett, 2002; Collett and Graham, 2004; Collett *et al.*, 2013). These environmental cues can be of different modalities, such as olfactory and visual, and tend to be used together to varying degrees depending on the species and the environment itself (Knaden and Graham, 2016). Because while

foraging animals need to perform an action – walk or fly towards a specific place – to be rewarded with food, foraging can be considered an operant conditioning paradigm.

In the laboratory, the most used paradigm in operant conditioning in insects is the y- or t-maze (Harris *et al.*, 2005; Dupuy *et al.*, 2006; Ali *et al.*, 2011; Avarguès-Weber and Giurfa, 2013). In this set up, the two arms of the maze differ on a particular cue, typically an odour or a visual cue, and if the animal finds a reward or a punishment when choosing to approach it. Although animals form an associative memory between a cue and a reward or punishment, similarly to what happens in classical conditioning, it is their own action that dictates the consequence of being rewarded or punished. Therefore, operant conditioning approaches are advantageous compared to classical conditioning because animals are in closed-loop with their environment, perceiving it, learning it, acting on it and integrating their own actions into the learning process. This less reductionist approach allows for complex neural processes to occur, which potentially facilitates memory formation and mimics the learning capabilities and behaviour in natural conditions.

Despite the advantages of observing naturalistic behaviours, in operant conditioning paradigms animals are in closed-loop with their environment and their interactions with it are complex and difficult to control. This lack of control over the characteristics of the environment being learnt and the time course of perceiving, acting and being rewarded or punished is one of the main disadvantages of these paradigms. Furthermore, access to the neural changes due to learning in a freely moving animal is not possible during the learning phase, being restricted to post-learning modifications.

Comparing classical and operant conditioning

Because these two paradigms are fundamentally different, one ought to question if the processes by which memory is formed through one type of conditioning are similar to the ones occurring during the other type of training. Based on studies from honey bees and locusts, we do know that memories acquired through classical conditioning can be transferred to an operant maze paradigm. Honey bees trained to associate an olfactory cue to a sugar reward (Sandoz *et al.*, 2000) or an electric shock (Carcaud *et al.*, 2009) through classical conditioning display preference or avoidance behaviour by flying more or less

often near the conditioned odour, respectively, when tested in a maze. Locusts that learnt the association between an odour and a food reward through classical conditioning also approach the arm with the conditioned odour more often than the other when tested on a y-maze (Simões *et al.*, 2011). Although these studies show that these memories are transferable between the different contexts to some extent, they do not compare the proportion of learners in one context and the other. Indeed, because of the fundamental differences in methodology between these two types of paradigm, the proportion of learners through one type of conditioning are not comparable to the proportion of individuals that learn through the other.

To our knowledge, only one study has addressed this issue, describing a set-up where a controlled comparison can be inferred between these two types of conditioning (Brembs and Heisenberg, 2000). This study was conducted in fruit flies *Drosophila melanogaster*, which were restrained inside a flight simulator where their yaw torque could be recorded. The recorded yaw torque of the fixed fly could then change the angular rotation of the flight simulator, in a closed-loop situation. Using this paradigm, Brembs and Heisenberg delivered an electric shock to the fly if it would turn in one direction but not the other. If this system was in closed-loop and the fly was able to rotate the environment in the desired direction, avoiding the direction that was paired with an electric shock, it would represent operant conditioning. If the experimenter controlled the direction by which the world moved and recorded the yaw torque of the fly in open-loop, learning would occur through classical conditioning. Because in these experiments flies learning through classical conditioning were replayed the exact same cues other flies experienced in closed-loop, this consisted in the only real comparison of operant and classical conditioning, showing that learning is facilitated if the animal's behaviour is involved in the process. Furthermore, the same paradigm was later used for imaging flies' neurons during learning through both types a conditioning (Liu *et al.*, 2006). However, comparisons for other insects and learning modalities have not yet been developed, and the neural basis of each type of learning have not been described, leaving this question mainly unanswered.

Virtual Reality systems for insects

To compare operant and classical conditioning, one needs a paradigm that, similarly to the Brembs and Heisenber's flight simulator, allows an animal to be able to navigate and learn in open and closed-loop with a virtual environment. In such system, the animal's movement (or intention to move when tethered) needs to be recorded and, in turn, change or not the virtual world. Although this was accomplished by Brembs and Heisenberg's flight simulator, and similar set-ups have been posteriorly used to investigate other aspects of learning in fruit flies (Tang and Guo, 2001; Brembs and Hempel de Ibarra, 2006; Peng *et al.*, 2007), to our knowledge no further research was conducted using this set-up to understand these two types of associative memory formation.

Posterior attempts to create a virtual reality system for insects, with full control over their visual surroundings, can be grouped in two main techniques, with freely moving or tethered insects. In the first scenario, animals can fly or walk in a virtual world as they would in a real arena. Thus, they are in closed-loop with their visual surroundings unless external manipulations of the virtual world are imposed, leading to an open-loop situation. Although these VR arenas can be used for several animal species (Stowers *et al.*, 2017), research with insects has typically used walking (Schuster *et al.*, 2002) or flying fruit flies (Fry *et al.*, 2008, 2009; Straw *et al.*, 2010), whose position was tracked in 2D or 3D, by one or multiple cameras, respectively. Tracking the fly's position has allowed experimenters to cancel self-induced motion of the world as the fly moves (Straw *et al.*, 2010) and even permit insects to instantly travel between different virtual worlds (Stowers *et al.*, 2017). Although these virtual reality paradigms have the advantage of eliminating confounding effects caused by tethering animals and have helped elucidating several aspects of fruit flies' visual system and motor control (Fry *et al.*, 2009; Straw *et al.*, 2010), the same limitations observed for free walking animals in real arenas are common to these paradigms.

Conversely, in the second scenario, animals are tethered and, therefore, in open-loop with their visual surroundings. Virtual reality systems allow for their intention to move to

be recorded and close the loop between the animal and the visual environment. Despite the earlier development of similar set-ups for mice and rats, typically used for studying spatial navigation (reviewed in Thurley and Ayaz, 2017), the establishment of techniques that could allow an insect to be restrained and interacting with a virtual world while walking was a step-by-step process, with the first contributions dating just a few years later than the flight simulator. In 2005, Hedwig and Poulet published a study addressing phonotactic steering in crickets (*Gryllus bimaculatus*), in which crickets were restrained on top of a light ball supported by air. As the animals attempted to walk, the ball moved underneath them, which was recorded by a computer optical mouse allowing the crickets' fictive path to be displayed (Hedwig and Poulet, 2005). Many studies on cricket audition have been published since then using adaptations of this set-up (Schöneich and Hedwig, 2010; Haberkern and Hedwig, 2016; Sarmiento-Ponce *et al.*, 2018), but experiments were always performed using auditory stimuli presented in open-loop. However, this trackball system set the path for the development of similar ones for fruit flies, honey bees and cockroaches, who have come much closer to true insect virtual reality systems. In these systems, the recordings of the trackball's movement were performed by one or more optical mice, or by a camera placed above the ball. At the same time, a virtual visual world was displayed to the animal, either using projections or LED lights. The fictive position of the animal was then able, to some extent, to change the virtual world accordingly. Moreover, because the animals were restrained while interacting with a fictive world, neural recordings or imaging could be performed in parallel (e.g. Seelig *et al.*, 2010; Tuthill *et al.*, 2011; Paulk *et al.*, 2014). All these studies are relevant for the understanding of the insect brain because of the methodology they developed or their findings on visual perception (Tuthill *et al.*, 2011), attention (Paulk *et al.*, 2014), vision modulation (Chiappe *et al.*, 2010), optomotor response (Seelig *et al.*, 2010), amongst others topics. However, some deserve special attention here for their contributions to the understanding of closed- and open-loop neural activity, memory formation or the display of naturalistic behaviours in related paradigms.

The first study to address here is the one of Takalo *et al.* (2012), who developed a

set-up for cockroaches to navigate on a projected virtual world in closed-loop. Although no particular questions were addressed in this study, it was the first one to demonstrate an insect truly navigating in a virtual world. They have shown that cockroaches, when placed on an air supported ball that controlled a virtual world, treat virtual objects as real, walking around projected trees. Similarly, Dahmen *et al.* (2017) have observed the naturalistic walking behaviour of another insect species, the desert *Catagliphis fortis*. Desert ants, as other species of eusocial insects, are able memorise the direction and distance they travelled from their nests to a food source and use that information to return to their colonies efficiently, a process called path integration. In this study, ants were trained to an artificial feeder and were placed on the trackball just before their homebound run. Thus, they performed this route on the ball, firstly running at high velocities and then displaying slower searching behaviour around the correct position of the fictive nest. Although in this study ants were not navigating in a virtual world and, therefore, the behaviour they displayed was in open-loop, to our knowledge this was the first time path integration was observed in tethered ants, on a system that allows such detailed analysis of the insect's behaviour.

Also to be acknowledged here is the study of Paulk *et al.* (2015), who investigated how neural activity of flies walking on an air supported trackball differed between observing a visual stimulus passively compared to observing the same visual cue while actively interacting with the virtual environment. To do so, they recorded the local field potentials (LFPs) within different areas of the flies' brain, which are transient electrical signals generated by the summed and synchronous activity of groups of neurons, captured extracellularly (Buzsáki *et al.*, 2012). They observed that the frequency at which the visual cue flickered was detected in the LFP recordings, when the fly was in closed- and open-loop with its environment. However, the coherence of the LFP signals within the fly's brain was increased when the fly was in closed-loop, actively interacting with the virtual cue, compared with when it was in open-loop, passively observing it. Although this was not the first demonstration of differential neural activity between passive and active interactions with the environment in insects (Palka, 1969), to our knowledge it was the first one

to do so in a an insect restrained on top of an air supported ball, walking in a virtual world.

Furthermore, two studies performed by Buatois and collaborators have addressed the learning abilities of honey bees when placed in a real y-mazed compared to learning when tethered on top of a trackball (Buatois *et al.*, 2017, 2018). In the first study, walking honey bees were subjected to an operant conditioning training on a y-maze, in which one arm was rewarded with sucrose and the other with an aversive quinine solution, depending on which visual cue was presented in that arm. After training, the preference for one visual cue or the other was tested on the same y-maze. In parallel, another group of bees was trained in a classical conditioning paradigm, while tethered on top of an air supported trackball. The same visual cues were presented to the bees, one at a time, with a sugar reward being delivered after one of the cues and the quinine solution after the other. After training, bees were allowed to fixate on one cue or the other, and their heading direction towards the chosen cue was recorded. Bees successfully learnt the association between each cue and its corresponding reward or punishment in both paradigms. Bees that were trained in a y-maze chose the arm where the visual cue associated with a reward was present, while bees that were trained on the trackball fixated and turned more towards the same cue, but learning was more successful in free walking bees. However, in the second study, while one group of bees learnt the association between similar visual cues and reward/punishment on a y-maze, the group of tethered bees was allowed to rotate the virtual cues and also learn through operant conditioning, being rewarded with sucrose or punished with a quinine solution depending on the cue they rotated to the center of the world and fixated on. In this paradigm, bees were in closed-loop with the virtual world in rotation, but in open-loop in translational movements and unable to walk towards the virtual cues. Although this resulted in similar learning abilities between free-walking and tethered bees, transferring to the opposite training paradigm led to differences in performance. Transferring bees from the virtual reality training to the y-maze resulted in an improvement of learning, while the reciprocal case led to a reduction in performance. Thus, although the visual cues were the same, the process by which memory was acquired was different, and both studies suggest, as in Brembs and Heisenberg (2000) experiments,

that active interactions with the environment facilitate learning.

Lastly, true navigation on a virtual world, in both translation and rotation, was also accomplished for fruit flies in the study of Haberkern *et al.* (2019). These authors showed that walking behaviour of tethered flies navigating in a virtual world is similar to free walking flies' in a real arena, showing object approach and fixating in both contexts, as well as similar walking speeds. The fixating behaviour was also similar between flies that controlled the motion of the visual world in rotation solely or also in translation, although the authors argue that only in full closed-loop their behaviour could be adequately described. Furthermore, Haberkern and colleagues made use of optogenetics to induce a fictive perception of a sugar reward or a heat stimulus and observed that pairing a virtual object with fictive-heat led to learning of this association, but pairing it with a fictive-sugar reward only led to searches around the reward location (similar to free walking flies) but no memory of the association between the location and reward.

Despite the crucial advances on developing virtual reality systems for insects, the question of how passive and active interactions with the environment change information perception, processing and ultimately learning and memory formation remains unanswered. Thus, the differences in neural activity while forming memories through classical and operant conditioning, the two most used paradimgs for studying learning and memory formation in all animal models, remain unobserved.

Aims and objectives

The main aim of this thesis was to investigate the differences in passive compared to active learning, through classical and operant conditioning, respectively, using wood ants as the selected insect model. To achieve this, we had two methodological and their corresponding experimental objectives:

1. Develop a visual classical conditioning paradigm for wood ants, using adaptations of previously established paradigms for classical conditioning in other insect species.

1.1. We intended use this new paradigm to investigate if wood ants, as honey bees, are able to form visual memories passively through classical conditioning and analyse memory acquisition and retention at different time points.

2. Develop a virtual reality set-up in which harnessed wood ants can navigate through a virtual environment in closed-loop and be replayed the same changes in the virtual world in open-loop.

2.1. To investigate if wood ants can perceive and interact with our virtual world, we intended to perform simple behavioural experiments based on naïve behaviours they display naturally.

3. Develop an automatic reward system that would provide a drop of sucrose solution to the ant when triggered by the position of the ant in the virtual world.

3.1. We intended to use this paradigm to train ants to associate a position in the virtual world, near a virtual object, with the presence of a sucrose reward. We intended to analyse the ants ability to form this memory actively and passively by comparing ants that arrive to the right virtual cue by themselves, in closed-loop as in operant conditioning, with ants that observe the same visual cues passively, in open-loop as in classical conditioning.

Summary of contributions and guide through Thesis

This thesis makes several contributions to the fields of insect behaviour, learning and neuroscience. Although not all aims have been met, contributions to other fields previously not envisaged are also achieved here.

In Chapter 2, we show that wood ants are able to form an associative memory between a visual cue and a sugar reward through classical conditioning (Fernandes *et al.*, 2018*a*,*b*). To our knowledge, it is the first time such paradigm is used in any form of classical conditioning in wood ants. Furthermore, most paradigms have been developed for olfactory learning and, to our knowledge, no other visual classical conditioning studies have been

reported for any ant species.

In Chapter 3, building upon this methodology, we show that wood ants, similarly to eusocial bee species, can show lateralisation of memory formation through classical conditioning. While bees recall olfactory memories at different time points when trained with the right compared to the left antenna, we show that wood ants recall visual memories differently when rewarded with a touch on one antenna or the other prior to presenting the reward to the mouthparts. We demonstrate that these time points are similar to the ones found with eusocial bees, with touching the right antenna producing short- and medium-term memories and touching the left producing long-term memories. To our knowledge, it is the first time lateralisation of memory retention is shown in an insect species except for bees and the first time it is shown for visual memories. This investigation contributes to the field of lateralisation and, more specifically, the evolution of lateralisation within the Hymenoptera, as well as the fields of learning and memory formation in insects.

In Chapter 4, we describe a new methodology for wood ant navigation with fixed rather than free moving animals, using a virtual reality system. In this paradigm, wood ants are able to walk on top of a polystyrene ball supported by air, which movements are recorded by an optical mouse and, in turn, change the position of the ant in the virtual world accordingly. This allows us to acquire very detailed descriptions of the animals' behaviour while navigating and interacting with a virtual world. Furthermore, we show that manipulations of the world can be perform to an extent that experiments with free moving animals do not, which would allow for an adequate comparison between classical and operant conditioning.

We validated this new paradigm by showing that wood ants interact with a virtual beacon by approaching and fixating it, as they do with similar real objects. We also demonstrate that wood ants show repeatable walking behaviour, walking approximately similar distances and during comparable times, at equivalent speeds, when placed in the same virtual world twice. To our knowledge, it is the first time repeatable naïve walking distance and time has been described in an insect. Furthermore, we show that self-induced optic flow is not necessary for reliability of walking, which is in accordance with previous

studies on other ant species. We also show that self-induced optic flow, when present, can be used to evaluate total distance walked and time spent walking, but it is not used to integrate the velocity at which ants move. Moreover, we show that wood ants change their behaviour when experiencing external optic flow, walking longer distances at faster speeds but during the same amounts of time. To our knowledge, it is the first time the use of optic flow cues in navigation is investigated in wood ants, the first time this is investigated in insects using naïve walking behaviour within a virtual world and the first time self-induced and external optic flow are directly compared.

Although our virtual reality paradigm was not yet used for comparing classical and operant conditioning, on Chapter 5 we discuss the potential neural mechanisms for memory formation through these two distinct learning paradigms. We base our models for passive learning, through classical conditioning, and for the integration of proprioceptive (internal) and exteroceptice (external) cues during walking behaviour based on our behavioural experiments and previous studies addressing the neural circuits involved in similar behaviours. Lastly, we propose several methodologies and experimental procedures that could shed a light on questions left open by this Thesis.

Visual associative learning in wood ants

Abstract

Wood ants are a model system for studying visual learning and navigation. They can forage for food and navigate to their nests effectively by forming memories of visual features in their surrounding environment. Previous studies of freely behaving ants have revealed many of the behavioural strategies and environmental features necessary for successful navigation. However, little is known about the exact visual properties of the environment that animals learn or the neural mechanisms that allow them to achieve this. As a first step towards addressing this, we developed a classical conditioning paradigm for visual learning in harnessed wood ants that allows us to control precisely the learned visual cues. In this paradigm, ants are fixed and presented with a visual cue paired with an appetitive sugar reward. Using this paradigm, we found that visual cues learnt by wood ants through Pavlovian conditioning are retained for at least 1 h. Furthermore, we found that memory retention is dependent upon the ants' performance during training. Our study provides the first evidence that wood ants can form visual associative memories when restrained. This classical conditioning paradigm has the potential to permit detailed analysis of the dynamics of memory formation and retention, and the neural basis of learning in wood ants.

Introduction

Learning and memory formation in insects has been studied extensively, most typically in foraging individuals from colonies of eusocial Hymenoptera (bees or ants) or adult fruit

flies (Giurfa, 2007; Davis, 2005). Learning associations between appetitive or aversive stimuli and the characteristics of the environment in which they occur enhances foraging efficacy (Dukas and Bernays, 2000; Simões *et al.*, 2013). Indeed, foraging insects display learning abilities that include the categorization of visual stimuli (Zhang *et al.*, 2004), contextual (Dale *et al.*, 2005) and sequence learning (Collett *et al.*, 1993), interval timing (Boisvert and Sherry, 2006) and sameness–difference rules (Giurfa *et al.*, 2001), amongst others (Chittka and Niven, 2009).

Visual memories are of particular importance to foraging insects including honey bees (*Apis mellifera*; e.g. Cartwright and Collett, 1983), desert ants (*Cataglyphis spp.*; e.g. Collett *et al.*, 1992), wood ants (*Formica rufa*; e.g. Nicholson *et al.*, 1999) and bumble bees (*Bombus terrestris*; e.g. Gumbert, 2000), which use them to navigate towards a feeder. In wood ants, visual memories of landmarks can enable ants to locate the position of a food source or the nest but can also provide directional cues along the entirety of the route (Collett and Collett, 2002; Durier *et al.*, 2003; Graham *et al.*, 2004; Harris *et al.*, 2005, 2007).

Although experimental paradigms with freely moving animals can allow them to reproduce behaviours they naturally display in the wild, experimenters typically lack control over the specific cues being learnt. Furthermore, for species in which optogenetic techniques are lacking, it is not possible to investigate the neural circuits underpinning learning and memory in detail unless the animals are fixed to permit electrophysiology. These issues can be overcome to some extent by the development of classical conditioning paradigms for appetitive learning in restrained insects (e.g. Takeda, 1961; Bitterman *et al.*, 1983) that reproduce an experimental paradigm for studying learning and memory developed more than 100 years ago by Pavlov (1897).

In appetitive classical conditioning, a conditioned stimulus (CS) is paired with an unconditioned stimulus (US), such as food or water, which evokes a strong, unconditioned response (UR). The majority of these studies in insects are based upon a paradigm developed originally for honey bees (Takeda, 1961; Bitterman *et al.*, 1983). In this paradigm, the honey bee performs the proboscis extension reflex (PER) when presented with su-

crose (US) paired with a CS, typically an odour. After only three trials, bees learn to associate the CS with the US, responding to the CS alone with a PER, and can form a long-term memory of this association (Wittstock *et al.*, 1993). Other species of bees (*Bombus terrestris*, *Melipona quadrifasciata*, *Scaptotrigona depilis*) and flies (*Musca domestica*, *Drosophila melanogaster*) also perform the PER to a CS after pairing it with an appetitive US (Fukushi, 1976, 1979; Laloi *et al.*, 1999; Chabaud *et al.*, 2006; Mc Cabe *et al.*, 2007). In insects that lack a proboscis, a similar paradigm can be used, though the response involves other mouthparts. For example, ants (*Camponotus aethiops* and *Camponotus fellah*) perform the maxilla–labium extension reflex (MaLER) (Guerrieri and d'Ettorre, 2010; Guerrieri *et al.*, 2011), and locusts (*Schistocherca gregaria*) perform the palp opening response (POR) (Simões *et al.*, 2011, 2012, 2013).

Most of these studies on associative memories using classical conditioning approaches on restrained insects use an odour as the CS, with only a few exceptions in flies (Fukushi, 1976) and honey bees (Hori *et al.*, 2007; Niggebrügge *et al.*, 2009; Balamurali *et al.*, 2015; Avarguès-Weber and Mota, 2016) using a visual cue as the CS. Although visual plus olfactory cues and olfactory cues alone produce comparable learning rates, using visual cues alone as the CS seems to produce weaker learning (Gerber and Smith, 1998).

In this study, we developed a visual appetitive learning paradigm through classical conditioning in the wood ant, *Formica rufa*. Wood ants have been shown to form visual memories while navigating and rely upon them while foraging (Collett and Collett, 2002; Durier *et al.*, 2003; Graham *et al.*, 2004; Harris *et al.*, 2005, 2007). We show for the first time that ants can acquire and retain short- and medium-term visual memories when harnessed, demonstrating visual learning through classical conditioning.

Methods and Materials

Animals

Experiments were performed using wood ants (*Formica rufa* L.) from two different colonies. Colonies were collected from Ashdown Forest, Sussex, UK (N 51 4.680, E 0 1.800) in

June and August 2016 and maintained indoors for 5 months prior to training, during which they were kept at 26°C, under a 12 h light:12 h dark cycle and fed with sucrose (333 g/L). These conditions were maintained throughout our experiments except that the colonies were starved for at least 2 days before the start of training. On the day of training, ants were selected from the surface of the colony and placed in a box with a glass slide upon which was a drop of sucrose solution (200 g/L). Only those ants that started to feed were selected for subsequent training, thereby ensuring that ants chosen were motivated to feed. To prevent ants becoming satiated, potentially reducing their motivation to learn during training, they were removed from the box as soon as they started to feed.

Harnessing

Ants were placed in the freezer for 1–2 min and then harnessed individually at the back of their head to an insect pin (Austerlitz Insect Pin®, Fine Science Tools GmbH, Heidelberg, Germany, 26000-40) with low melting point wax. The insect pin was then fixed in modelling clay (Plasticine®). The ant was allowed to stand on a custom-made plastic holder embedded in the same modelling clay, thereby ensuring it maintained a typical standing posture and that the legs were free to move (figure 1A). Ants were left fixed in a dark room for at least 2 h prior to training.

Training and testing

Experiments were conducted in a transparent Perspex box (50*50*50 cm) covered in white paper, open to the front to permit experimenter access (figure 1A). A camera (eo-13122M, Edmund Optics Inc., Barrington, NJ, USA) with a macro lens (EF 100 mm f/2.8 L Macro IS USM, Canon, Surrey, UK) was placed directly above the ant, viewing it through a hole in the upper surface of the box. This ensured that the ant's head stayed within the camera's field of view throughout the whole experiment. To reduce extraneous visual input, the box was back-lit by two 26 W light sources, and the rest of the room was kept in darkness. The camera was attached to a computer and recordings were performed using uEye64 software (IDS Imaging Development Systems GmbH, Obersulm, Germany). The visual cue (CS) was a bright blue cardboard rectangle (width: 60 mm;
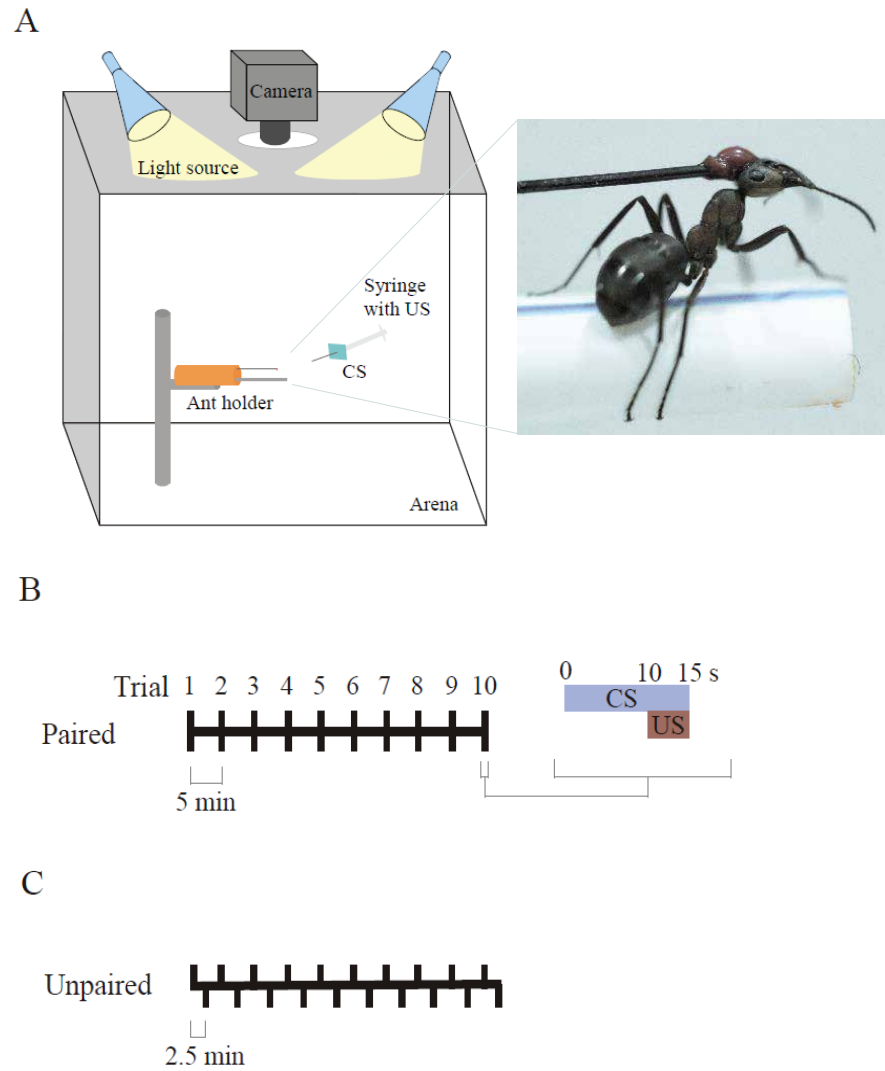
Figure 1: Experimental set-up and training scheme. A) The ant was placed inside a Perspex box illuminated by two light sources, directly underneath the camera. It was fixed to an insect pin attached to a Plasticine cylinder (orange) supported by a holder, ensuring that it retained a naturalistic stance (see Materials and methods). The conditioned stimulus (CS) and unconditioned stimulus (US) are represented by the blue rectangle (CS) attached to the syringe with the sugar reward (US). Inset: a close-up view of an ant in the holder. The ant's head is fixed with wax. Ants were subjected to two types of training, B) paired or C) unpaired. Vertical black lines represent the US and CS, which were temporally dissociated in unpaired training (see Materials and methods).

height: 45 mm) attached to the syringe in which the US (sucrose 200 g/L) was manually delivered to the ant (figure 1A).

Ants were subjected to paired (N=51) or unpaired (N=29) training. These two types of training were conducted in parallel, in a randomized order. During paired training, the syringe plus cardboard was held a few millimetres in front of the ant for about 10 s. The syringe with a drop of sucrose was then moved next to the mouthparts and antennae so ants could detect its presence and feed on it for about 5 s. Therefore, ants were presented with the CS for 15 s, of which the last 5 s overlapped with the sucrose reward. Ants were subjected to 10 trials, separated by 5 min (figure 1B). During unpaired training, the CS (cardboard plus syringe) and the US (drop of sucrose from the syringe) were presented to the ants but temporally dissociated from each other. Between the presentation of either the US or the CS, ants rested for 2.5 min. This training consisted of 10 CS presentations and 10 sucrose deliveries, which ensured that ants in the paired and unpaired training groups were subjected to the CS and US the same number of times. The duration of each US and CS presentation was the same as in the paired training: 5 and 10 s, respectively (figure 1C). Ants were tested for memory retention either 10 min (paired: N=15; unpaired: N=15) or 1 h (paired: N=15; unpaired: N=14) after the last trial. In these tests, the visual cue (cardboard plus syringe) was presented to the ant for about 10 s, and their response recorded. The US (drop of sucrose) was then delivered to ensure ants were still motivated to feed and respond. The ants' response was recorded in every trial and test. For all the cases, the duration of the CS and US presentations varied slightly because they depended on how long ants took to start feeding. Ants that did not feed on every trial (33 paired and 7 unpaired) were discarded.

MaLER scores

Ants' responses during the presentation of the visual cue in training trials and tests were recorded and separated into three types of behaviour: full extension with movement (FEM), as if feeding; full extension without movement (FE); or partial extension (PE) of the maxilla–labium or maxillary palps (figure 2) (Paul *et al.*, 2002). A positive response was counted every time an ant performed any type of MaLER (Guerrieri and d'Ettorre,
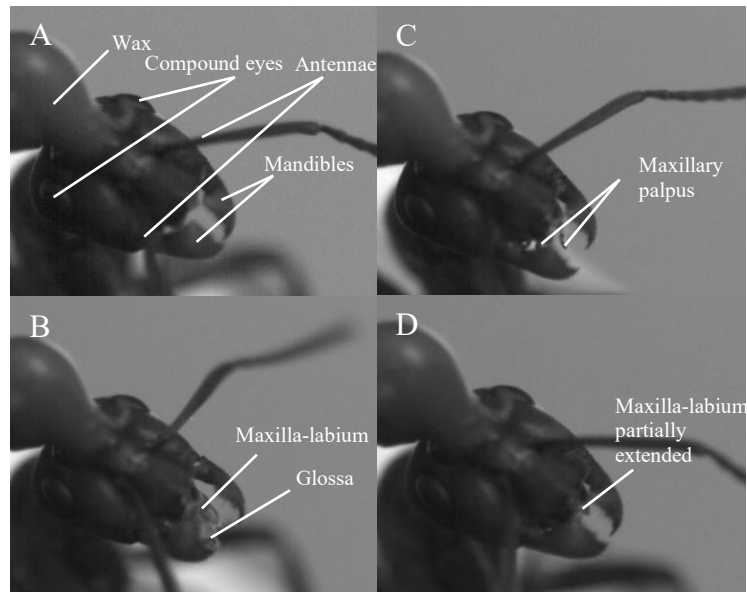
Figure 2: The maxilla–labium extension response (MaLER) of wood ants. Individual frames from video recordings show ants' mouthpart movements during training. A) No response; B) full extension of the maxilla–labium that terminate in the glossa; C) partial extension with only the maxillary palpus visible; and D) partial extension of the maxilla–labium structures.

2010) during the CS presentation (data available from figshare: https://doi.org/10.25377/sussex.5794386). If ants were extending or moving their mouthparts spontaneously just prior to a trial, we postponed training or testing for a few seconds. Ants that continuously moved their mouthparts were excluded from our analysis.

Statistical analysis

The distribution of the number of responses from each type of MaLER was analysed using a G-test for goodness-of-fit with a prior expectation of one-third of the responses from each type. This was performed with grouped classes (one, two and three or more responses) to avoid classes with a low number of responses and adjusted with a William's correction (Sokal and Rohlf, 1995).

The influence of the type of training (paired or unpaired) and of trial number in the ant's response to the CS during training was analysed using logistic regression with mixed effects (Bates, 2010). For this, MaLER was scored as a binomial variable, with positive responses being counted as 1 and no response as 0. To compare the proportion of ants responding in each training trial and in each retention test between paired and unpaired

types, we performed G-tests of independence, adjusted with William's correction, except for the first trial, which was analysed using Fisher's exact test because of the low number of responses (Sokal and Rohlf, 1995). The responses on the first trial were included in all statistical analysis, to account for spontaneous responses to the CS.

The correlation between the proportion of ants that responded to the CS in the retention tests and the number of times they responded during training was analysed using Spearman's rank correlation. Logistic regression was also performed to determine the number of trials in which ants needed to show a response during training to predict whether they would respond in the test (Bates, 2010).

G-tests were performed in Excel (Excel 2010, Microsoft, Redmond, USA), using bespoke code (Sokal and Rohlf, 1995). All other statistical analysis was performed in R (v3.5.1, R Foundation for Statistical Computing, Vienna, Austria) using RStudio (v1.1.463, RStudio Inc, Boston, USA). For the logistic regression, the 'lme4' package was used (Bates, 2010).

Results

The wood ant MaLER can be conditioned to a visual stimulus

During each paired training trial, ants were presented with a visual stimulus (CS) and sucrose (US). On those training trials in which paired ants responded, they did so with one of three different types of response that varied in the degree to which the mouthparts were extended and moved: FEM, FE or PE of the maxilla–labium or maxillary palps (see Materials and methods). Typically, ants performed FEM or PE more often than they performed FE. In most cases, ants showed little consistency among trials in the type of MaLER, though a few ants did consistently perform FEM or PE (figure 3). The distribution of the occurrence of each of the three MaLER types across the 10 training trials differed significantly (G-test of independence, N=51, d.f.=6, $G_{adjusted}$=17.3, P<0.01). Consequently, we display the three types of MaLER separately in subsequent figures, though we considered the MaLER as a single response when analysing responses to the CS. Statistical analysis
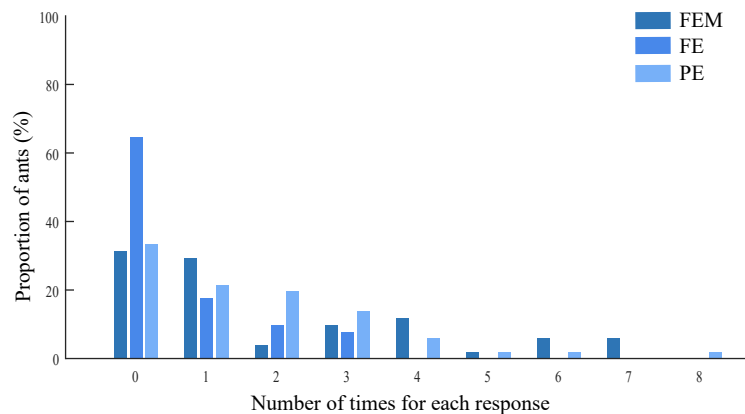
Figure 3: Consistency in the MaLER to the CS during paired training. Ants (N=51) performed full extension with movement (FEM; dark blue), full extension (FE; medium-blue) or partial extension (PE; light blue).

and learning curves for each type of MaLER are included in the Appendix A (figure A1, table A1).

Ants were exposed to 10 paired (N=51) or unpaired (N=29) training trials. Naïve ants showed a low tendency to perform MaLER when presented with the visual stimulus (figure 4); on the first paired or unpaired trial, prior to training, just 3–4% of the ants performed MaLER. There was no significant difference in the spontaneous MaLER rate between paired and unpaired naïve ants (table 1). Increasing the number of training trials led to a significant increase in the occurrence of MaLER in the paired ants, which plateaued at around 50% from the third trial onward (figure 4A; logistic regression, N=51, d.f.=507, z=5.949, P<0.01). Conversely, there was no increase during training for unpaired ants (figure 4B; logistic regression, N=29, d.f.=287, z=0.758, P=0.45). Individual ants showed substantial variation in the number and type of MaLER that they displayed during training (figure 4C, D). Considering all trials together, the proportion of ants that responded to the visual cue was significantly higher during paired than during unpaired training (logistic regression, N=80, d.f.=796, z=5.306, P<0.01). After the first trial, each subsequent trial differed significantly between paired and unpaired ants (table 1). Taken together, these results suggest that ants associated the visual cue with the sucrose reward

contingent upon the timing of presentation of the CS and US (i.e. whether paired or unpaired).

Paired training evokes short- and medium-term appetitive memories

To assess whether wood ants had formed a memory following training, we tested them by presenting the CS alone. Each ant was tested either 10 min or 1 h after the last training trial, which is indicative of a short-term or a medium-term form of memory, respectively (Guerrieri *et al.*, 2011). After 10 min, 53.3% of ants that had received paired training responded during testing compared with just 13.3% of ants that had received unpaired training (figure 5A). Likewise, after 1 h, 40% of ants that had received paired training responded during testing compared with 7.48% of ants that had received unpaired training (figure 5B). In both tests, the proportion of ants responding to the CS was significantly higher if they had undergone paired rather than unpaired training (figure 5; G-test of independence, 10 min test: N=30, d.f.=1, $G_{adjusted}$=5.5, P<0.05; 1 h test: N=29, d.f.=1, $G_{adjusted}$=4.42, P<0.05). Each test was followed by the US (sucrose) to ensure that the ants were still motivated to feed. For both tests, every ant fed. Thus, following paired but not unpaired training, ants acquired short-term and medium-term visual associative memories.

Table 1: Comparison of the frequency of ants showing the maxilla–labium extension response (MaLER) between paired and unpaired ants for each trial. The number of ants (N), degrees of freedom (d.f.), G-test of independence (G) and P-value are shown (*P<0.05, **P<0.01). The first trial was analysed with Fisher's exact test.

Trial	N	d.f.	G(adjusted)	P
1	80	n.a.	n.a.	>0.1
2	80	1	3.86	<0.05*
3	80	1	8.41	<0.01*
4	80	1	6.63	<0.01*
5	80	1	8.41	<0.01**
6	80	1	7.5	<0.01**
7	80	1	10.69	<0.01**
8	80	1	11.76	<0.01**
9	80	1	17.13	<0.01**
10	80	1	17.13	<0.01**

We assessed whether those ants that responded to the presentation of the visual stim-
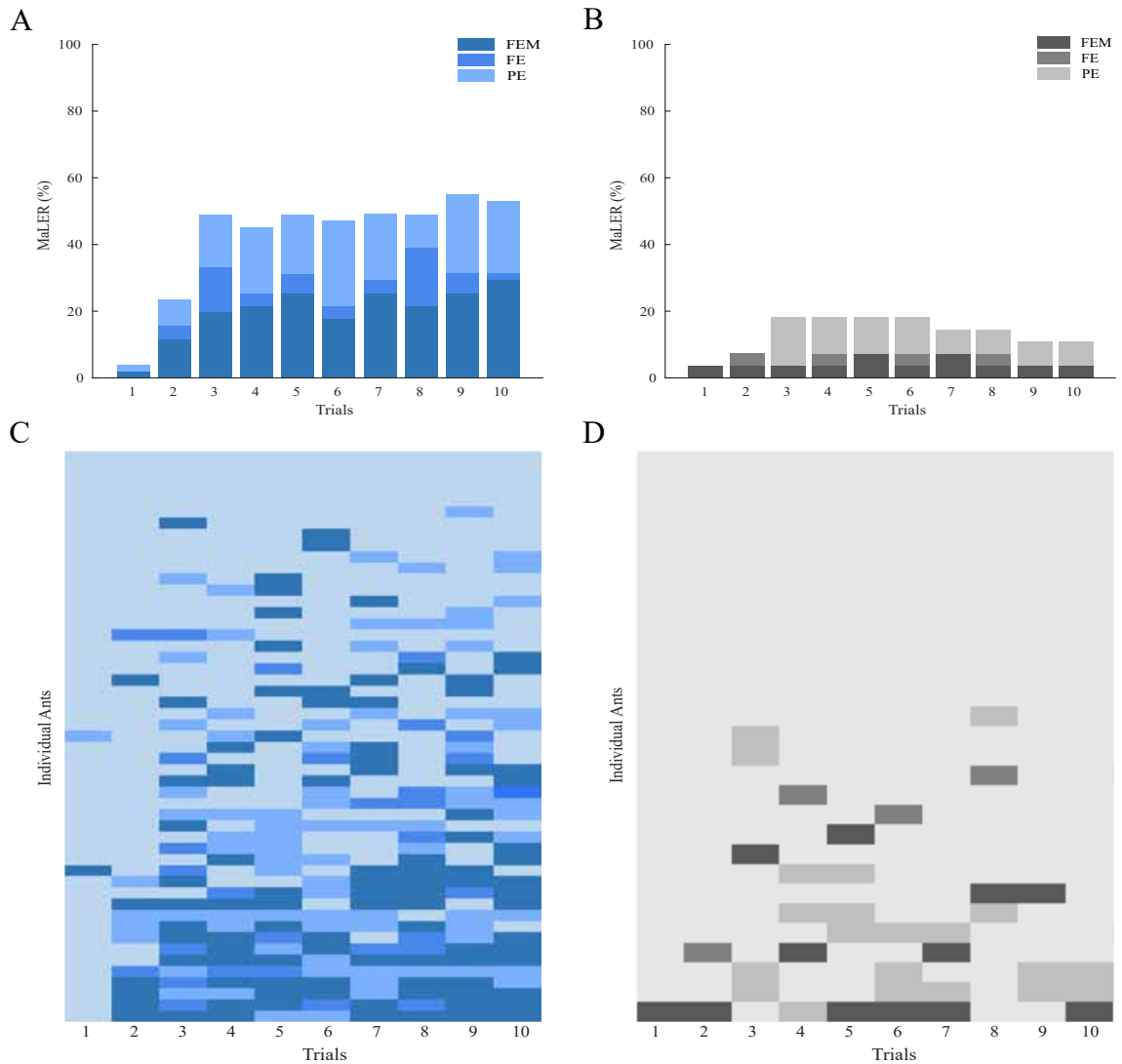
Figure 4: Wood ants learn to associate a visual cue with a paired sugar reward. A) The percentage of ants (N=51) performing the MaLER following the CS presentation significantly increased throughout paired training. B) The percentage of ants performing the MaLER did not increase significantly throughout unpaired training (N=29). C) Individual performance of paired ants, and D) unpaired ants during training. The three types of MaLER are represented in dark (FEM), medium (FE) and light (PE) blue or grey.
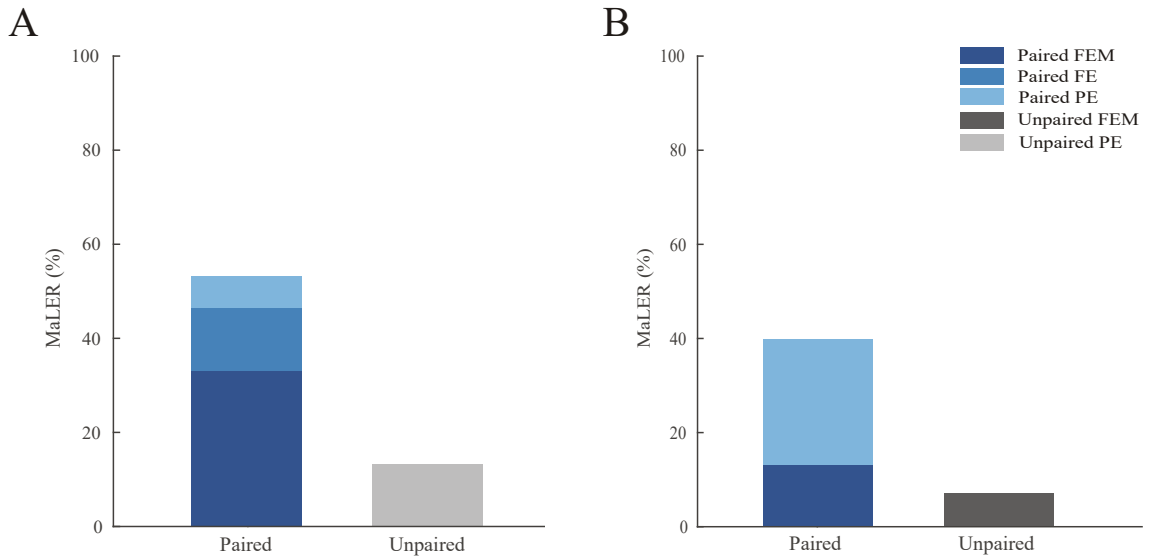
Figure 5: Wood ants retain appetitive memories for at least 1 h. Ants were tested A) 10 min (paired: N=15; unpaired: N=15) or B) 1 h (paired: N=15; unpaired: N=14) after the last training trial. The percentage of ants responding to the CS alone after paired training is shown in blue, and the percentage responding to the CS alone after unpaired training is shown in grey. The three types of MaLER are represented in dark (FEM), medium (FE) and light (PE) blue or grey.

ulus (CS) with MaLER during training were the same subset of ants that subsequently responded during testing. We pooled the 10 min and 1 h tests, separated ants that responded during testing from those that did not, and determined whether they had responded during training (figure 6); 64.3% of the paired ants that responded during testing also responded during training in at least half of the trials, whereas only 18.8% of the ants that failed to respond during testing responded in at least half of the trials during training (figure 6A). Indeed, ants that responded during testing also responded significantly more during training compared with ants that did not (logistic regression, N=30, d.f.=306, z=3.775, P<0.01). In contrast, only three ants of the 29 ants that had undergone unpaired training responded during testing, and just one of those three ants had performed MaLER on at least half the trials during training (figure 6B). Therefore, ants' behaviour during training was similar to that during testing; however, there was a higher number of responses during paired training and testing than during unpaired training and testing.

Next, we analysed whether the number of trials on which an ant responded during paired training was correlated with their response during testing. Combining the two tests together, we observed that the percentage of ants that responded during testing correlated with the number of trials on which they responded during training (figure 6C; Spear-

Table 2: The probability of ants responding during memory tests is predicted by the number of training trials on which they responded to the CS. The number of ants (N), degrees of freedom (d.f.), logistic regression (z) and P-value are shown (*P<0.05, **P<0.01).

N° of responses in training	N	d.f.	z	P
1 or more	31	30	0.008	0.994
2 or more	31	30	0.007	0.994
3 or more	31	30	0.009	0.993
4 or more	31	30	2.752	0.006**
5 or more	31	30	2.774	0.006**
6 or more	31	30	2.392	0.017 *
7 or more	31	30	2.554	0.011*
8 or more	31	30	2.29	0.022*
9 or more	31	30	0.008	0.994

man's rank correlation, N=10, d.f.=9, ρ=0.812, P<0.01).We also examined whether the number of trials on which an individual ant responded during training could predict its response during testing. The probability of performing MaLER during testing increased significantly with the number of trials in which ants responded during training (figure 6D; logistic regression, N=31, d.f.=29, z=2.79, P<0.01). Furthermore, ants that responded on four or more trials during training were significantly more likely to respond during testing than those that did not (logistic regression, N=30, d.f.=29, z=2.725, P<0.01; table 2), which is indicative of a threshold during training for short- and medium-term memory formation.

Discussion

Our aim was to develop a classical conditioning paradigm to analyse the acquisition and retention of visual associative memories in restrained wood ants. Here, we have shown that this is possible by making use of the MaLER, using a paradigm modified from earlier ones designed for appetitive olfactory classical conditioning in other ant species (Guerrieri and d'Ettorre, 2010; Guerrieri *et al.*, 2011). We showed that also in wood ants MaLER can be used as the UR in classical conditioning experiments. This response to a visual CS is initially low but increases when paired with an appetitive US, sucrose. After three or more training trials, 50% of the ants responded to the CS before sucrose was given. To ensure ants learnt the intended CS and not any other feature, we used an unpaired control

in which all the CS and US presentations were temporally dissociated from each other. Because ants in the unpaired training group did not increase their responsiveness to the CS, we conclude that the key visual feature learnt by paired ants was the intended CS.

In classical conditioning experiments, it is important to have a detailed description of the behaviour we consider to be the UR because this directly affects how learning and memory are quantified. Our analysis identified different degrees of MaLER expression, in contrast with previous studies that have reported a single type of response (Bitterman *et al.*, 1983; Guerrieri and d'Ettorre, 2010), but is similar to the palp opening reflex of desert locusts, which also includes flicking, palpation or outward opening of the maxillary palps (Simões *et al.*, 2011).

Our experiments also showed that wood ants can form a memory of the association between the visual CS and the US, and retain it for at least 1 h. This memory was contingent upon the timing of the CS and US presentations; ants that were trained with unpaired presentations of CS and US did not show memory formation. Furthermore, the propensity of an individual ant to retain a memory of the CS/US association for 10 min or 1 h after training depended upon the number of training trials on which they responded. Previous studies have interpreted these times as short- and early medium-term memory, respectively (Guerrieri *et al.*, 2011). Assessing long-term retention of memories 24 h after training was not possible because restrained ants did not survive long enough for testing. This may indicate that restrained animals are subjected to high levels of stress, which is known to negatively influence learning (Bateson *et al.*, 2011). However, our method of restraint allows ants to adopt a natural stance and to move their limbs and antennae freely (figure 1), whilst ensuring that the CS and US presentations as well as the number of trials and the inter-trial interval are controlled, and that associative memories are acquired only by Pavlovian association. Thus, it seems unlikely that restraint adversely affects the rates of learning we report.

Despite differences in methodology, our training paradigm produced similar rates of learning (50%) to previous studies using classical conditioning of a visual cue performed with honey bees (Hori *et al.*, 2007; Balamurali *et al.*, 2015; Niggebrügge *et al.*, 2009).
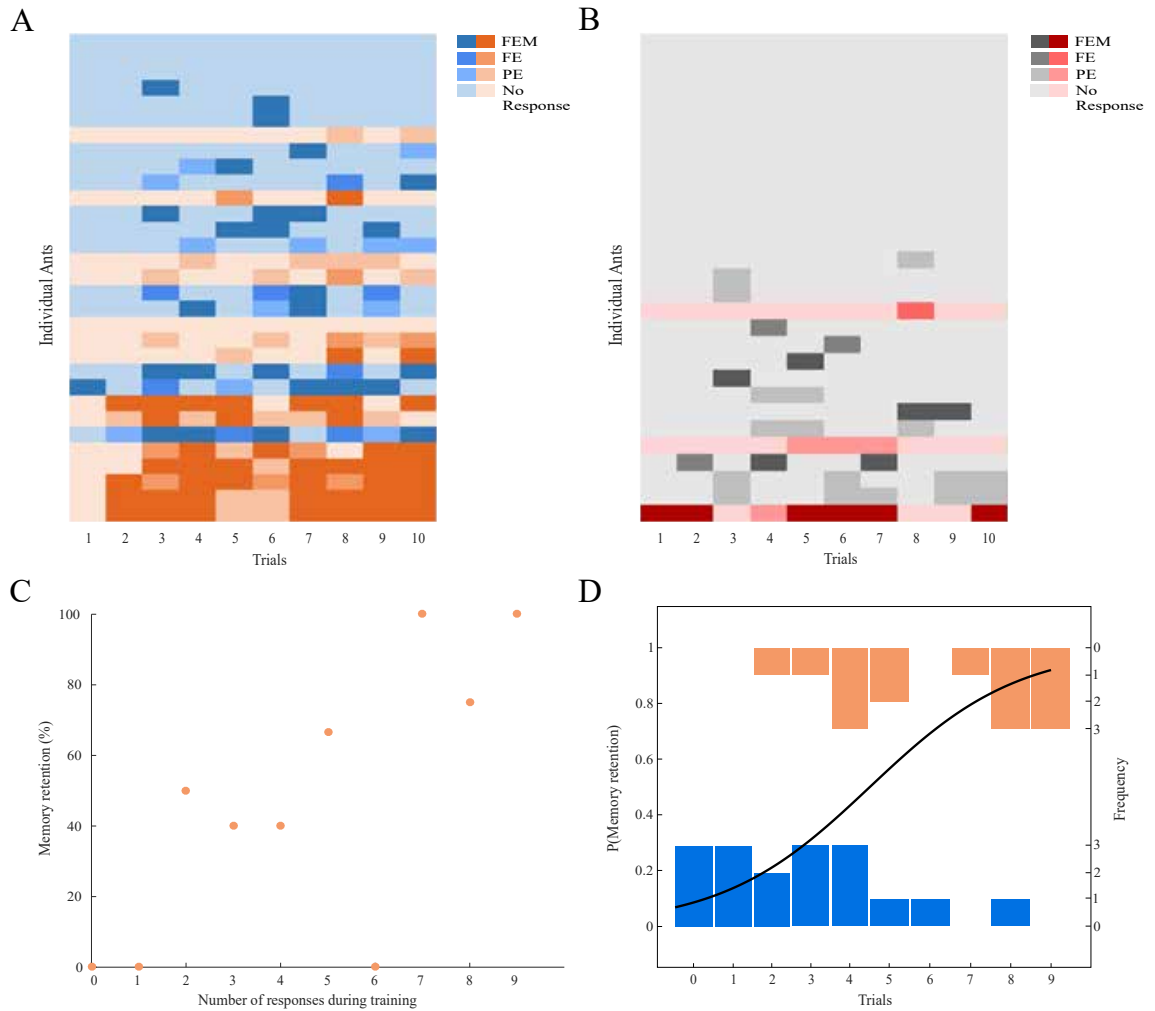
Figure 6: Wood ants' responses during memory tests are predicted by their behaviour during training. A) Responses of individual ants during paired training (N=30). Those that did not respond during subsequent testing are shown in blue, whereas those that responded are shown in orange. B) As in A but for unpaired training (N=29). Ants that did not respond are shown in grey whereas those that did respond are shown in red. C) The percentage of paired ants that performed MaLER following the CS in the tests correlates significantly with the number of times they responded to the CS during training. D) The more trials on which an ant responded during paired training, the higher the probability (P) of responding in the test (black line). Ants that did not respond during testing (blue) cluster around lower numbers of responses during training, while ants that did respond (orange) cluster around higher numbers of responses.

Hori *et al.* (2007) and Balamurali *et al.* (2015) achieved a learning rate of 40–50% in restrained honey bees. Niggebrügge *et al.* (2009) achieved higher response rates of 60–80% with a visual cue in restrained honey bees but ablated the bees' antennae, in contrast to our methodology and that of Balamurali *et al.* (2015) in which the antennae were intact (for further comparison of methodologies for visual classical conditioning in honey bees, see Avarguès-Weber and Mota, 2016). It is possible that higher rates of learning could be achieved in wood ants with the antennae ablated. Yet, despite the different paradigms for producing visual classical conditioning, wood ants, like honey bees, can form associative memories. This ability of wood ants, despite their relatively small compound eyes and visual systems compared with those of honey bees (Perl and Niven, 2016; Jander and Jander, 2002), emphasises that this is not restricted to insects with a substantial investment in vision, but is more widespread among the Hymenoptera.

Previous studies of learning in wood ants have employed freely moving animals (e.g. Collett and Collett, 2002; Graham *et al.*, 2004; Harris *et al.*, 2005, 2007). In these experiments, ants move in a specific direction to acquire sucrose, which reflects their natural foraging strategy; ants leave the nest and walk to locate aphids, which are a source of honeydew, a sugar-rich secretion upon which they feed. To feed directly on aphid honeydew, wood ants forage through cluttered environments along foraging trails that can extend for 100 m (for a review, see Robinson, 2005). Whether searching for sucrose rewards in reduced experimental paradigms or foraging in the natural environment for honeydew, ants are exposed to numerous visual cues, but how the sequences of visual cues encountered upon a foraging route are associated with the final reward from the feeder or an aphid remains unclear. It is unlikely that our learning paradigm, in which a single visual cue is presented briefly prior to obtaining a reward, captures the process of learning and memory formation along foraging routes fully. Instead, it is more akin to the final moments of foraging immediately before the reward is received. Whether sequences of visual cues with increased duration between the cues and the reward that more closely resemble natural foraging can be learnt within our experimental paradigm remains unclear. Nevertheless, our study provides the first evidence that wood ants can form visual associative memories

even when restrained and lacking the context of navigating through the environment.

Wood ants have been used extensively as a model system for studying navigation and visual learning in insects, producing many insights into mechanisms underpinning these behaviours (e.g. Collett and Collett, 2002; Graham *et al.*, 2004; Harris *et al.*, 2005, 2007). However, these studies have used freely moving ants, so insights into the neural circuits underlying the formation of memories in wood ants are restricted to insights that can be inferred from behavioural tests (e.g. Fernandes *et al.*, 2015). Our study provides a method for analysing visual learning in restrained ants, which opens up new experimental possibilities for determining the neural basis of navigation and visual learning in wood ants. Although removed from wood ants' natural foraging strategies, our paradigm permits control over the timing and duration of learnt cues and rewards, as well as the number of trials individual ants are subjected to, allowing a more detailed understanding of how precisely wood ants form the visual memories upon which their foraging depends.

Lateralisation of short-, medium- and long-term visual memories in an insect

Abstract

The formation of memories within the vertebrate brain is lateralised between hemispheres across multiple modalities, however, in invertebrates evidence for lateralisation is restricted to olfactory memories, primarily from social bees. Here we use a classical conditioning paradigm with a visual conditional stimulus to show that visual memories are lateralised in the wood ant, *Formica rufa*. We show that a brief contact between a sugar reward and either the right or left antenna (reinforcement) is sufficient to produce a lateralised memory, even though the visual cue is visible to both eyes throughout training and testing. Reinforcement given to the right antenna induced short- and medium-term memories whereas reinforcement given to the left antenna induced long-term memories. Thus, short-, medium- and long-term visual memories are lateralised in wood ants. This extends the modalities across which memories are lateralised in insects and suggests that such memory lateralisation may have evolved multiple times, possibly linked to the evolution of eusociality in the Hymenoptera.

Introduction

Brain regions and the neural circuits they contain can show considerable lateralisation, even in the brains of bilaterally symmetric animals that show little asymmetry in their gross morphology. Such lateralisation appears to be important in the formation of mem-

ories in humans across many sensory modalities (for review see Rogers, 2014). For example, cortical areas of the left hemisphere are more likely to be involved in storing memories of verbal information, whereas right cortical areas are more engaged in storing visual information (Gazzaniga, 2000). Spatial memories in humans are also lateralised, being transferred from the right hippocampus to the right side of the prefrontal cortex (reviewed in Burgess *et al.*, 2002). Lateralisation of memory formation is not restricted to humans, however, having been extensively demonstrated in chicks (Moorman and Nicol, 2015), and with some evidence of lateralisation in both zebra fish (Rogers and Andrew, 2002) and rodents (Jordan and Pytte, 2017).

Whether lateralisation of memory formation across different modalities is a common feature of brains and nervous systems remains unclear. Few studies have demonstrated functional lateralisation associated with memory formation within the numerous phyla commonly termed the invertebrates (Frasnelli, 2013). Apart from a study on the lateralisation of olfactory memory formation in the procerebrum of the terrestrial slug, *Limax valentianus* (Matsuo *et al.*, 2010), most evidence comes from the insects. Nevertheless, the majority of these studies have focussed upon a single modality, on appetitive classically conditioned olfactory memories in bees (Letzkus *et al.*, 2006; Rogers and Vallortigara, 2008; Anfora *et al.*, 2010; Frasnelli *et al.*, 2011; Rigosi *et al.*, 2011; Haase *et al.*, 2011; Guo *et al.*, 2016), though one study has found lateralisation of aversive olfactory memories in groups of fruit flies (Pascual *et al.*, 2004).

The association between lateralisation and olfactory memory has been studied in both solitary mason bees (Anfora *et al.*, 2010) and social bees from three tribes within the Apinae, namely honeybees (Letzkus *et al.*, 2006; Rogers and Vallortigara, 2008), stingless bees (Frasnelli *et al.*, 2011), and bumble bees (Anfora *et al.*, 2011). Lateralisation is evident in the formation of short-term olfactory memories in social bees, which form stronger memories following training with the right antenna. Conversely, long-term memories in social bees are lateralised so that stronger memories are formed in response to inputs from the left antenna (Letzkus *et al.*, 2006; Rogers and Vallortigara, 2008; Frasnelli *et al.*, 2011; Anfora *et al.*, 2011).

To determine whether such lateralisation of short and long-term memory formation in insects occurs within sensory modalities other than olfaction, we assessed whether visual memories are lateralised. To our knowledge, no studies have investigated the lateralisation of the retention of visual memories in insects, despite their importance to numerous aspects of insect behaviour (e.g. Collett and Collett, 2002; Collett *et al.*, 2003, 2013; Collett and Zeil, 2018; Knaden, 2019), though honeybees are known to learn an association with a visual cue more effectively when trained with only the right eye than those trained with only the left (Letzkus *et al.*, 2007). We used wood ants because they are a model system for studying visual navigation (Graham and Collett, 2002; Graham *et al.*, 2004; Buehlmann *et al.*, 2016) and have been shown to form visual memories in a classical conditioning paradigm (Fernandes *et al.*, 2018*a*). Using this paradigm, we trained ants to form an associative appetitive visual memory, in which the visual cue was presented to both eyes followed by a touch with the sugar reward in one of the antennae (lateralised reinforcement). This training produced no lateralisation in learning but revealed that short- and medium-term visual memories were preferentially formed following right antennal reinforcement whereas long-term memories were preferentially formed following left antennal reinforcement. To our knowledge, this is the first demonstration of lateralised visual memory formation in an insect, with broad implications for our understanding of visual memories in insects and the evolution of lateralisation in memory formation.

Methods and Materials

Animals and preparation

Red wood ant workers (*Formica rufa* L.) from three different colonies were used for the experiments. These colonies were collected from Ashdown Forest, Sussex, UK (N 51 4.680, E 0 1.800) in June 2017 and June and August 2018 and maintained indoors for 2 months prior to the experiments commencing, during which they were kept at 26°C, under a 12 h light:12 h dark cycle and fed with sucrose (333 g/L). Prior to training, colonies were starved for at least 2 days to increase motivation for feeding. Ants were selected from the

colony on the day of training and harnessed in a custom-made holder as described in (Fernandes *et al.*, 2018*b*, Chapter 2).

Training

Ants were trained to associate a visual cue with a sucrose reward. A sucrose reward (drop of 200 g/L sucrose solution) given to the ant through a syringe was the unconditional stimulus (US). A blue cardboard rectangle (width: 60 mm; height: 45 mm) attached to the syringe was the conditional stimulus (CS).

We performed six different types of training to investigate the role of the antennae during visual associative learning. During paired training, ants were presented with the CS (attached to the syringe) for 10 seconds, followed by a brief touch on one of the antenna with the US immediately before it was manually presented to the mouthparts for 5 seconds (Appendix B: figure B1). This was repeated 10 times, every 5 minutes. Thus, each ant was trained for approximately 50 minutes. Only ants that fed on every training trial were used for analysis (Fernandes *et al.*, 2018*b*, Chapter 2). Paired Right (PR) trained ants experienced right antenna touching by the US prior to contact with the mouthparts, whereas Paired Left (PL) trained ants experienced left antenna touching by the US prior to contact with the mouthparts.

Ants subjected to unpaired training were presented with the CS for 10 seconds and two and half minutes later were presented with the US to one of their antennae before the US was touched to the mouthparts for 5 seconds. Consequently, these ants were exposed to the CS and US the same number of times as ants subjected to paired training but separated temporally, being also trained for approximately 50 minutes. Unpaired Right (UPR) ants experienced right antenna touching by the US prior to contact with the mouthparts, whereas Unpaired Left (UPL) ants experienced left antenna touching by the US prior to contact with the mouthparts. An additional unpaired group, Randomized Unpaired Left (RUPL), was added in which the same number of CS and US presentations were performed as in UPL but the CS and US were presented in a randomized order and ants only experienced left antenna touching by the US. As an additional control, CS Only (CSO), we presented the CS (plus syringe) but without the US to the antennae or the

mouthparts.

Although presenting the visual cue manually may activate different modalities with odours being potentially detected by the ant at the same time as the CS and US were presented, all cues were common between all training types and for both CS and US trials, with the exception of the visual cue which was either paired or not with the sugar reward. Thus, differences in learning between paired and control groups can be only attributed to learning the association between the visual cue and US. For all types of training, half of the ants were trained with their right side facing the experimenter and the other half with the left, to account for potential environmental asymmetries that could be learnt during training. In both cases the visual stimulus faced the ant and was seen by both eyes.

Testing

We tested PR, PL, UPR and UPL groups in the same set-up with a 10 second CS presentation either 10 minutes, 1 hour or 24 hours after training, similar time points selected on previous studies with honey bees (Rogers and Vallortigara, 2008). The remaining groups, RUPL and CSO, were tested in the same manner but only at 1 hour or 24 hours after training. For every group, ants that were tested at 10 minutes were not tested at any other time points, but all ants that were tested at 24 hours were also tested at 1 hour. All ants were kept harnessed until the last test was over. Ants harnessed overnight for 24 hour testing were kept in a dark and humid environment. After testing, ants were presented with the sugar reward to assess whether they were still motivated to feed. Ants that did not feed after testing were excluded from analysis.

Bilaterally trained and tested ants

Data from (Fernandes *et al.*, 2018*a*, Chapter 2) were used for comparing unilateral versus bilateral touch to the antenna. These ants were subjected to a paired training, similar to the PR and PL groups from the current study, but during which the contact between the US and the antenna was not controlled, being variable for each ant but frequently bilateral (PB group). These ants were tested either 10 minutes or 1 hour after training, as described above.

Scoring and statistical analysis

We scored whether ants performed the maxilla-labium extension response (MaLER) during the 10 second presentation of the conditioned stimulus (CS) for each training trial and test (Fernandes *et al.*, 2018*b*, Chapter 2). All statistical analysis was performed in R (v3.5.1, R Foundation for Statistical Computing, Vienna, Austria) using RStudio (v1.1.463, RStudio Inc, Boston, USA). To analyse whether the propensity to perform MaLER increased during training and whether it differed among different training groups, we ran a logistic regression model with mixed effects (Bates, 2010), using the package 'lme4'. Models were implemented using the `glmer` command incorporating fixed (trial number and training type) and random (individual) effects (see Appendix B). We used a Barnard's test (Barnard, 1989) to compare the proportion of responses during each test between groups with the 'barnard' R package. One-tailed or two-tailed Barnard's tests were applied depending on the specific test (see Appendix B). To determine if those ants responding during testing were also those that learnt, we used a classification of 'learners' or 'non-learners' established in (Fernandes *et al.*, 2018*a*, Chapter 2); those individuals that responded on 4 or more of the 10 training trials were 'learners'. Bonferroni corrections (Bonferroni, 1936) were applied when appropriate (see Appendix B).

Results

Unilateral antennal reinforcement is sufficient to learn the association between a visual cue (CS) and a sugar reward (US)

During each paired training trial, ants were presented with the CS (visual cue) for 10 seconds, followed by a brief contact between the US (sucrose reward) and one antenna (Appendix B: figure B1 A,B), which we hereafter refer to as reinforcement. The ant was then allowed to feed from the US for 5 seconds, during which both antennae made contact with the US (Appendix B: figure B1 C). Each training bout was separated from the next by a five minute interval (see Material and Methods). The majority of ants did not perform the Maxilla Labium Extension Reflex (MaLER) when presented with the visual cue on

the first trial but during training performance increased until it plateaued at approximately 30 to 40% for those ants that were trained with reinforcement given to the right antenna (PR; figure 1A). The same training regime but with reinforcement given to the left antenna led to the performance of MaLER in approximately 20 to 30% of ants (PL; figure 1A). We used a logistic regression to compare the probability of ants performing MaLER in the PR and PL training regimes (see Material and Methods). There was no significant difference in the percentage of ants performing MaLER in the PR and PL training regimes (logistic regression, N=180, z=1.26, p=0.21), and the performance of MaLER in response to presentation of the CS increased significantly during both PR and PL training (logistic regression; PR: N=88, z=6.17, p<0.01; PL: N=92, z=3.48, p<0.01). Furthermore, the direction that ants faced during training did not influence learning (logistic regression, N=180, z=0.32, p=0.75; see Appendix B).

To determine whether unilateral antennal contact with the US during a specific brief period of each training trial produced differences in learning performance in comparison to bilateral antennal contact, we compared PR and PL trained ants with bilaterally trained counterparts (PB ants) (data from Fernandes *et al.*, 2018*a*, Chapter 2). These PB ants were subjected to paired training during which antennal contact with the US was not controlled, was variable between ants and frequently bilateral. Both PR and PL ants performed MaLER in response to the visual cue significantly less during training than PB ants (logistic regression; PR-PB: N=231, z=-3.57, p<0.01; PL-PB: N=231, z=-4.67, p<0.01; figure 1A). Thus, paired training with reinforcement given to a single antenna is sufficient for ants to form an association between the visual cue (CS) and the sugar reward (US), but reinforcement to both antennae during training results in a greater number of ants learning the association.

Paired CS-US single antennal reinforcement is necessary for associative learning

To ensure that ants had learnt the CS-US association, we performed unpaired training in which the CS and US were presented in alternation with a two and a half minute interval
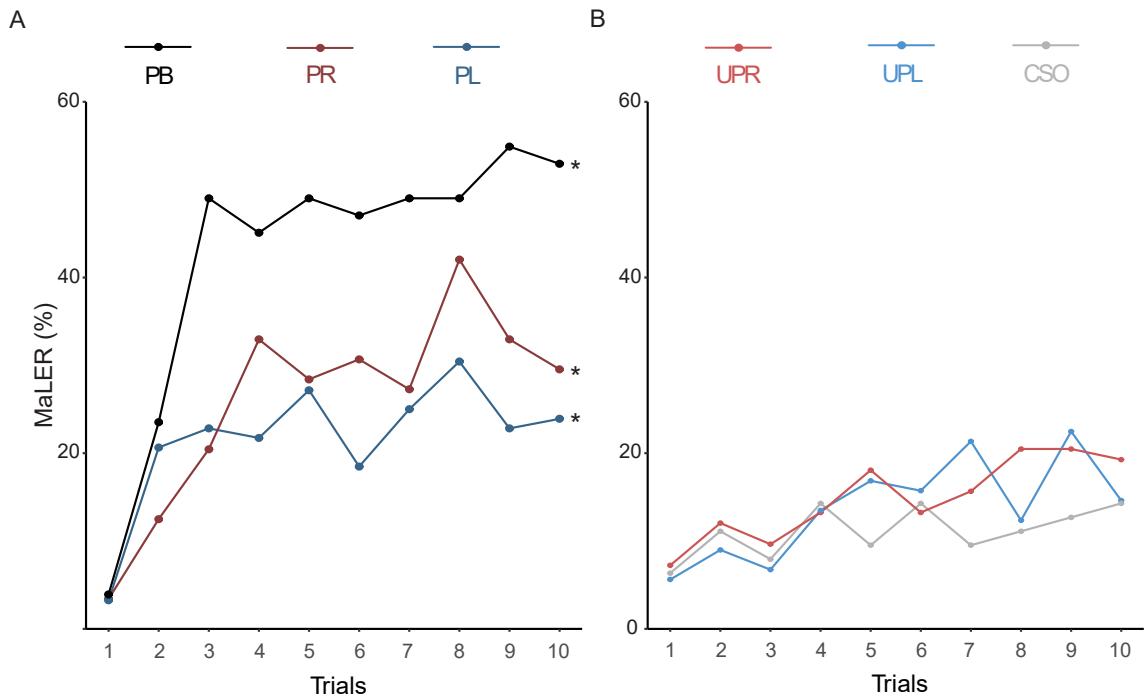
Figure 1: Ants subjected to a paired training with unilateral and bilateral reinforcement of antennal touch with sugar learn the association between a visual cue and a sucrose reward. A) The performance of ants subjected to any type of paired training increases significantly during training. Responses of paired ants with a reinforcement on the right antenna (PR, dark red; N=88) and the on the left (PL, dark blue; N=92) show no significant difference, but are both significantly lower than paired ants with a reinforcement on both antenna (PB, black; N=51). B) Unpaired training with a reinforcement on the right (UPR, light red; N=83) or left antenna (UPL, light blue; N=89) produces weaker MaLER performance during training. The responses of ants that visualize the CS but do not touch the sugar reward with neither antennae nor mouth parts (CSO, gray; N=63) do not increase with training trial. There is no significant difference between CSO and the unpaired groups, but MaLER during PR and PL training is significantly higher than the corresponding unpaired groups and CSO ants. Asterisks indicate a significant increase of MaLER performance during training.

rather than paired with US following the CS with no interval (see Material and Methods). The percentage of ants performing MaLER when subjected to unpaired training with reinforcement given to the right antenna (UPR) or left antenna (UPL) increased to a lesser extent than during paired training (figure 1B). Indeed, the percentage of ants performing MaLER was significantly lower during unpaired training compared to the equivalent paired training (logistic regression; PR-UPR: N=171, z=-3.35, p<0.01; PL-UPL: N=181, z=-2.68, p<0.01).

To determine whether the increase in MaLER observed during paired or unpaired training was greater than expected if no CS-US association was formed, we performed a further control in which the CS was presented without the US, a CS-only control (CSO; figure 1B). CSO ants did not show any significant increase in the percentage of MaLER performed throughout their training (logistic regression, N=63, z=1.36, p=0.17). Ants subjected to any paired training regime performed a significantly higher percentage of MaLER than did ants subjected to the CSO training regime (logistic regression, PR-CSO: N=151, z=4.39, p<0.01; PL-CSO: N=155, z=3.35, p<0.01). However, ants subjected to either unpaired training regime (UPR, UPL) showed no significant increase in the percentage of MaLER they performed in comparison to ants subjected to the CSO training regime (logistic regression, UPR-CSO: N=146, z=1.5, p=0.13; UPL-CSO: N=152, z=0.91, p=0.36). Thus, paired training is necessary to learn the association between the visual cue (CS) and the sugar reward (US).

Right antennal reinforcement is necessary for short- and medium-term visual memory formation

We tested whether ants retained a short-term memory 10 minutes after the end of each of the training regimes. Ten minutes after training, approximately 60% of the PR ants performed MaLER when presented with the CS (figure 2A). Ten minutes after UPR, PL and UPL regimes only 10-20% of ants performed MaLER (figure 2A). The proportion of ants performing MaLER following PR training was significantly higher than those ants subjected to PL training at this time point (Barnard's test, PR-PL: N=74, df=72, p<0.01). The

proportion of ants performing MaLER following PR training was also significantly higher than ants subjected to UPR training (Barnard's test; PR-UPR: N=73, df=72, p<0.01). In contrast, the proportion of ants performing MaLER following PL training did not differ significantly from ants subjected to UPL training (Barnard's test, N=75, df=74, p=0.18). Thus, the paired presentation of CS and US coupled with right antennal reinforcement is necessary and sufficient for the formation of short-term memory.

To assess whether ants retained a medium-term memory, we tested ants from each of the training regimes one hour after training ended. A pattern similar to that observed at 10 minutes was evident at one hour after training (figure 2B). Ants subjected to the PR training were the only cohort to perform a significantly higher proportion of MaLER responses in comparison to those ants subjected to CSO training (Barnard's test, PR: N=113, df=112, p<0.01; PL: N=117, df=116, p=0.03; UPR: N=169, df=168, p=0.03; UPL: N=113, df=112, p=0.23; figure 2B). Furthermore, the proportion of PR ants that responded to the CS 10 minutes or 1 hour after training was significantly higher for ants classified as 'learners' during training (performed MaLER in 4 or more of the 10 training trials; see Material and Methods) than ants that were not (Barnard's test; 10 min: N=37, df=36, p=0.036; 1h: N=50, df=49, p=0.003). Thus, paired CS-US presentation coupled with right antennal reinforcement is necessary and sufficient for the formation of short- and medium-term memory.

Furthermore, PR ants did not differ in the extent to which they performed MaLER in comparison to PB ants at 10 minutes or 1 hour (Barnard's test, 10 min: N=52, df=50, p=0.96; 1h: N=66, df=64, p=0.98). This suggests that although the percentage of responses to CS presentation during training was significantly lower for ants that were subjected to unilateral antennal contact with the US in comparison to bilateral contact, unilateral right antennal contact is sufficient for memory recall at short- and medium-term.

Left antennal reinforcement is sufficient for long-term visual memory formation

To detect a long-term memory, we tested ants from each of the training regimes 24 hours after training was completed. In contrast to the short- and medium-term memories tested for 10 minutes and one hour after training, less than 10% of ants subjected to PR training responded to the CS at 24 hours. Indeed, the percentage of ants performing MaLER in response to CS presentation 24 hours after PR training did not differ significantly from that of the cohort of ants subjected to CSO training (Barnard's test, N=87, df=86, p=0.31; figure 2C). Ants subjected to PR training also performed MaLER in response to CS presentation at 24 hours in similar proportions to the UPR trained ants (Barnard's test, N=78, df=77, p=0.37). Of the 39 PR ants that were tested at both 1 and 24 hours, 33% responded to CS presentation by performing MaLER on the first test (1 hour) but not the second (24 hours), while only 0.5% responded at 24 but not 1 hour and 0.2% of the ants responded in both tests. There was a significant reduction in the performance of MaLER in response to CS presentation from 1 to 24 hours after training for the PR ants (Barnard's test, N=39, df=38, p<0.01).

Conversely, around 30% of those ants subjected to PL training performed MaLER upon CS presentation (figure 2C). The percentage of PL trained ants performing MaLER was significantly higher than those ants subjected to CSO training (Barnard's test, N=87, df=86, p<0.01). Of those PL ants that were tested both at 1 and 24 hours, 28% performed MaLER on the second test (24 hours) but not the first (1 hour), while only 1% responded to the CS at 1 hour but not at 24 hours and 0.5% in both tests. There was a significant increase in the performance of MaLER in response to CS presentation between 1 and 24 hours (Barnard's test, N=40, df=39, p<0.05). Furthermore, the proportion of ants that responded to the CS at 24 hours was significantly larger for ants that were classified as 'learners' during training (performed MaLER in 4 or more trials; see Material and Methods) than ants that were not (Barnard's test, N=40, df=39, p=0.014).

At 24 hours, UPL ants also performed MaLER upon presentation of the CS significantly more than the CSO trained ants (Barnard's test, N=88, df=87, p<0.01), and in-
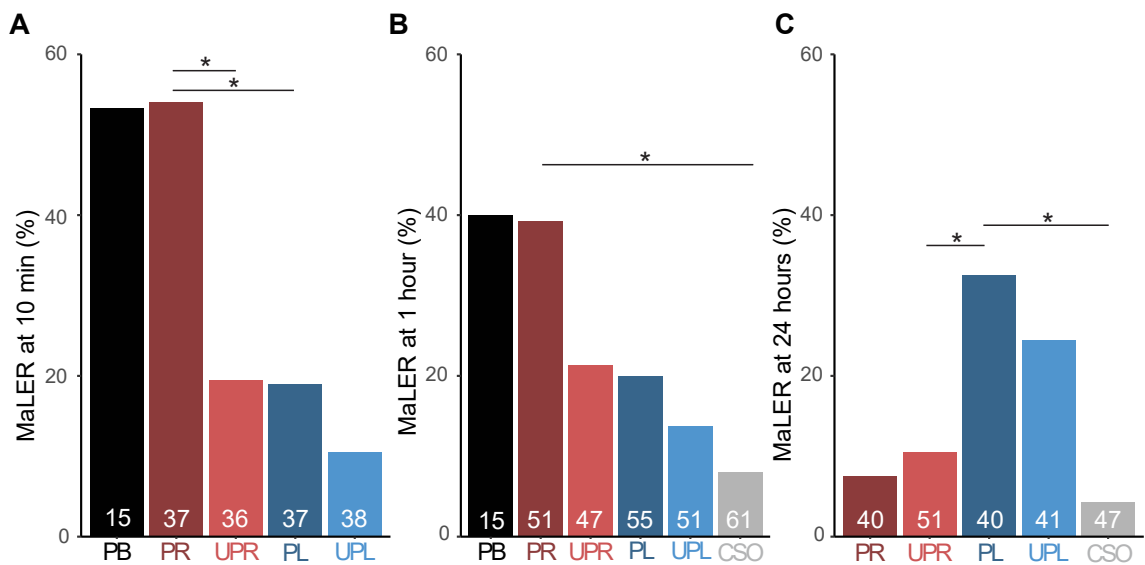
Figure 2: Wood ants retain visual associative memories and recall those memories either 10 minutes and 1 hour or 24 hours after training, depending on which antenna was reinforced during training
A) Percentage of MaLER performance to the CS at 10 minutes. PR ants are not significantly different than PB ants, but are significantly higher than UPR and PL. PL ants do not respond significantly more than UPL ants. B) Percentage of MaLER performance to the CS at 1 hour. PR ants' responses are not different than PB but are significantly higher than the baseline CSO, while none of the remaining unilateral reinforcement trainings produced significantly more responses than CSO. C) Percentage of MaLER performance to the CS at 24 hours. PL ants were the ones that showed highest number of responses, being significantly higher than the PR and CSO, but not UPL ants. Numbers inside each bar indicate the number of ants (N) of the corresponding group. Asterisks indicate significant differences in MaLER percentages during testing, between training types.

distinguishably from PL ants (Barnard's test, N=81, df=80, p=0.27). This suggests that the formation of long-term memory produced by PL training is less sensitive to the separation of CS and US than is short- or medium-term memory produced by PR training. To test this, we exposed ants to unpaired randomised training (RUPL) in which the order of CS and US presentation is randomised. RUPL ants showed greater variability during training without a clear trend for an increase of MaLER performances with training (logistic regression, N=44, z=0.079, p=0.94; Appendix B: figure B2 A). The RUPL ants were indistinguishable from UPL ants in their performance of MaLER in response to the CS presentation during testing 1 hour (Barnard's test, N=96, df=95, p=0.29; Appendix B: figure B2 B). At 24 hours, 20% of RUPL trained ants performed MaLER, which was not significantly different than PL and UPL (Barnard's test; RUPL-PL: N=81, df=80, p=0.03; RUPL-UPL: N=82, df=81, p=0.29; Appendix B: figure B2 C), nor CSO trained ants (Barnard's test; N=88, df=87, p=0.099).

Discussion

We investigated whether visual memories are lateralised within an insect brain by using an associative learning paradigm applied to workers of the red wood ant, *Formica rufa*. We found that visual memories are indeed lateralised; a brief contact with the right antenna (reinforcement) produces a short-term memory that is present 10 minutes after the end of training and persists for at least one hour (figure 2A, B). We also showed that wood ants can form long-term classical conditioned visual memories that are also lateralised; left antennal reinforcement produces a long-term memory that is present at 24 hours despite no memory being present at 10 minutes or one hour after training (figure 2C). The strength of memory formed by unilateral antennal contact was indistinguishable from that formed by bilateral antennal contact at 10 minutes and 1 hour after training. The lateralisation of memory produced by right or left unilateral antennal reinforcement arises even though there are no differences in learning (figure 1A), and despite both antennae contacting the US (sucrose reward) whilst the ant was feeding (Appendix B: figure B1).

Relevance and reliability of our measures

We evoked associative visual memories in wood ant workers using an appetitive classical conditioning paradigm (Pavlov, 1897). Such paradigms have been widely used to evoke associative memories in insects (e.g. Bitterman *et al.*, 1983; Guerrieri and d'Ettorre, 2010; Simões *et al.*, 2011), coupling a reward (US) with a stimulus (CS) that later becomes sufficient to evoke a response alone. Our training paradigm did not test the specific feature that ants learnt. By presenting the CS without the US during training or by temporally decoupling CS and US through unpaired training, we distinguished associative memories from other non-associative memory processes, and ensured that ants responded to the visual CS. Although manual delivery of the CS and US has a slim possibility that other associated cues are also present during the CS and US trials, the unpaired ants' responses during CS presentations are significantly lower than those of paired trained ants, so we can conclude that the cue being learnt by paired ants is the visual CS. Furthermore, paired ants' MaLER performance increases during the CS presentation alone, before being touched with the sugar reward in the antenna. Thus, the asymmetrical touch of one antenna reinforces the processing and retention of the visual information acquired by both eyes, and is not the component being learnt in our paradigm. Nevertheless, we did observe a high proportion of UPL ants performing MaLER to the CS 24 hours after training, which was not visible for the UPR ants at the 10 minutes and 1 hour tests. This implies that short- and medium-term memories are more sensitive to the temporal separation of the CS and US presentations during training, compared to long-term memories. To account for this, we included another control group with randomized order of CS and US trials which, indeed, responded less at the 24 hour test. We also ensured that asymmetries in the immediate environment did not influence the lateralisation of visual memories by placing individual ants with either their right or left eye facing the experimenter during each training condition and always presenting the visual cue to both eyes. Ants were harnessed in the same way in both conditions, again ensuring consistency and avoiding introducing asymmetries from additional sources.

We used the maxilla-labium extension response (MaLER) as the unconditioned re-

sponse (UR), which was originally described for olfactory learning (Guerrieri and d'Ettorre, 2010; Guerrieri *et al.*, 2011) but which has been subsequently used in a visual learning and memory paradigm (Fernandes *et al.*, 2018*a*, Chapter 2). The response to the visual CS was initially low but increased when paired with an appetitive US, sucrose, up to 50% of the ants responding per trial. The visual cue was visible to both eyes and lateralisation was produced through right or left antennal reinforcement, emphasising the robustness of this lateralisation. Despite differences in methodology, our training paradigm yielded similar learning rates to previous studies with honeybees using classical conditioning of a visual cue (Hori *et al.*, 2007; Balamurali *et al.*, 2015; Niggebrügge *et al.*, 2009). We did not ablate or cover the antennae during training or testing, though paradigms for visual classical conditioning in honeybees have involved removing the antennae ostensibly to improve learning rates (Hori *et al.*, 2007; Niggebrügge *et al.*, 2009 ; but see Balamurali *et al.*, 2015). To our knowledge, only one study has tested memory recall at long time periods in honeybees with 30% responding to the visual cue after 24 hours (Niggebrügge *et al.*, 2009), a similar percentage to our observations for ants trained with a reinforcement on the left or both antennae.

Functional relevance of memory lateralisation

Short- and medium-term visual memories in wood ants are consistently lateralised to the right, whereas long-term visual memories are consistently lateralised to the left. Our testing paradigm involved presenting the visual stimulus (CS) to both eyes simultaneously. Consequently, memory transfer between sides, which has been reported for olfactory memories in honeybees (Sandoz and Menzel, 2001; Malun *et al.*, 2002; Sandoz *et al.*, 2003; Komischke *et al.*, 2005), cannot account for the right to left switch in the lateralisation of visual memories. Instead, our results are consistent with short- and medium-term memories formed on the right disappearing whilst long-term memories are formed on the left without prior formation of short- and medium-term memories. This memory lateralisation is likely to have functional implications at the level of the neural circuits encoding the memory. The lateralisation of memory formation may permit the different cellular mechanisms responsible for the coding of short- and medium-term memories versus long-

term memories to occur in parallel, avoiding the pronounced gaps in memory that occur in the transitions between these different memory types as observed in pond snails (Marra *et al.*, 2013). Moreover, lateralisation may be advantageous because it avoids the storage of a memory of the same association in parallel in the two hemispheres of the brain. This would reduce redundancy within neural circuits and would also save energy (for review see Niven and Laughlin, 2008; Burns *et al.*, 2010). Such features of lateralised memory formation may be particularly important within relatively small brains such as those of ants with small numbers of neurons and low energy availability (Niven and Farris, 2012).

The lateralisation of visual memories in wood ants is revealed only through unilateral antennal reinforcement. Consequently, we do not expect that there will be deficits in visual memory formation during behaviours in which both antennae are used. This expectation is supported by bilateral antennal reinforcement during training, which produces short- and medium-term memories of equivalent strength to unilateral training in wood ants. Although the precise patterns of contact made by antennae during feeding on aphid honeydew, which is a major food source for wood ants (Robinson, 2005), are unknown, antennal movements are biased during trophallaxis in wood ants, the receiver using their right antenna more often (Frasnelli *et al.*, 2012). Although both the left and right antennae of the receiver are used during trophallaxis and that this consists in an interaction between individuals, it is possible that similar antennal asymmetries are be present in other behaviours with a more pronounced learning component.

Implications for memory lateralisation in the insects

Visual memory lateralisation among the wood ant workers within our study, which were taken from multiple colonies within the same region raising the possibility that they could be genetically related, implies population- rather than individual-level lateralisation, in which the lateralisation of a particular feature varies among individuals. Both individual- and population-level lateralisation are well-documented within the insects. In species such as the desert locusts (Bell and Niven, 2014), fruit flies (Buchanan *et al.*, 2015), and antlions (Miler *et al.*, 2017) several behaviours that do not involve interactions with other individuals are lateralised at the individual level, with very few exceptions of population-

level lateralisation (Cooper *et al.*, 2011; Kight *et al.*, 2008). Substantial evidence for population-level lateralisation of such behaviours is derived mostly from eusocial insects such as leaf cutting ants (Jasmin and Devaux, 2015) and bumble bees (Kells and Goulson, 2001), although some of these behaviours are also lateralised at the individual level. Conversely, population-level lateralisation is always observed during interactions between individuals of both eusocial and solitary species (Kamimura, 2006; Frasnelli *et al.*, 2012; Lang and Orgogozo, 2012; Kamimura *et al.*, 2014; Rogers *et al.*, 2013; Romano *et al.*, 2016a,b; Benelli *et al.*, 2017a,b). Niven and Bell (2018) have argued that whether a trait is lateralised at the individual- or population-level depends upon specific selective pressures to which the behaviour is subjected. Consequently, if different selective pressures act on distinct behaviours, it is possible for an individual to show individual- or population-level lateralisation in distinct behaviours. Thus, social behaviours that benefit from coordinated actions are commonly found lateralised at the population-level amongst several insect species. This coordination of lateralisation in the same direction across most of the individuals within a population could drive lateralisation of neural circuitry, depending on the strength of sociality within the species. In turn, this could drive non-social behaviours to be equally lateralised, even when they do not confer a specific advantage to the individual or group of individuals, though evidence for this is lacking. In eusocial insect species, the low levels of competition between individuals is likely to promote population-level lateralisation of social behaviours and may promote lateralisation of non-social behaviours alike.

Whether the population-level lateralisation of visual memories, as opposed to individual-level lateralisation, confers specific advantages remains unclear. The population-level lateralisation of short-, medium- and long-term of visual memory formation in wood ants shows similarities to that of olfactory memories in honeybees, bumble bees and stingless bees (Letzkus *et al.*, 2006; Anfora *et al.*, 2010, 2011). Yet not all bees show similar patterns of lateralisation; solitary mason bees (*Osmia bicornis*) do not show olfactory memory lateralisation (Anfora *et al.*, 2010). One explanation for this pattern is that honeybees, bumble bees and stingless bees are eusocial whereas mason bees are solitary: the

evolution of population-level lateralisation of olfactory memories in bees being due to the specific advantages of lateralisation conferred by eusocial environments in which conflict between individual workers is low (Anfora *et al.*, 2010 ; for review see Niven and Frasnelli, 2018). Our discovery of visual memory lateralisation in wood ants supports this framework and extends it in several ways. All previous studies of memory lateralisation focussed on olfactory memories so the lateralisation of visual memories shows that this is not restricted to a specific modality. Furthermore, wood ants are eusocial like honeybees, bumble bees and stingless bees but as members of the Formicidae they are thought to represent an independent evolution of eusociality within the order Hymenoptera (sawflies, wasps, ants, bees) (Peters *et al.*, 2017). Thus, the presence of visual memory lateralisation in wood ants is the first evidence of memory lateralisation outside one family of bees (Apidae). Because of the absence of information about lateralisation of memory formation in more basal lineages within the Hymenoptera, we cannot be certain whether memory lateralisation has evolved independently in ants and eusocial bees or is present in more basal lineages but has been lost in solitary mason bees.

Distance, speed and time integration of tethered wood ants walking on a virtual world

Abstract

Animal behaviour is a closed-loop process between the brain, body and environment, dependent upon the integration of both internal and external cues. For instance, the integration of proprioceptive cues, such as a stride integrator, for estimating distance walked has been observed in foraging desert ants, while self-generated motion of the visual world (optic flow) is used for distance and speed integration typically in bees and flies. However, most of these studies were performed in free moving individuals and, consequently, full control over visual cues perceived and detail analysis of the animals' behaviour was not always achieved. Furthermore, some studies do not distinguish self-induced from external optic flow, which could hinge confounding effects. Here we investigated walking behaviour of wood ants workers, *Formica rufa*, using a newly developed virtual reality paradigm in which the visual motion of the world can be fully controlled and presented in closed-loop with the ants movement (thus self-generated) or externally imposed in open-loop. We've shown that wood ants, tethered on top of an air supported ball, interact with a virtual world and demonstrated, for the first time, that they show consistent walking behaviour when placed twice in the same virtual world, walking repeatable distances, at similar median speeds and for equivalent amounts of time. This consistency is not dependent upon optic flow but can be modified by it. Moreover, self-induced and external optic flow modify walking parameters differently, supporting the idea their integration is dependent upon different mechanisms. Distance, speed and time consistency also seems

to be a particular feature of translational walk, not being present in rotatory behaviour which, instead, is increased in novel virtual environments compared to familiar ones.

Introduction

For animals to produce adaptive behaviour, they require a controlled feedback between their motor system, the central generator of motor commands and the integration of sensory inputs from their environment (Todorov, 2004). Sensory inputs can be generated by the external environment alone but can also be generated by the animals' own movements. Animals can produce local movements, such as eye movements and in-hand manipulation of objects, but they can also move through their surroundings (Rossignol *et al.*, 2006). Although this has been widely investigated in humans and other vertebrates (McCrea, 2001), insects have also been used as models for investigating sensorimotor control during locomotion. Insects display a series of locomotion behaviours, such as collision avoidance (Yakovleff *et al.*, 1995), approach (Graham *et al.*, 2003), predation (Rossel, 1991), adjusting height relative to the ground (Portelli *et al.*, 2010), landing (Srinivasan *et al.*, 2000), mate following (Boeddeker *et al.*, 2003), amongst many others, that require precise control between their own movement, the sensory inputs produced by their movement and external cues (Huston and Jayaraman, 2011).

Central place foraging, the ability of an animal to maximize the efficiency of its foraging trips, has also been observed in some insect species (Bell, 1990). Social insects, such as ants and bees, although not the only insects with such capabilities, are the most well studied central place foragers. Central place foraging imposes specific demands on animals, requiring that they not only control their locomotion but do so to specific goals - food or nest (Dyer, 1998). While foraging for food to bring to their nests, they can use a multitude of external cues to locate themselves within their surroundings, such as odours (Buehlmann *et al.*, 2015), pheromone trails laid by themselves or other individuals (David Morgan, 2009) and landmarks in their visual surroundings (Harris *et al.*, 2005), which vary depending on the ecology of the species. Some species of social insects are also able to integrate information about the direction and distance they are from the nest

as they travel and subsequently use that vector-based information to return to the nest and later to the feeders' location, a process known as path integration (Collett and Graham, 2004). Thus, the ability to integrate information about the environment during locomotion goes beyond momentary processing and reaction to changes in the surroundings. This information needs to be stored and accessed when necessary. Additionally, in the case of honey bees, the integrated distance and direction between their nest and other locations can also be communicated to other individuals through the waggle dance (von Frisch, 1966).

The study of the mechanisms underpinning outward and return foraging trips in social insects has shown that both ants and bees can use external environmental cues such as polarized light or direct light from the sun for integrating direction (Srinivasan, 2015). However, distance integration seems to be dependent upon a combination of external and internal cues of the animal. Heran and Wanke (1952), working with honey bees *Apis mellifera*, proposed the first hypothesis to explain how an insect can assess the distance it travelled. They observed that some bees that found a feeder while flying uphill, thus expending a large amount of energy to reach it, reported longer distances through their waggle dances, compared to bees that found a feeder while flying downhill, thus expending less energy travelling. They proposed that honey bees have an internal mechanism of perceiving and integrating energy expenditure that could then be translated into distance. Although other early studies supported this hypothesis (Scholze *et al.*, 1964; Frisch *et al.*, 1967), they were later considered inconclusive or unrepeatable (reviewed in Esch and Burns, 1996). Indeed, later studies that manipulated energy expenditure in both honey bees (Goller and Esch, 1990; Esch *et al.*, 1994; Esch and Burns, 1995) and desert ants *Cataglyphis fortis* (Schäfer and Wehner, 1993) showed that individuals were still able to integrate the correct distance, suggesting that distance integration is not dependent upon this internal mechanism.

Experimental evidence seems to favour two other hypothesis for distance estimation: internal proprioceptive (idiothetic) cues, such as a locomotor activity integrator, and self-generated optic flow, i.e, the motion of the visual world caused by one's own movement

(Esch and Burns, 1996). Studies with desert ants returning from a feeder to their nests through channels with patterns on the floor or walls, perceived by the ventral or lateral portion of the eye, respectively, suggest that ventral but not lateral self-induced optic flow could be used by desert ants to regulate the distance walked (Ronacher and Wehner, 1995; Ronacher *et al.*, 2000), although distance was still estimated correctly if optic flow cues were not available (Ronacher *et al.*, 2000). Thus, although desert ants can make use of external visual cues and the self-generated motion of the visual environment to estimate distance, other mechanisms such as the use of proprioceptive cues could be available in the absence of optic flow. Wittlinger and collaborators (2006) addressed this by manipulating ants, at the feeder position, to have shorter (stumps) or longer legs (stilts). They observed that ants with stumps and stilts walked shorter and longer distances, respectively, compared to normal ants returning from the feeder back to their nest (Wittlinger *et al.*, 2006). Although it is possible that the results could hinge on some unintended side-effect of the manipulation (Collett *et al.*, 2006), a thorough analysis of the walking behaviour of these ants revealed that the walking behaviour was unaffected by the manipulation, except stilts produced longer strides but with slower frequency and an overall lower walking speed, whereas the opposite was observed for ants on stumps. Therefore, it seems that changes in the distance walked on the homeward bound path could be accounted for mainly by the number of steps, i.e., some internal stride integrator (Wittlinger *et al.*, 2007). However, Wittlinger and collaborators (2007) also suggest the use of optic flow by walking desert ants to explain part of the variation they observed on the stride integrator experiments. Furthermore, a study with a species that engages in carrying behaviour, the desert ant *Cataglyphis bicolor*, has shown that these ants can estimate the distance they've travelled while being carried, when proprioceptive cues are unavailable, using exclusively optic flow (Pfeffer and Wittlinger, 2016).

The interaction between optic flow and stride integrator cues for distance estimation during the homebound travel of *C. fortis* ants was later addressed by Wolf *et al.* (2018). Ants with their ventral eyes covered while being trained to a feeder, possessing solely odometer cues, were able to estimate the correct homeward distance, but ants that had

both cues available during training but no optic flow when tested estimated shorter distances. Furthermore, ants that had the eye caps removed after performing their homebound journey perform a second homebound journey using the stored optic flow memory. Thus, optic flow and stride integrator seem to operate independently and interact with each other, with their interaction varying depending on which cues are available and other specific contexts."

Conversely, honeybees flying through a tunnel with head- or tail-wind, thus differing on the number of wing beats they performed, still estimate similar distances, suggesting that proprioceptive cues do not play a role in distance integration in this species (Srinivasan *et al.*, 1997). To estimate distance correctly, flying honey bees seem to use of optic flow (Esch and Burns, 1995; Esch *et al.*, 2001; Si *et al.*, 2003; Tautz *et al.*, 2004). Bees flying at high altitudes above ground experience slower self-induced optic flow compared to those flying closer to the ground and, consequently, they report shorter travelled distances compared to their counterparts when performing waggle dances (Esch and Burns, 1995). Similarly, bees that fly above water experience slower self-induced optic flow and thus report shorter distances than bees flying above land (Tautz *et al.*, 2004). Lateral optic flow was mostly studied in free flying bees inside tunnels with patterned walls. Narrow tunnels, with higher lateral optic flow, led to increased reported distances on waggle dances compared to wider tunnels (Esch *et al.*, 2001), which was unaffected by contrast and spatial frequency of the visual stimuli (Si *et al.*, 2003). Furthermore, ventral and lateral optic flow both influence the perceived distance to a similar extent (Si *et al.*, 2003).

The distance between one location and another can be assessed if one holds the information of the time spent moving between the two locations and/or the velocity at which one moves. However, in walking desert ants, speed and time changes due to carrying heavy loads on the homeward journey do not seem to affect distance integration (Zollikofer, 1994). Furthermore, Wittlinger and collaborators (2006, 2007) have shown that the changes in speed and walking time produced by the leg manipulations cannot account for changes in distance estimation. Similarly, bees flying similar distances during different time periods and at different velocities estimated similar distances (Srinivasan

et al., 1997), while bees flying different distances during similar amounts of time report different distances through their waggle dances (Barron *et al.*, 2005). Thus, for both flying honeybees and walking desert ants, time and speed do not seem to influence distance estimation.

Although distance perception does not seem to be dependent upon time and speed, the ways by which each of these locomotion parameters is perceived could still depend upon similar cues to those used for distance estimation. To our knowledge, time integration in insects has only been studied in bumble bees *Bombus impatiens* (Boisvert and Sherry, 2006). Although these experiments were performed using a classical conditioning approach and not during goal-directed foraging, bumble bees do seem to be able to integrate time intervals, which suggests that foraging insects might be able to do so too, even if it is not used for distance estimation. Nevertheless, no studies have demonstrated time integration in foraging insects and, therefore, it is not known if this would occur using similar cues as for distance integration.

Conversely, velocity integration has been widely investigated, and has benefited from a characteristic common to several walking and flying species - the ability to regulate speed and keep a steady average velocity while travelling, present in ants (Zollikofer, 1994), ladybirds (Zanker and Collett, 1985), bees (Heran and Lindauer, 1963; Baird *et al.*, 2010) and flies (David, 1982; Kern *et al.*, 2012), amongst others. How speed control is achieved was first addressed in walking insects by Zanker and Collett (1985) working with ladybirds (*Coccinella septempunctata)*, who found no effect of optic flow or energy expenditure in speed control. Similarly, desert ants travelling on top of patterns moving at different speeds, thus experiencing different optic flow, walk at equivalent velocities (Ronacher and Wehner, 1995). Conversely, optic flow does seem to influence speed control in walking honey bees (Schöne, 1996). In flying insects, ground speed control was first investigated in fruit flies, *Drosophila melanogaster*, by David (1982), using a wind tunnel with patterned ground and walls. Flies were able to control ground speed using optic flow rather than air flow cues, although the use of air flow has been observed in later studies (Sherman and Dickinson, 2004; Budick *et al.*, 2007) and recent manipulations of

the flies' visual world with virtual reality systems contradict the use of optic flow for reg-ulating altitude (Straw *et al.*, 2010). The use of optic flow for ground speed control has also been observed in blow flies *Lucilia spp* (Kern *et al.*, 2012), honey bees (Baird *et al.*, 2005; Barron and Srinivasan, 2006; Portelli *et al.*, 2010, 2011) and bumble bees *Bombus terrestris* (Baird *et al.*, 2010; Baird and Dacke, 2012; Linander *et al.*, 2015, 2016, 2018; Frasnelli *et al.*, 2018; Lecoeur *et al.*, 2019). Honey bees flying in patterned tunnels can keep their speed constant regardless of the contrast or type of pattern (Baird *et al.*, 2005), and even compensate for strong headwinds (Barron and Srinivasan, 2006). Both honey bees and bumblebees adjust their flying velocity depending on the speed of the optic flow they perceive (Baird *et al.*, 2010; Portelli *et al.*, 2010, 2011; Linander *et al.*, 2017; Lecoeur *et al.*, 2019). Furthermore, the use of optic flow in velocity regulation seems to be present regardless of visual cues being natural or artificial, in two or three dimensions, and ex-periments being performed in the laboratory or in the wild (Baird and Dacke, 2012) and is found in related locomotion behaviours such as landing (Srinivasan *et al.*, 2000) and learning flights (Linander *et al.*, 2018).

Taking into account that the methodologies of previous studies differed in multiple ways, their results seem to be consistent: flying insects favour the use of optic flow to in-tegrate distance and velocity, whereas walking insects use proprioceptive cues, as well as ventral optic flow when available. However, in all the studies reported here, experiments were performed with free moving animals, which limits, to some extent, the ability to fully control the cues being perceived. For instance, some of the experiments addressing optic flow use in distance and velocity integration used moving patterns, which not only modifies self-induced visual motion but adds an external optic flow that could have differ-ent effects on the animals' behaviour (Zanker and Collett, 1985; Ronacher and Wehner, 1995; Schöne, 1996). Furthermore, insects' behaviour tends to be analysed in detail only during very short observations (Wittlinger *et al.*, 2007). To address this issue, Dahmen and colleagues (2017) developed an experimental set-up in which a restrained desert ant could run on top of an air supported ball. Training ants to a feeder and testing their homeward bound runs on top of the ball, they showed that ants can display naturalistic behaviour and

make use of path integration in such circumstances. Although this study did not make use of manipulated visual cues, similar walking trackballs have been developed for fruit flies and bees making use, to some extent, of virtual reality to investigate other fields within insect vision (Tuthill *et al.*, 2011; Paulk *et al.*, 2014; Chiappe *et al.*, 2010; Seelig *et al.*, 2010).

In this study, we aimed to investigate how optic flow is used during walking behaviour of wood ants *Formica rufa*, widely used for studying insect navigation (e.g. Harris *et al.*, 2005; Graham *et al.*, 2003; Buehlmann *et al.*, 2016; Woodgate *et al.*, 2016; Fernandes *et al.*, 2015). To achieve this, we developed a new methodology in which the visual cues perceived by ants can be fully controlled and manipulated while the ants' walking behaviour can be recorded and analysed at all times. In our experimental set-up, a tethered ant can walk on top of an air supported ball and navigate in a virtual world that changes in accordance with the ant's movement on top of the ball. To achieve this, we made use of methodological considerations described in Dahmen *et al.* (2017) and of a virtual reality engine designed for studying navigation in harnessed rodents (Aronov, 2014), thus closing the loop between the ants behaviour and changes in the visual world. We show here that ants interact with the virtual world and that they display repeatable behaviour when placed twice in the same virtual environment, walking similar distances, at similar median speeds and for equivalent time periods. We then use this paradigm to evaluate how manipulations of the optic flow, both self-induced and external separately, can change the repeatability of the ants walking behaviour.

Materials and Methods

Animals

Red wood ants (*Formica rufa* L.) from three different colonies were used for the experiments. These colonies were collected from Ashdown Forest, Sussex, UK (N 51 4.680, E 0 1.800). The oldest colonies were collected between June and August 2018 and maintained in the laboratory for around 9 months prior to the experiments. The newest colony

was collected in April 2019 and kept indoors for around 1 month before experiments commenced. Ant colonies were kept at 26°C, under a 12 h light:12 h dark cycle and fed with sucrose (333 g/L).

Tagging, pinning and body size measurements

Ants were selected from their nests based on their relatively large size and high activity. Before being placed in the set-up described below, each individual ant was marked with a distinct pattern of coloured paint spots (Humbrol Enamel, Hornby Hobbies Ltd, Kent, UK) on its thorax and abdomen for posterior identification. Tagged ants were left in a box with inside walls covered in fluon (ASC 109, Blades Biological Ltd, Edenbridge, UK) for at least 30 minutes before pinning for the paint to dry.

Ants were then anaesthetized with ice and placed under a microscope (210444, Olympus Corporation, Tokyo, Japan). A fine minutien pin (0.1 mm diameter, 1 cm length; Fine Science Tools GmbH, Heidelberg, Germany), with the top third bent around 45° was attached to the most posterior segment of their thorax using fast dry UV glue (5SF-MC12/6, 5 Second Fix). Painted and pinned ants were left inside the same fluoned box until being restrained in the virtual reality set-up.

Prior to pinning, one group of ants was selected for registering body weight, using a fine scale balance (AV264C, OHAUS, Nänikon, Switzerland). The same group of ants was selected for measuring the length of the right hind femur, as an approximation for overall size. For this, legs were removed and photographed after experiments were completed and photographs were taken into ImageJ where a line was fit along the femur, excluding joints. Head width, another approximation for overall size, was measured using frames from the experimental video recordings (described below) of the same group of ants. Individual frames were taken to ImageJ where a line was fit from the top of one eye to the top of the other.

Virtual reality set-up

In short, our virtual reality (VR) system consisted on a walking platform which movement was recorded and in turn changed the coordinates of the projected virtual world accord-

ingly. This created a closed-loop between the ants' movement and the visual stimuli, allowing them to navigate in a virtual environment (figure 1).

Harnessing

Ants were positioned on top of walking platform using a similar methodology to Dahmen *et al.* (2017), where it was shown that *Cataglyfis fortis* ants had to be able to adjust their height from the ground to display walking behaviour. In our set-up, the wood ant was restrained using the minutien pin glued to its thorax, which was placed through a glass rod (OD = 1.0 mm, ID = 0.75 mm, TW100F-3, World Precision Instruments LLC, Florida, USA), with extremities rounded using a torch to decrease friction, attached to a needle (0.8 mm width, 120 mm length, B Braun^{TM} 466564/3, Fisher Scientific, Leicestershire, UK). Two custom made plastic structures were glued to the needle, just behind the tip of the rod (2.5 x 2.5 cm and 0.5 mm thick). This way, the pin could enter the glass rod and the bent tip kept in between the two plastic structures, so that the ant could adjust its position up and down and initiate but not continue turning (figure 1). The other tip of the needle was attached to a micromanipulator (M-3333, Narishige International Ltd, London, UK) that allowed precise positioning of the ant in the centre of the walking platform.

Walking platform and movement recordings

The platform on top of which ants walked consisted on a 4 cm diameter polystyrene ball (Craftmill, Stockport , UK). A custom made metallic cup (4 cm diameter, 4 cm height), placed underneath the polystyrene ball, was perforated in the centre (2 mm diameter) and connected to an air pump (Charles Austen, ET 80, SepticsDirect, Weston-super-Mare, UK) so that the ball was supported by an air cushion. The air flow was regulated by a flow meter valve (30 lpm, Futuris), being kept at around 2 lpm. This allowed the ball to move underneath the ant when the ant walked or turned.

The movement of the trackball was recorded by an optical mouse (M500, Logitech, Lausanne, Switzerland) sensor, connected to the computer (Yoga 720, NVIDIA® GTX 1050, Intel® Core^{TM} i7, Lenovo Technology United Kingdom Limited, Basingstoke, UK). The optical mouse sensor was attached to the trackball's support and positioned

within 2 mm away from the center of the cross between the ball's equatorial and meridian lines (figure 1A). Thus, movements of the ball produced movements of the cursor on the screen, corrected using a custom written linear acceleration function.

Virtual reality

The virtual world was designed and rendered in Malab (R2019a, Mathworks, Massachusetts, USA) using the package Virtual Reality Matlab Engine (VIRMEn; Aronov and Tank, 2014). VIRMEn contains a graphical user interface where a virtual world can be designed and it performs 3D rendering of the virtual environment for display. The designed world can contain objects, walls and floors of various colours, textures and shapes. Visual features of the designed worlds varied depending on the experiment (see below).

VIRMEn is also composed of three different Matlab files (.m format) that can be customized. A movement file contains information about how the animal's movement is recorded and how that movement changes its view of the virtual world. We customized the movement file to record the position of the optical mouse cursor on the screen, which moved according to the ants' movement on top of the ball. The position of the cursor was reset to the center of the screen at every iteration without resetting the changes in the virtual world. Forwards, backwards or sideways (translational) movements of the ant on the ball changed the ant's position in the world in the x and y direction and rotating (angular movements) changed the ant's view angle (facing direction). Thus, ants could navigate in closed-loop in the custom designed virtual world. Movement functions were calibrated by rotating the ball 360 and observing a 360 rotation of the virtual environment. Further modifications of the movement file were performed for each experiment (see below).

Other customizable files include transformation functions, which allow for an immersive view adapted to different display surfaces. We used a cylindrical transformation function, with a perspective view angle, which had to be further corrected (see below).

Experiment files contain an initialization, a runtime and a termination function. We have customized these functions to create, run in every iteration (at around 30 Hz) and terminate a DAT and an Excel file containing the ant's position on the world for every experiment, as in x, y (location of the ant in the virtual world), z (height, constantly at

zero) and radians (direction of the world the ant was facing). The experiment file was also customized separately for the case of open-loop experiments (see below). In those cases, VIRMEn used as input a DAT file with the coordinates of another trial, which could be played without the optical mouse recordings of the ant's movement affecting the motion of the world.

Display

VIRMEn's virtual world was projected onto a tracing paper, wrapped around two custom made 3D printed rings (OpenSCAD; Cura 3.6; Ultimaker B.V., Geldermalsen, NL), creating a cylinder with 5 cm of diameter and 20 cm high. The cylinder was attached around the ball's support, creating a chamber opened only at the top (from where the ants were filmed) and the back (from where they were held on the ball).

The VR was projected at around 120 Hz, by a projector (ML750ST, Optoma, Watford, UK) placed 35 cm from the tracing paper and trackball chamber, above the frequency we think wood ants integrate visual information (Miall, 1978).

To further reduce distortions caused by projecting onto a cylindrical surface, we used Immersive Display Pro software (v3.1.0, Fly Elise-ng, Einhoven, NL), creating a projected image 10 cm high and with 2.1 rad (approximately 120°). This did not cover a large portion of the wood ant's horizontal visual field, which should be similar (potentially smaller) than *Melophorus bagoti* (Schwarz *et al.*, 2011) and *Cataglyphis* ants (Zollikofer *et al.*, 1995), who possess horizontal visual fields of approximately 300 degrees (150 degrees on each side) or larger, respectively.

To reduce the presence of visual cues between the ant and the projection, a 3D printed custom made floor was placed above the polystyrene ball, with a 2 cm diameter hole in the centre where the ant was placed (figure 1). However, based on the visual properties of *Cataglyphis* ants (Zollikofer *et al.*, 1995), wood ants should have a blind region starting from below their horizon and of approximately 50 degrees wide (25 degrees on each side or potentially larger). We did not measure the standing height of wood ants, i.e., the distance between the ant's eyes and the ground. Assuming it would range between 1 mm and 5 mm, the angle between the ant's eyes and the edge of the custom made floor's hole,

on the horizontal axis, would be between around 168 degrees (84 degrees on each side) and less than 126 degrees (63 degrees on each side), highly above the angle of the ants' blind region. Thus, wood ants would still be able to receive visual information from the trackball's movement, as well as static information from the custom made floor, with the ventral portion of their eyes.

Video recordings

Above the open top of the trackball chamber, we placed a high speed camera (eo-13122M, Edmund Optics Inc., Barrington, USA) with a macro lens (EF-S 60 mm f/2.8 Macro USM, Canon, Surrey, UK). We connected the camera to a second computer (Yoga 720, NVIDIA® GTX 1050, Intel® Core™ i7, Lenovo Technology United Kingdom Limited, Basingstoke, UK) and recorded the ant's behaviour at around 73Hz (software: uEye64, IDS Imaging Development Systems GmbH). Thus, the restrained ant on top of the ball was visible at all times (figure 1).

Experiments

Each tagged and pinned ant was place in the VR set-up as explained above and allowed to run in the virtual world for around 10 minutes. After the first 10 minute trial, ants were placed in a fluoned box with other ants from the same nest, from 2 to 4 hours, until they were placed for the second time in the set-up and allowed to navigate on the virtual world for another 10 minute trial. Ants were recorded from above throughout the whole 10 minutes of each trial. Ants that did not seem healthy, that seemed stuck and not able to adjust their vertical position on the ball or that touched the rod, syringe or floor with their legs were excluded from the experiments. After running in the environment twice, all ants were discarded.

For all experiments, trials started at the center of the visual world, at (x,y) coordinates (0,0), but their starting facing direction varied between experiments (see below). During each 10 minute trial, they were allowed to rotate and navigate in any direction of the world.
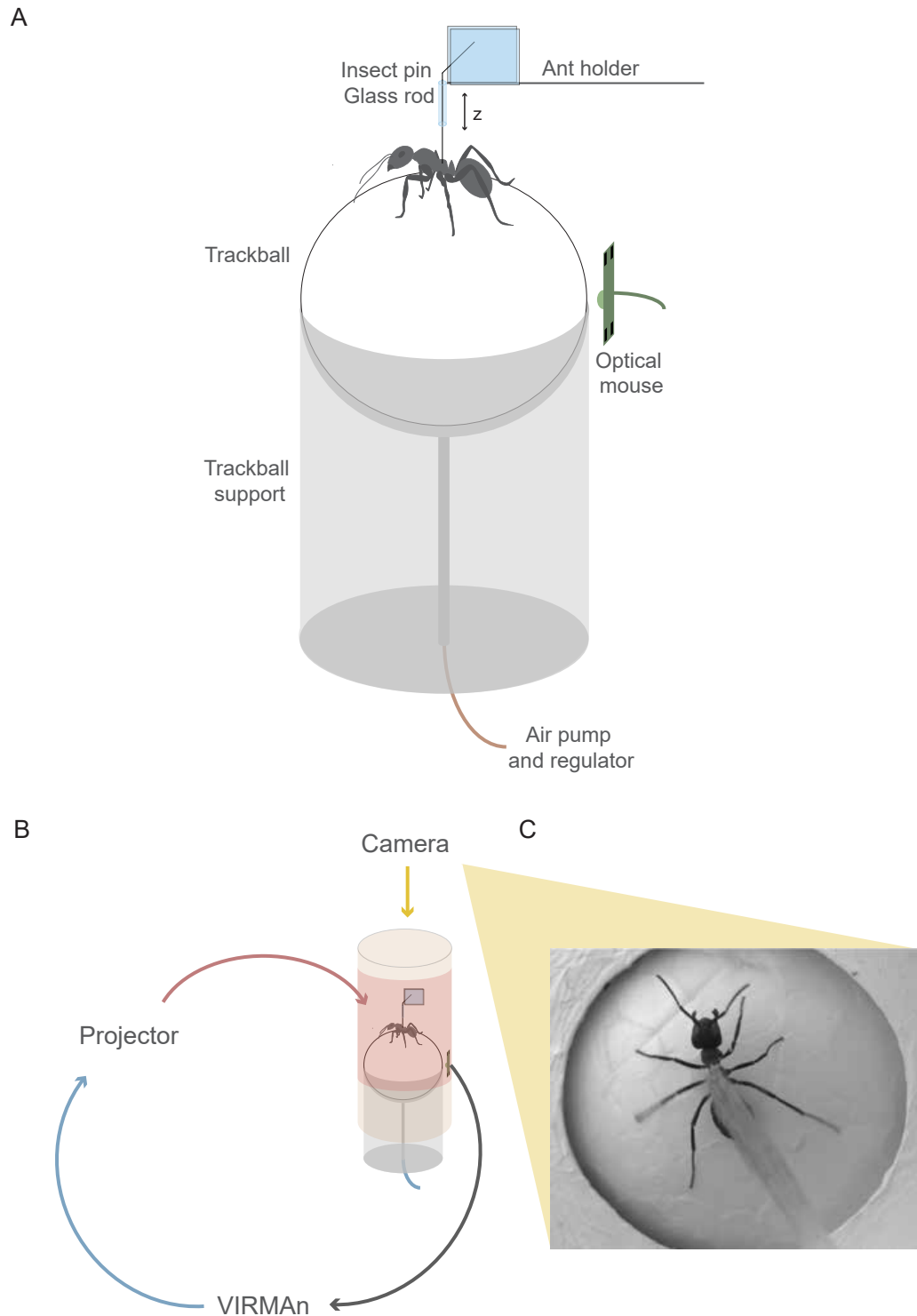
Figure 1: Schematic diagram of the virtual reality paradigm. A) Wood ants are placed on top of a polystyrene ball (trackball), seating on a metallic cup (trackball support) perforated inside to allow pressure regulated air flow from an air pump to support the trackball. The ant is restrained by an insect pin glued to the posterior portion of the thorax. The insect pin is kept in place by a glass rod and two custom made plastic structures that allow the ant to move up and down (z) but not rotate on top of the ball. As the ant walks, the air supported trackball moves underneath, which is recorded by an optical mouse. B) The optical mouse is connected to a computer running the Matlab package VIRMEn. VIRMEn creates a virtual world and changes it according to the optical mouse recordings. The virtual world is displayed to the ant through a projector connected to the same computer onto a tracing paper cylinder surrounding the ant, trackball and support. C) A camera placed above the set-up records the ant at all times. A portion of the trackball is covered by a custom made white floor to reduce the presence of visual cues between the ant and the virtual world.

Experiment 1: Interaction with the virtual world

To evaluate if ants would engage with the virtual world, we allowed them to experience two different environments with white background and a virtual object, one in which the object was visible and one in which it was invisible. The virtual object was a rectangular parallelepiped, placed with its center at (x,y) coordinates (30,30) (width: 20, length: 20, height: 40 virtual units; figure 2A). When visible, two of its walls were black and two were dark gray. Ants were not able to cross the walls of the visible or invisible object. The order by which they walked in one or the other environment was randomized. To avoid ants starting a trial always facing the object, the direction they were facing at the start of the experiment (different between ants but the same for each) was also randomized.

Experiment 2: Consistency with and without virtual optic flow

To determine if ants showed consistent and reliable naïve walking behaviour, we allowed ants to run twice on the same virtual world, with a white background and no virtual visual cues. To analyse the consistency of ants' walking behaviour with virtual optic flow cues present, we allowed a separate group of ants to run on the VR twice in the same environment, but this time with a virtual floor composed of black dots that gave a sense of motion when the ant turned or walked (figure 2B).

Experiment 3: Optic flow manipulations

To analyse the importance of optic flow in ants' walking behaviour on the VR, we allowed ants to run once with virtual optic flow (virtual dotted floor; figure 2B) and once without (only white background), in randomized order. Ants were divided in three groups, which differed in the amount of gain added to their movement, i.e., how much their movement on top of the ball changed the virtual world. Thus, self-generated optic flow was different depending on the set gain. This was acquired by changing VIRMEn's movement file and performed only for translational movement (x and y) and not for angular rotation. A group of ants walked in both environments with the original gain (same as for previous experiments), hereafter called normal optic flow. Another group of ants walked on the ball with 3 times less gain (slow optic flow), acquired by dividing the velocity in which x

A
B
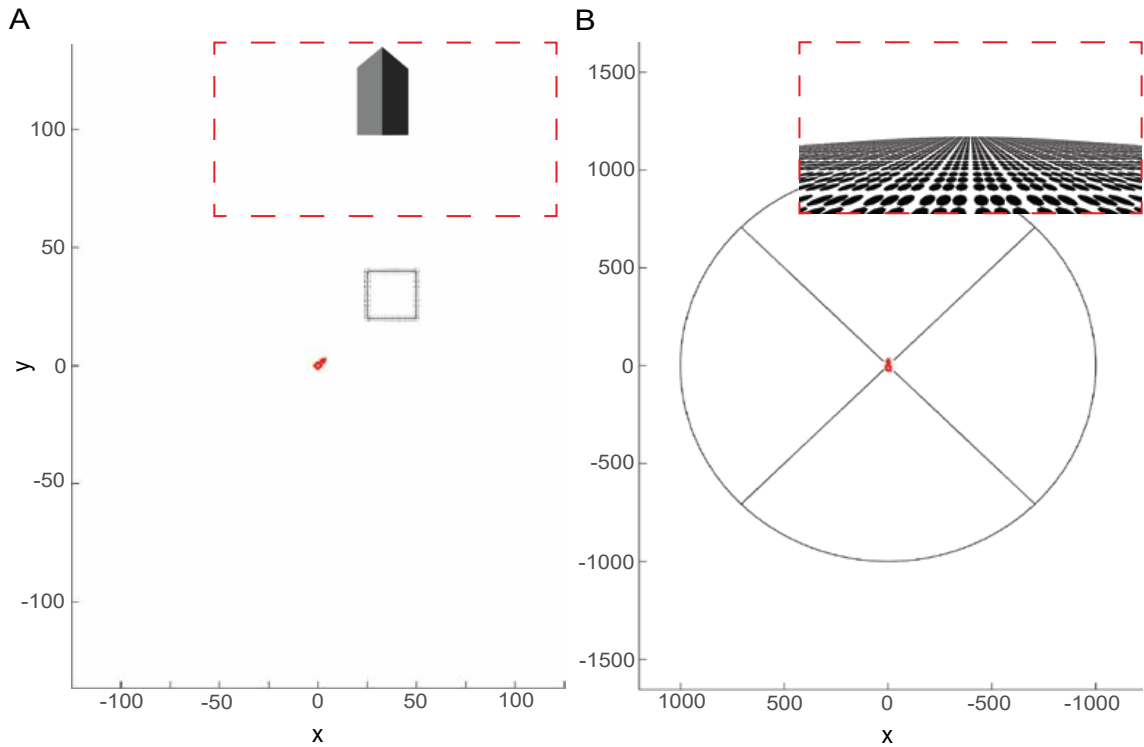
Figure 2: Schematic diagram and ant's view of the virtual world. A) Design of the virtual world used for Experiment 1, with a white background and an object, viewed from above. The starting point is represented at the center of the world in red. The arrow represents one of the random directions an ant could have been facing at the start of the trial. If the object was visible, the ants' view of the world on the position and direction represented by the red arrow would correspond to the image surrounded by the dashed red line. B) Design of the virtual world used for all other experiments, viewed from above. All ants started at the center of the world facing forward (red arrow). If the floor was visible and the ants experienced optic flow, the ants' view of the world on the position and direction represented by the red arrow would correspond to the image surrounded by the dashed red line. If the object floor were invisible, ants experienced a white environment with no visual cues.

and y in the virtual world are changed by the balls movement by 3. Another group walked with 3 times more gain (fast optic flow), acquired by multiplying the velocity in which x and y in the virtual world are changed by the ball's movement by 3. Thus, ants' movement on the ball moved them in the virtual world 3 times slower or faster, respectively.

Experiment 4: Open-loop optic flow

In previous experiments, optic flow was always generated by the ants' own movement. To determine if external optic flow would have the same effect on ants' behaviour, we allowed a group of ants to walk once on a virtual world with optic flow in closed-loop, as in previous experiments, and once in open-loop. We kept the visual input equivalent between the two trials by replaying on the second trial the cues they had observed on the first. The optical mouse recordings were performed using another computer, thus not

affecting the motion of the visual world.

Data analysis

The ants' x and y position on the virtual world, as well as their facing direction (in radians), were recorded by VIRMEn at around 30Hz, saved automatically and later edited in Excel (Excel 2010, Microsoft, Redmond, USA). All analysis was performed in R (v3.5.1, R Foundation for Statistical Computing, Vienna, Austria) using RStudio (v1.1.463, RStudio Inc, Boston, USA). For all experiments, a set of additional variables was calculated. Time at each frame was calculated (in seconds) by dividing the frame number by the VIRMEn's frame rate, 30 Hz. Distance walked in x and y between each frame, as well as the total distance ($\sqrt{xdistance^2 + ydistance^2}$), were obtained using the original x and y positions. Total distance was multiplied by 0.71 to transform distance walked in the world in centimeters. This ratio was calculated by rotating the ball in y, keeping the its meridian aligned with the optical mouse and verifying to which distance in the virtual world did it correspond to. Velocity at each frame was calculated by multiplying the total distance walked by 30 Hz (cm/s). The same calculations were performed for angular distance and velocity, using the recorded angle (in rad and rad/s, respectively). Further analysis was divided in "translational", every frame in which ants move in x and y (even when there was still an angular change at the same time), and "angular", every frame in which ants do not move in x and y but rotate in the same position. By default, if no distinction is mentioned, parameters refer to translational distance, speed or time walked, otherwise stated angular. The first minute of data was excluded to eliminate potential side effects of ants just having been placed on the set-up. To avoid analysing walking parameters using a very limited number of frames, ants that walked less than one second in at least one of the trials were excluded from analysis.

Experiment 1

To analyse if wood ants would interact with the virtual reality, we calculated two parameters for all frames: their distance to the center of the virtual object and the direction they faced. In this experiment, distance in the virtual world was considered, rather than real

distance in centimeters. We compared the median distance to the center of object between the two conditions, when object was either visible or invisible, using a Wilcoxon signed-rank test. The average angle ants faced was compared between the two conditions using a Watson-Wheeler test for circular data.

Experiment 2

To analyse ants' naïve behaviour, we used total distance walked, median velocity and total time spent walking. Because all variables were composed of positive numbers only and some show higher variation for larger in comparison with smaller variables, all regression analysis performed was based on general linear models using a gamma function. We analysed if there was a correlation between distance walked and speed and/or time by performing a regression analysis using the R command `glm` with `family = Gamma(link=identity)`, from the R package 'glm'.

We also performed a regression analysis using either the body weight, head width or right hind femur length (body size parameters) as the independent variable, to analyse how did ants' body size and weight influence their total distance walked and median speed (dependent variables), using the same command.

To analyse the consistency in ants' walking behaviour, we performed a regression analysis of the translational distance walked, median speed and time spent walking between the first and second trials in the same white background environment, using the same R command and package. We also compared the median distance walked, median speed and median time walked between the two trials using Wilcoxon signed-rank tests. The same analysis was performed for ants that were subjected to two trials with optic flow (dotted floor) present. The correlations and comparison of medians for angular distance, median angular velocity and time spent turning between the first and second trials were also analysed, using the same models, for both conditions.

Experiment 3

To analyse how optic flow modulated walking behaviour, we focused on the same parameters as previously described: distance walked, median velocity and time spent walking.

We performed a regression analysis for each of these parameters between the between the trial with flow (dependent variable) and the trial without flow (independent variable), using the gain as the covariate, with the same R command and package. The model was releveled to the medium gain (normal optic flow) and therefore we compared how faster and slower optic flow (high or low gain) change the correlation of the behaviours between the two trials. Wilcoxon signed-rank tests were performed to compare the median distance, velocity and time walking between the trial with optic flow and the trial without. Rotatory behaviour was analysed in the same manner.

Experiment 4

To determine if ants' behaviour changed due to external optic flow, we used the same parameters as previously described, distance walked, median velocity and time spent walking, and performed a regression analysis for each parameter between the first and the second trials. We used the same R command and package. We also compared the median distance, speed and time walked between the two using a Wilcoxon signed-rank test. Angular distance, speed and time spent rotating were analysed similarly.

Results

Wood ants respond to and interact with virtual reality

Wood ants were tethered on top of an air supported polystyrene ball and surrounded by a projected virtual world. Ants walked on top of the trackball and their position within the virtual world was recorded (figure 3A, B). To determine whether ants interact with the projected virtual world, we placed them in two different environments for 10 minutes each, one in which a black and gray object, shaped as a parallelipiped, was visible and one in which it was invisible. The presence of the visible object changed the ants trajectories; ants walked significantly closer to the object when it was visible, with the median distance to the object being larger and more variable when ants could not visualize it (Wilcoxon test, N=29, df=27, W=316, p=0.03; figure 3C).

The presence of the visible object also affected the direction the ants faced whilst
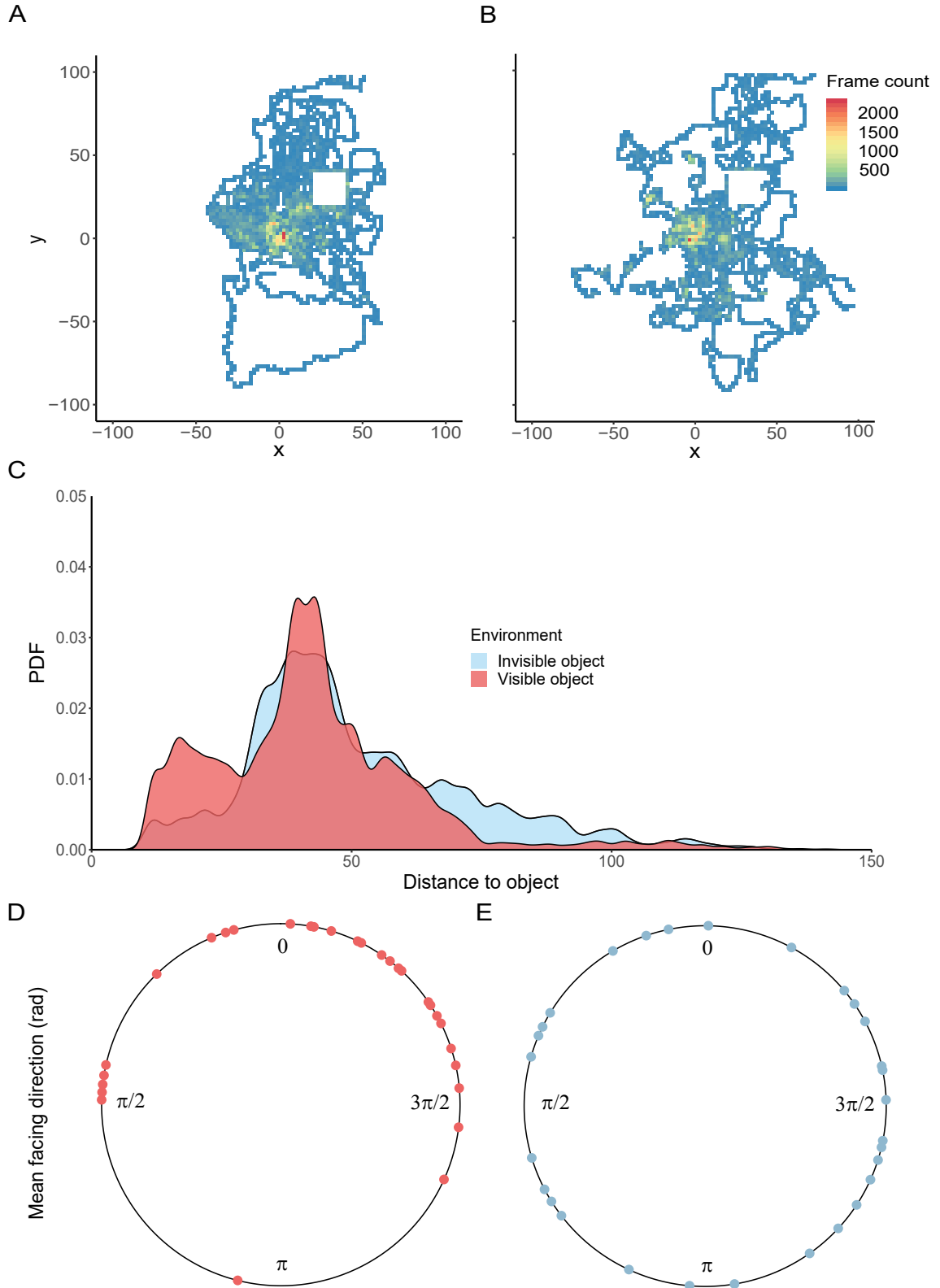
Figure 3: Wood ants interact with a projected virtual world by approaching and facing a virtual object. Ants' paths on the virtual world differ depending if a black and gray object is A) visible or B) invisible. The number of frames ants stayed on each x and y position within the virtual world is represented by colours from blue (few occurrences) to red (many occurrences). C) Probability density function of the distance between the virtual object and the ants' position on the virtual walk. D) Mean facing direction of each ant relative to the object when it was visible or E) invisible. An angle of 0 rad means ants are directly facing the object. The curvature of the screen occupies 2.1 rad, which means ants facing any direction from approximately π/2 to 3*π/2 have the object in the visible projected world.
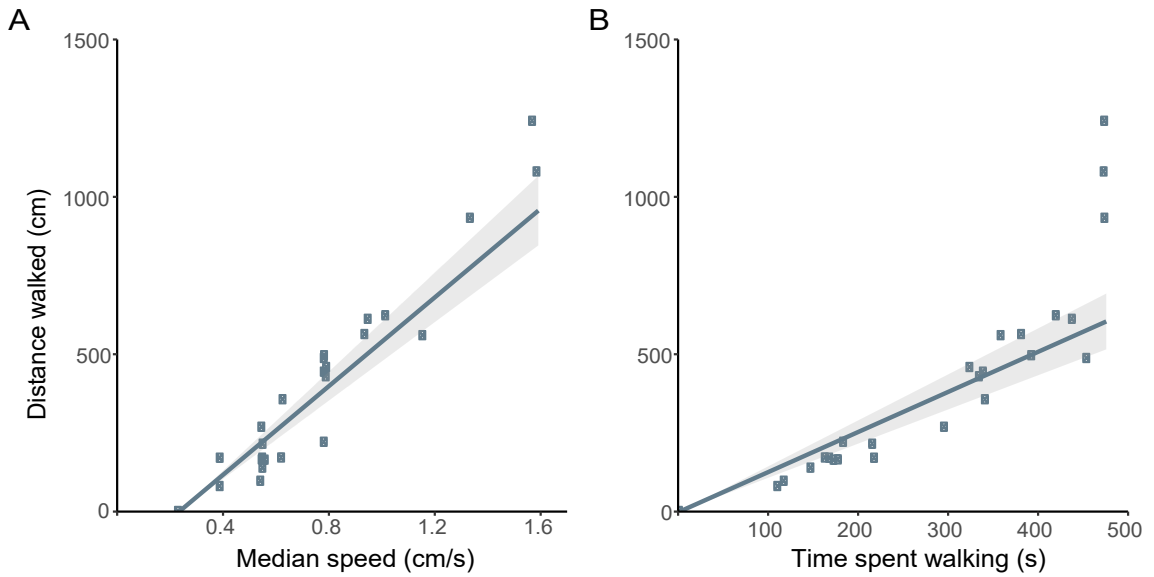
Figure 4: Distance walked by ants on a white virtual world correlates with the speed they walk at and the time they spent walking. Distance walked correlates with A) median speed and B) time spent walking. Lines and grey shading represent the estimated regression line and confidence intervals of the generalized linear model.

walking in the virtual world. Indeed, ants tended to fixate on the virtual object when it was visible but faced different directions when it was invisible (figure 3D, E). The direction ants faced whilst walking was significantly different between the two conditions (Watson-Wheeler test, N=29, df=27, W=8.42, p=0.01). Thus, the presence of a visible object within the virtual environment changed the ants walking behaviour, compared to their behaviour when navigating on a virtual environment with white background and no visible cues. This suggests that ants can recognise visual cues within a virtual world and adapt their walking strategies accordingly.

Individual workers show reliable and repeatable walking behaviour

To observe the naïve walking behaviour of wood ant workers, individual ants were tethered on the trackball in a virtual world with no visual cues. Ants displayed different walking strategies, reaching variable distances from their starting position (figure 4A, B). Those individuals that walked greater distances also walked at higher velocities (generalized linear models, N=25, df=22, t=3.55, p<0.01; figure 4A), and for longer times (generalized linear models, N=25, df=22, t=2.46, p=0.02, figure 4B).

To determine if there were morphological characteristics of the ants that could influ-

Table 1: Smaller and lighter ants travel longer distances and at faster speeds compared to larger and heavier ants. Regression analysis (generalized linear models) of distance walked and median speed with head width (mm), right hind femur length (mm) and body weight (mg). The number of ants (N), degrees of freedom (d.f.), t-statistics and P-value are visible for each combination of parameters analysed (*P<0.05).

	Walking parameter	N	d.f.	t	P
Head width	Distance	25	23	-2.05	0.05
	Medium Speed			-2.19	0.04*
Hind femur length	Distance	17	15	-2.4	0.03*
	Medium Speed			-2.46	0.03*
Body weight	Distance	17	15	-2.28	0.04*
	Medium Speed			-2.16	0.048*

ence their walking behaviour, we measured the ants' head width, right hind femur length (both approximations for body size), and weight. The distance ants walked and median velocity at which they walked were negatively correlated with both approximations for body size and with weight (table 1; figure 5). Thus, smaller and lighter ant workers walk longer distances at faster speeds than their larger and heavier counterparts.

When placed on the trackball in a white virtual environment for the second 10 minute period, wood ant workers behaved in a similar way to their previous trial. Indeed, there was a correlation between the distances they walked on the first and second trials (figure 6A, B; table 2). Furthermore, the median velocity at which they walked (figure 6C, D; table 2) and the time they spent walking (figure 6E, F; table 2) were also correlated across the two 10 minute trials. Moreover, there were no significant differences on the medians of any walking parameter between the first and the second trials (table 2).

However, rotating behaviour did not show similar consistency (fig 7; table 2). Ants tended rotate for longer times and angular displacements on the first trial than the second, but median angular speed did not change nor it correlated between trials. Taken together, ants show consistent behaviour in distance walked, median speed and time spent walking when placed twice in the same white virtual environment. Although it is unclear if they estimated distance by integrating the speed they walked at or the time they spent walking, larger and heavier ants walked reliably less and slower compared to smaller and lighter ants. Furthermore, translational movement differs from rotating, which does not show consistency to the same extent and seems decreased on the second trial despite visual world being the same.
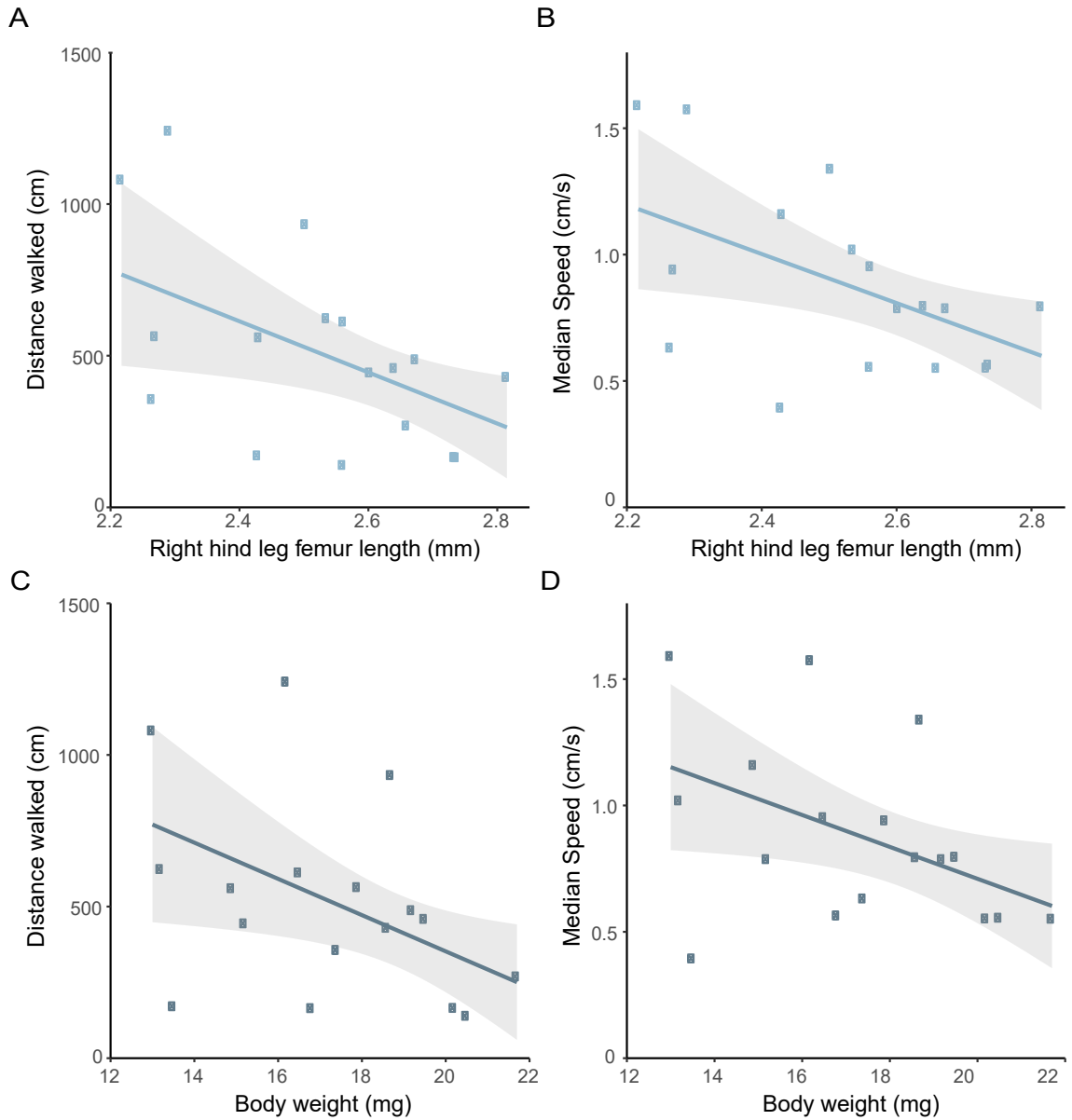
Figure 5: Smaller and lighter ants walk longer distances and at higher median speeds. A) Distance walked and B) median speed correlate with right hind femur length, an approximation for overall body size. C) Distance walked and D) median speed correlate with body weight. Lines and grey shading represent the estimated regression line and confidence intervals of the generalized linear model.
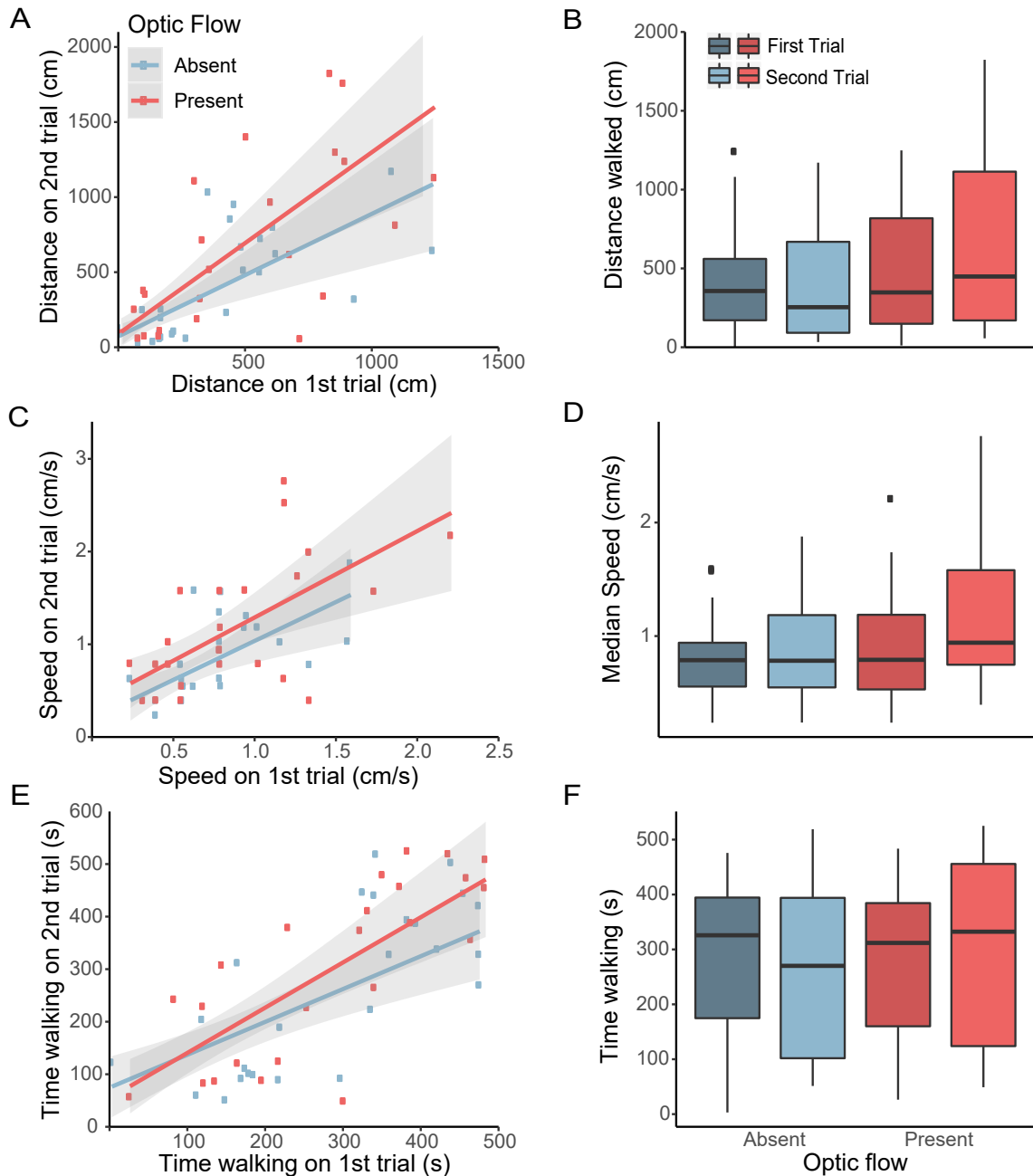
Figure 6: Ants show consistent walking behaviour when placed twice on the same environment. Distance walked A) correlates between the two trials and B) there are no differences in the median distance walked between the two trials. The speed ants walk at C) correlates between trials and D) there are no differences in the median speeds between the two trials. The time spent walking E) correlates between trials and F) there are no differences in the median time walked between the two trials. Lines and grey shading represent the estimated regression line and confidence intervals of the generalized linear model. Ants that were twice in a white virtual environment with no optic flow are represented in blue and ants that were twice in a virtual environment with optic flow are represented in red.
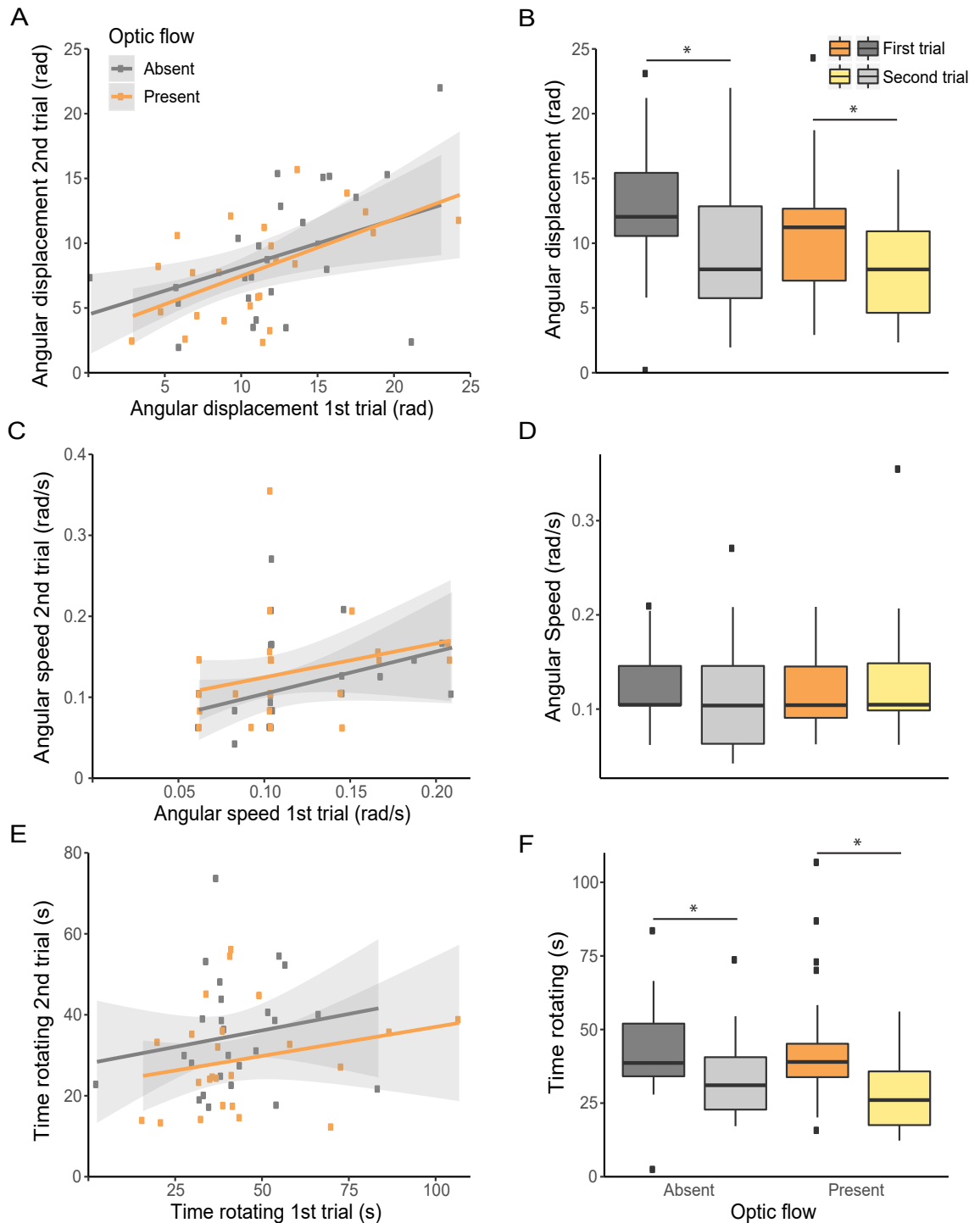
Figure 7: Ants rotate longer angular displacements and for longer time periods on the first trial compared to the second. Angular displacement A) correlates between two environments with or without flow and B) is higher for the first compared to the second trial. Median angular speed C) does not correlate nor D) changes between trials. Time spent rotating E) does not correlate between trials but F) is higher for the first compared to the second trial, regardless of which environment ants experience. Lines and grey shading represent the estimated regression line and confidence intervals of the generalized linear model. Ants that were twice in a white virtual environment with no optic flow are represented in gray and ants that were twice in a virtual environment with optic flow are represented in orange/yellow.

Optic flow does not alter reliable behaviour

To assess the effect of optic flow from the virtual world upon ants' naïve walking behaviour, they were tethered on the trackball for two 10 minute trials (as described above) in a virtual environment in which the ground was covered with black dots, which provided self-generated optic flow as the ant walked. This group of ants was allowed to navigate in the virtual world with optic flow on both 10 minute trials. Optic flow did not change the repeatability of the wood ant workers' walking patterns; the distance walked, median velocity and time spent walking on the first and second 10 minute trials were all correlated (figure 6; table 2). There were also no differences in the medians of any walking parameters between the first and second trials (figure 6; table 2).

Comparing this with the previous group of ants that had walked in a virtual world with no optic cues twice, there were no differences between the two conditions in terms of distance walked (no flow: 294.99 +/- 473.78 cm; flow: 370.572 +/- 717.591 cm; Wilcoxon test N=98, df=96, W=1001, p=0.16), speed (no flow: 0.78 +/- 0.479 cm/s; flow: 0.79 +/- 0.79 cm/s; Wilcoxon test, N=98, df=96, W=967, p=0.1) nor the time spent walking (no flow: 325.77 +/- 219.67 s; flow: 315.15 +/- 274.88 s; t-test, N=98, df=96, t=586.5, p=0.88).

However, consistency of rotating behaviour was still not observed when optic flow was present (fig 7, table 2). As when optic flow was absent, ants also tended to rotate for longer time periods and longer angular displacements on the first trial than the second. Furthermore, median angular speed did not correlate between trials. In summary, the walking patterns of ants that experienced self-generated optic flow from the virtual world were similar to the ones who did not, with ants travelling comparable distances, at similar median speeds and spending analogous amounts of time walking but not displaying consistent rotatory behaviour.

Table 2: Translation walk and rotating behaviour in ants experiencing the same environment twice. Regression analysis (generalized linear models) of distance walked, median speed, time spent walking, angular displacement, angular median speed and time spent rotating between two trials in identical environments, either both with optic flow (N=24, df=22) or both without (N=25, df=23). The estimated slope and intercept values, t-statistics and P-value are visible for each parameter analysed. The median values for each parameter are compared between the first and second trials, with W representing the Wilcoxon sign rank test and P the P-value (*P<0.05, **P<0.01).

			Estimate	t	P		W	P
No Flow	Distance	Slope	0.82	4.09	<0.01**		319	0.91
		Intercept	71.97	1.83	0.08			
	Median Speed	Slope	0.84	3.38	<0.01**		301	0.83
		Intercept	0.19	1.21	0.24			
	Time walking	Slope	0.62	4.51	<0.01**		354	0.43
		Intercept	74.52	2.48	0.02*			
	Angular Displacement	Slope	0.37	2.63	0.02*		438	0.01*
		Intercept	4.49	2.83	<0.01**			
	Angular Speed	Slope	0.52	1.57	0.13		364	0.32
		Intercept	0.05	1.39	0.18			
	Time rotating	Slope	0.16	0.86	0.4		417.5	0.04*
		Intercept	27.98	3.44	<0.01**			
Flow	Distance	Slope	1.21	4.87	<0.01**		248	0.42
		Intercept	88.35	1.86	0.07			
	Median Speed	Slope	0.93	3.54	<0.01**		226	0.21
		Intercept	0.36	1.92	0.07			
	Time walking	Slope	0.86	5.53	<0.01**		267	0.68
		Intercept	54.49	1.85	0.08			
	Angular Displacement	Slope	0.44	2.86	<0.01**		392	0.03*
		Intercept	3.09	2.11	0.046*			
	Angular Speed	Slope	0.41	1.22	0.23		261	0.59
		Intercept	0.08	2.19	0.04*			
	Time rotating	Slope	0.14	0.998	0.32		431.5	<0.001**
		Intercept	22.64	3.52	<0.01**			

Changes in optic flow speed alter distance and time spent walking but not speed

To further assess how optic flow modulates the ants' walking behaviour, we tethered ants on the trackball allowing them to walk once in an environment with optic flow present and once in an environment without virtual optic flow. For this group of ants, the velocity of the optic flow was the same as for previous experiments, hereafter called 'normal OF'. Ants experiencing two different environments, with and without normal OF, still show repeatable behaviour; ants travelled similar distances, at equivalent speeds and during consistent amounts of time (figure 8; table 3).

We then tethered two additional groups of ants on the trackball and exposed them once to a virtual environment without optic flow and once to a virtual environment with optic flow at one of two different speeds, slower or faster than the previously used normal optic flow. These changes in gain were implemented solely for translational movements. In the slow OF situation, we decreased the gain of the ants' movement three-fold so that each movement of the ant on the ball produced optic flow three times slower. In the fast OF situation, the gain was three-fold higher so that movements of the ants generated optic flow three times faster. For ants that were exposed to a slow OF, all walking parameters were correlated between trials (figure 9; table 3). Although these ants walked at similar velocities in both trials, the overall distance they walked and the time they spent walking were significantly higher when the slow OF was present, compared to when optic flow was absent (figure 9; table 3).

Walking parameters were also correlated between the two trials for ants that walked once with fast OF and once without virtual optic flow. For these ants, there were no differences in the overall distance travelled, median velocity and time spent walking with fast OF in comparison with these parameters obtained from the virtual environment without optic flow (figure 9; table 3).

We compared the slopes and intercepts of these regression lines between the three different optic flow speeds (slow, normal and fast) to assess the effect of optic flow in ants walking behaviour. There were no differences on slopes (generalized linear model, N=80,

Table 3: Translational walk and rotating behaviour for each self-generated optic flow speed. Regression analysis (generalized linear models) of distance walked, median speed, time spent walking, angular distance, angular speed and time spent rotating between a trial with and a trial without optic flow, for normal (N=25, df=23), slow (N=24, df=22) and fast (N=31, df=29) optic flow. The estimated slopes and intercepts, t-statistics and P-values are visible for each combination of parameters analysed. The median values for each parameter are compared between the first and second trials, with W representing the Wilcoxon sign rank test and P the P-value (*P<0.05, **P<0.01).

			Estimate	t	P	W	P
Normal OF	Distance	Slope	1.12	4.63	<0.01**	294	0.73
		Intercept	23.12	1.57	0.13		
	Median Speed	Slope	0.82	3.68	<0.01**	240	0.16
		Intercept	0.15	1.64	0.11		
	Time walking	Slope	0.76	4.51	<0.01**	327	0.79
		Intercept	43.9	2.12	0.045*		
	Angular Displacement	Slope	0.26	1.64	0.12	388	0.15
		Intercept	9.07	3.57	<0.01**		
	Angular Speed	Slope	0.33	2.02	0.06	315	0.97
		Intercept	0.08	4.19	<0.01**		
	Time rotating	Slope	-0.005	-0.04	0.97	384	0.17
		Intercept	50.21	5.52	<0.01**		
Slow OF	Distance	Slope	2.4	6.45	<0.01**	181	0.03*
		Intercept	-2.4	-2.72	0.013*		
	Median Speed	Slope	1.26	3.8	<0.01**	214	0.13
		Intercept	0.04	0.34	0.74		
	Time walking	Slope	1.71	5.75	<0.01**	189	0.04*
		Intercept	-0.38	-0.06	0.95		
	Angular Displacement	Slope	0.37	1.85	0.08	229	0.23
		Intercept	10.16	3.72	<0.01**		
	Angular Speed	Slope	0.31	1.2	0.24	231.5	0.25
		Intercept	0.09	3.12	<0.01**		
	Time rotating	Slope	0.48	2.53	0.02*	273	0.77
		Intercept	32.6	3.12	<0.01**		
Fast OF	Distance	Slope	0.69	2.11	0.04*	407	0.31
		Intercept	144.83	2.79	0.009**		
	Median Speed	Slope	0.61	2.51	0.02*	389	0.2
		Intercept	0.31	2.64	0.013*		
	Time walking	Slope	0.71	3.74	<0.01**	430	0.48
		Intercept	92.13	3.19	0.003**		
	Angular Displacement	Slope	0.76	5.83	<0.01**	515	0.63
		Intercept	3.36	2.99	<0.01**		
	Angular Speed	Slope	0.52	2.79	<0.01**	459	0.77
		Intercept	0.07	3.33	<0.01**		
	Time rotating	Slope	0.63	4.64	<0.01**	524	0.55
		Intercept	17.98	3.26	<0.01**		
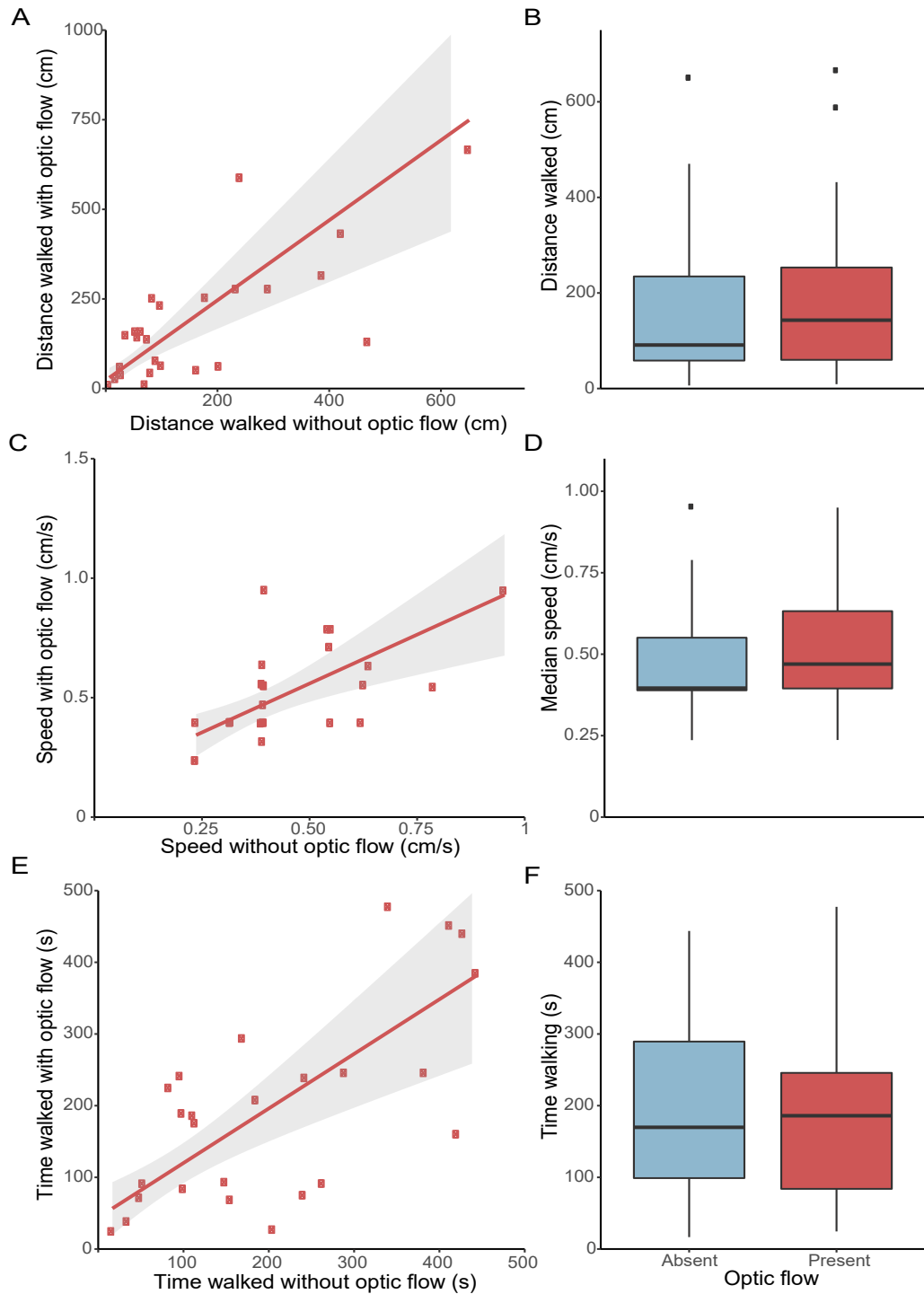
Figure 8: Ants show consistent walking behaviour when placed once in a virtual environment with optic flow and once without optic flow. Distance walked A) correlates between the two trials and B) there are no differences in the median distance walked between the two trials. The velocity ants walk at C) correlates between trials and D) there are no differences in the median speeds between the two trials. The time spent walking C) correlates between trials and D) there are no differences in the median time walked between the two trials. Lines and grey shading represent the estimated regression line and confidence intervals of the generalized linear model. The median values on trials in which optic flow was absent are represented on the blue box-plots and present on the red box-plots.

df=74, slow-normal: t=0.97, p=0.34; fast-normal: t=-0.54, p=0.59) nor intercepts (generalized linear model, N=80, df=74, slow-normal: t=-0.64, p=0.52; fast-normal: t=0.99, p=0.32) between the three conditions for the median velocity ants walked at, demonstrating that self-induced optic flow speed did not affect the velocity at which ants walked. However, the distance travelled by ants in the slow OF condition was significantly higher in comparison to the distance that ants walked in the normal OF condition (slope: generalized linear model, N=80, df=74, t=2.54, p=0.01). Conversely, ants that walked in the fast OF condition produced a regression with equivalent slope compared to the normal OF (slope: generalized linear model, N=80, df=74, t=-1.07, p=0.29) but walked significantly longer distances overall (intercept: generalized linear model, N=80, df=74, t=2.57, p=0.01). Furthermore, the fast OF condition did not alter the time spent walking (generalized linear model, N=80, df=74, slope: t=-0.2, p=0.84; intercept: t=1.23, p=0.22), but the slow OF condition produced a significantly higher slope compared with the normal OF (generalized linear model, N=80, df=74, t=2.97, p<0.01).

Rotating behaviour, however, did not correlate between trials for medium and slow optic flows but it did so for fast optic flow. Nevertheless, there were no differences in median angular displacement, angular speed and time spent rotating between present or absent optic flow in any of the conditions (fig 10; table 3). Thus, as previously observed, rotating behaviour is considerably different than translational walking regardless of the presence, absence or speed of the self-induced optic flow. Because of the inconsistency of rotatory behaviour, we did not compare how different speeds of optic flow affected angular parameters. Taken together, wood ant workers maintained their consistency in all translational walking parameters when experiencing two different types of environment differing on the presence or absence of virtual optic flow. However, altering the speed of the self-generated optic flow led to some differences in the distance travelled and the time spent walking. This suggests that ants used optic flow cues to measure the distance they walked and, to some extent, the time they spent walking in order to keep consistency, but velocity control was not dependent upon self-generated optic flow.
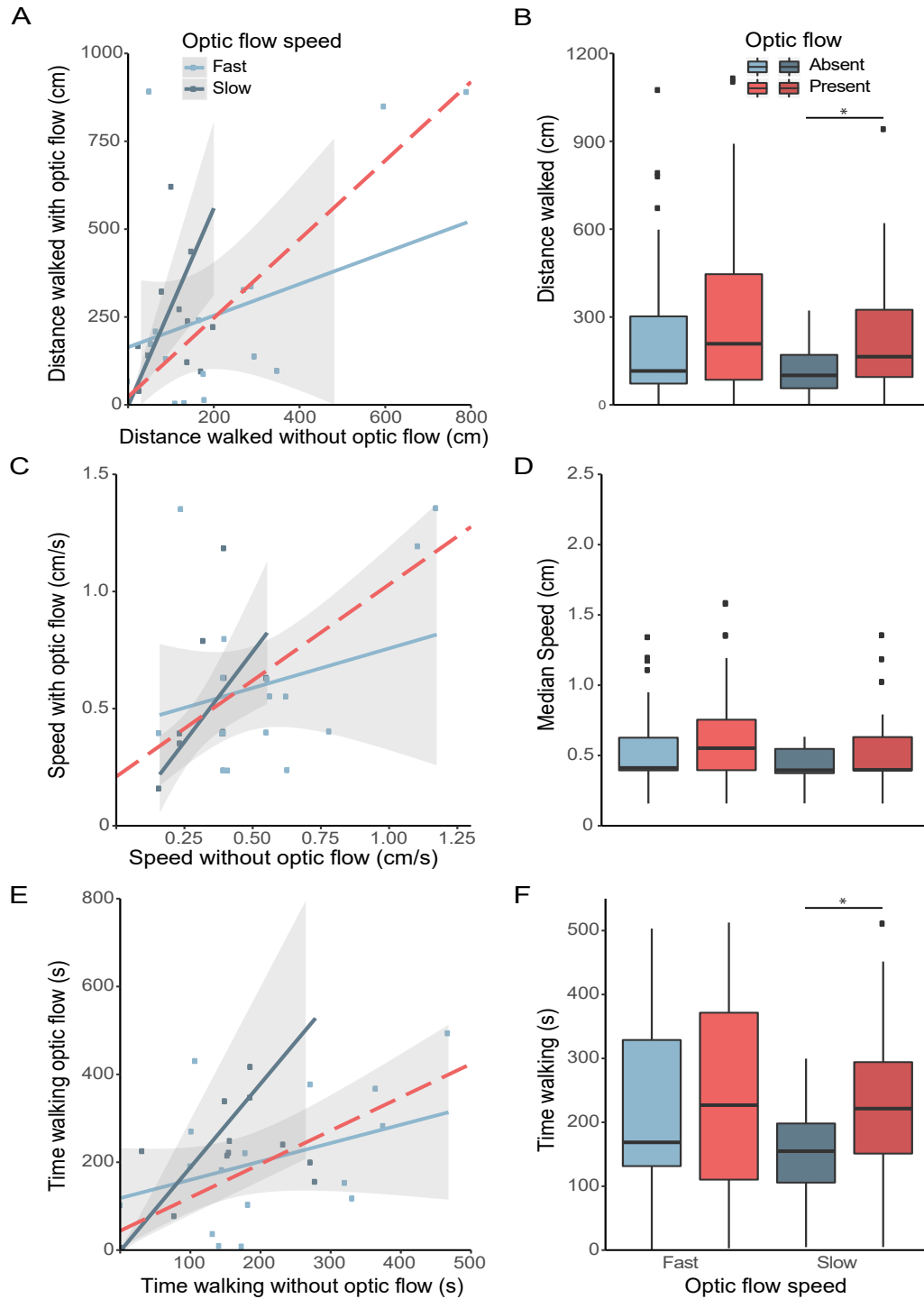
Figure 9: The velocity of self-generated optic flow modifies distance and time spent walking, but not walking speed. A) The distance walked by ants that walk in two different environments, with and without optic flow, correlates between the two environments. The slope of the estimated regression line is different depending on the optic flow speed. B) The median distance walked in the presence of slow optic flow is higher then when optic flow is absent, but not with faster optic flow. C) The median speed ants walk at correlates between the two environments and D) is not different between trials for any optic flow speed. E) Time spent walking correlates between the two environments and the slope of the estimated regression line depends on the optic flow gain. F) The median time spent walking is different between environments for slow gain but not for fast gain. Lines (slow: dark blue; fast: light blue) and grey shading represent the estimated regression lines and confidence intervals of the generalized linear model. The regression line estimated for the original optic flow gain is represented by the dashed red line. The median values on trials in which optic flow was absent are represented on the blue box-plots and present on the red box-plots (slow: dark colours; fast: light colours).
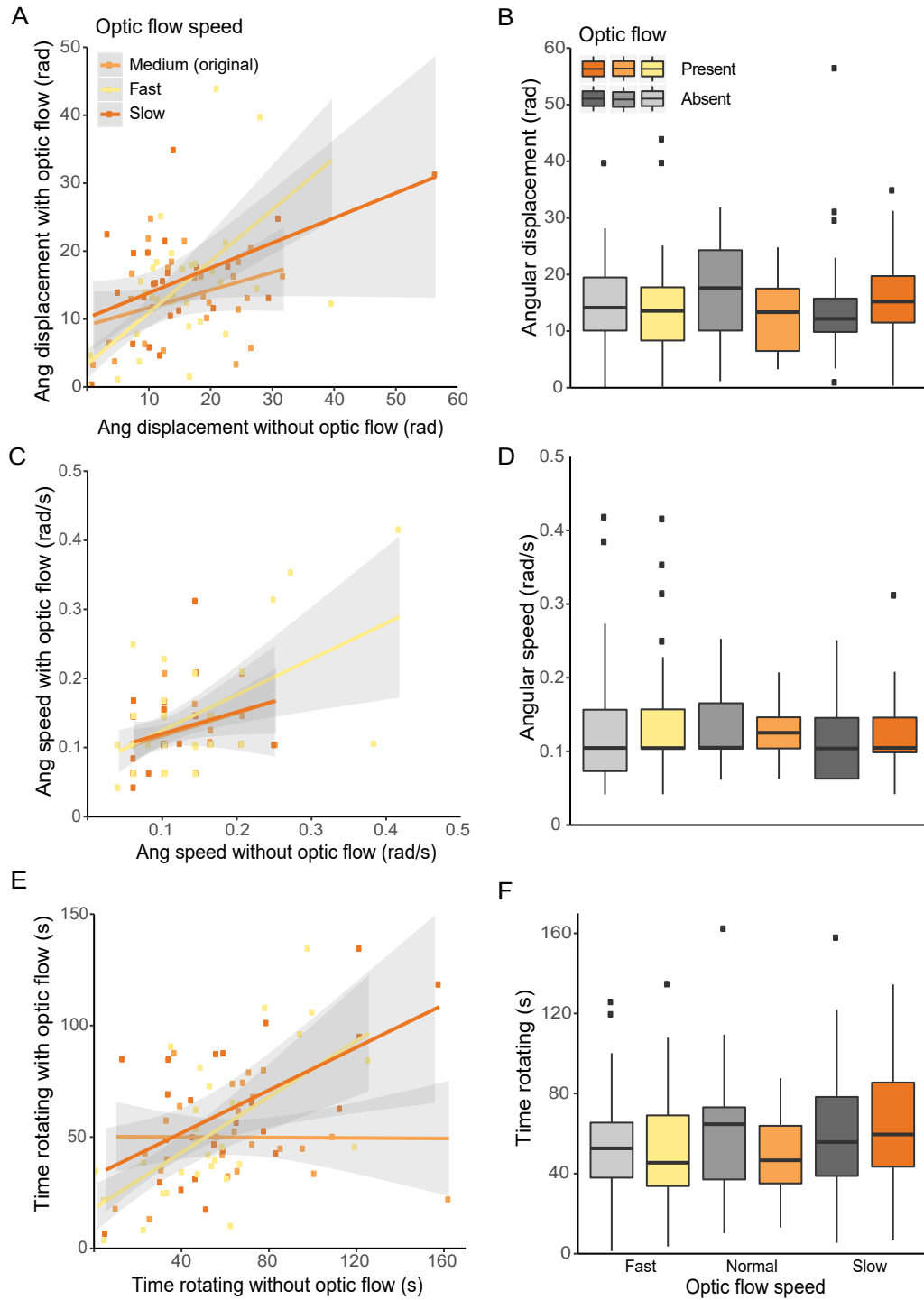
Figure 10: Rotating behaviour does not show significant differences between trials with or without self-induced optic flow, regardless of its speed. Angular displacement A) does not correlate between trials for normal and slow but it does for fast OF, although B) its median is not different between trials for any of the conditions. Median angular speed C) does not correlate between trials for medium and slow but it does for fast optic flow, although C) its median is not different between trials for any of the conditions. Time spent rotating E) does not correlate between trials for medium and slow but it does for fast optic flow, although F) its median is not different between trials for any of the conditions. Lines and grey shading represent the estimated regression lines and confidence intervals of the generalized linear model. The median values on trials in which optic flow was absent are represented on the gray box-plots and present on the orange/yellow box-plots (slow: dark colours; normal: intermediate colours; fast: light colours).

Open-loop optic flow largely increases distance and walking speed, but not time spent walking

We exposed an additional group of ants to the same virtual environment as above in which optic flow was present in both trials but either in closed-loop or in open-loop. To achieve this, ants experienced a first trial on an environment with the ground patterned by black dots in closed-loop so that their movement changed their position on the virtual world as in previous experiments. The visual output of this trial was saved and replayed to each ant when tethered on the ball for the second trial so that in this trial the ants' movements were recorded in open-loop and did not change the ants position on the virtual world.

Walking in an open-loop virtual environment did not disrupt the reliability of ants' walking behaviour. Indeed, all walking parameters were correlated between the closed- and an open-loop environments; ants that walked longer distances, at faster speeds and for longer time periods when able to generate optic flow in closed-loop also did so in open-loop conditions (figure 11; table 4). However, ants did walk overall longer distances and at faster speeds when in open- compared to closed-loop conditions, though no differences were observed in the time they spent walking (table 4). The replayed visual cues in open-loop consisted in external flow not only in a translational direction but also angular, which were not separated here. Interestingly, rotational behaviour showed a trend for longer distances, at faster speeds and longer times spent rotating in closed-loop, to which they were subjected on the first trial, compared to open-loop, to which they were subjected on the second trial (figure 12, table 4). Taken together, external optic flow led to significantly longer distances walked and faster speeds, although time spent walking was not affected. On the contrary, external flow led to ants rotating significantly shorter angular distances and at slower angular speeds, spending also less time rotating.

Discussion

We developed a virtual reality paradigm for harnessed wood ant workers, *Formica rufa*, showing that when they are restrained on top of an air supported ball they display walking
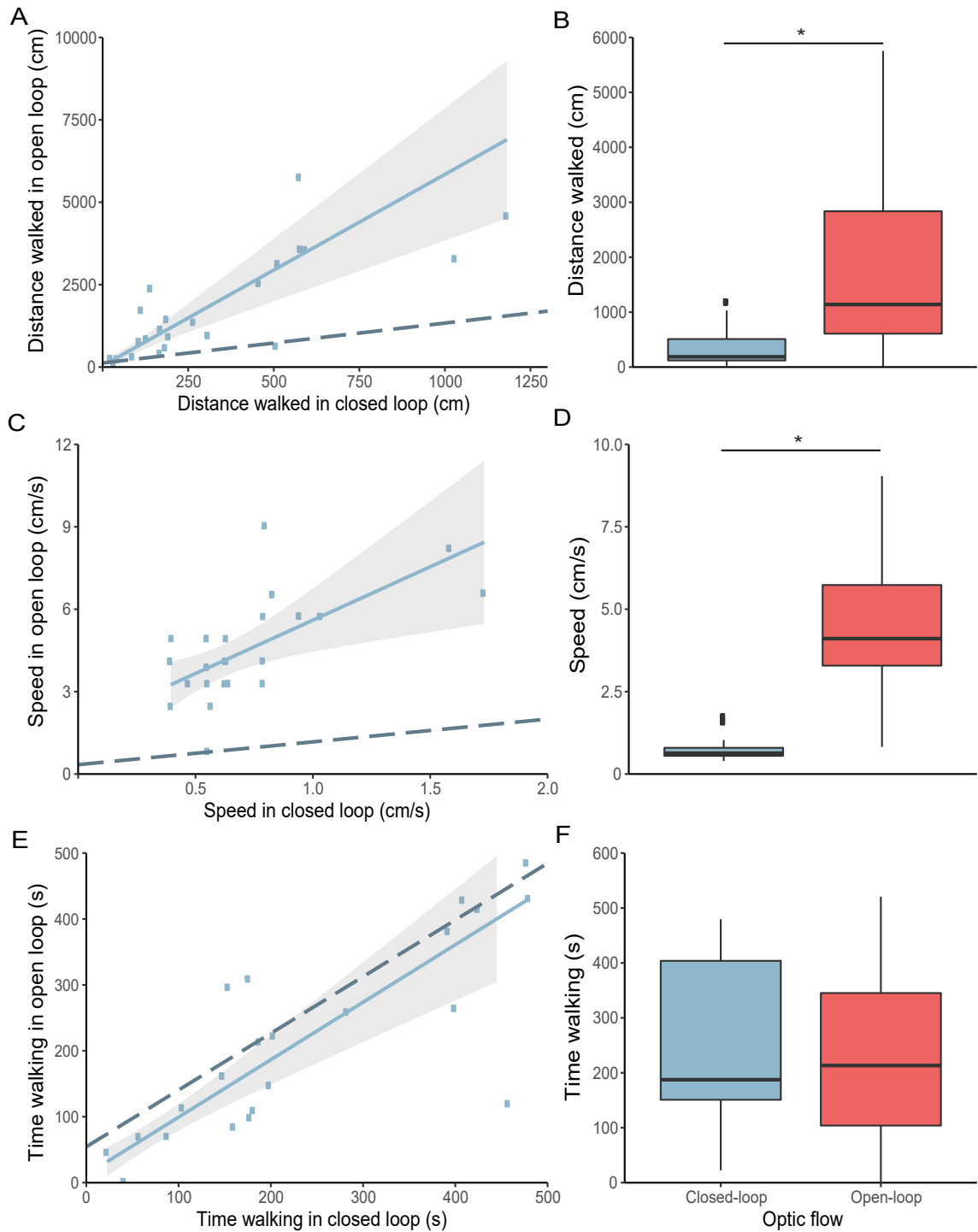
Figure 11: Open-loop optic flow significantly increases distance walked and median speed, but not time spent walking. A) The distance walked by ants that walk in two different environments, with closed-loop self-generated and open-loop external optic flow, correlates between the two environments. B) The median distance walked in open-loop is higher than in closed-loop. C) The median speed ants walk at correlates between the two environments and D) is higher in open-loop than in closed-loop. E) Time spent walking correlates between the two environments and F) is similar between open- and closed-loop. Lines and grey shading represent the estimated regression lines and confidence intervals of the generalized linear model. The regression line estimated for the ants that walked in two environments in closed-loop (figure 6) is represented by the dashed grey line. The median values on trials in which optic flow was external are represented on the red box-plots and self-generated on the blue box-plots.
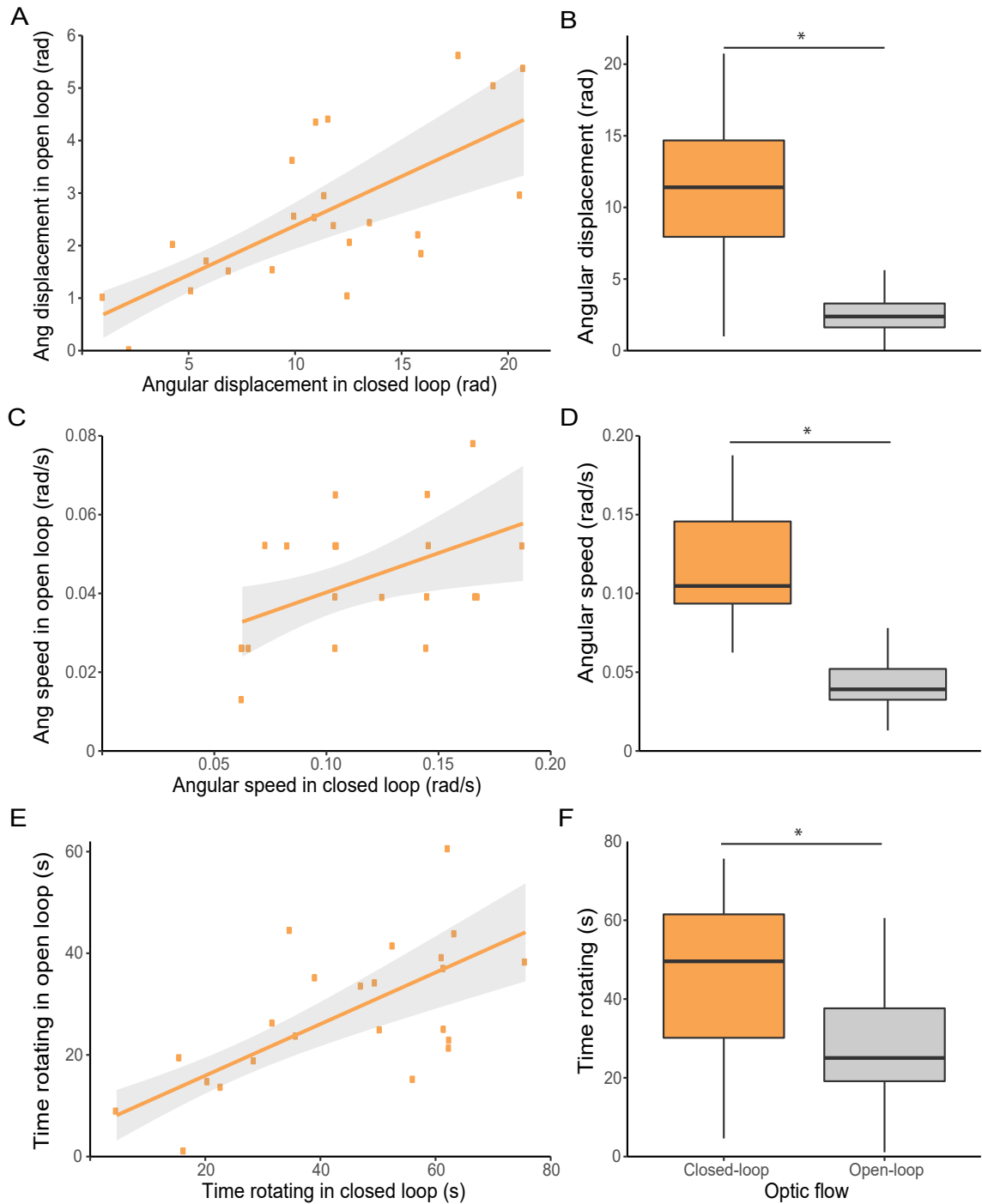
Figure 12: Ants rotate less in the presence of open-loop compared to self-induced optic flow. Angular displacement A) correlates between trials and B) is higher in the closed-loop condition. Median angular speed C) correlates between trials and D) is higher in the closed-loop condition. Time spent rotating E) correlates between trials and F) is higher in the closed-loop condition. Lines and grey shading represent the estimated regression lines and confidence intervals of the generalized linear model. The median values on trials in which optic flow was external are represented on the gray box-plots and self-generated on the orange box-plots.

Table 4: Translational walk and rotating behaviour for ants experiencing self-generated optic flow followed by an open-loop replay. Regression analysis (generalized linear models) of distance walked, median speed, time spent walking, angular displacement, angular speed and time rotating between a trial with open and closed-loop optic flow (N=23, df=21). The estimated slopes and intercepts, t-statistics and P-value are visible for each parameter analysed. The median values for each parameter are compared between the first and second trials, with W representing the Wilcoxon sign rank test and P the P-value (**P<0.01).

		Estimate	t	P		W	P
Distance	Slope	5.81	5.36	<0.01**		73	<0.01**
	Intercept	39.68	0.41	0.68			
Median Speed	Slope	3.89	2.91	<0.01**		5	<0.01**
	Intercept	1.71	1.94	0.06			
Time walking	Slope	0.89	7.34	<0.01**		283	0.7
	Intercept	11.32	0.91	0.37			
Angular Displacement	Slope	0.19	5.55	<0.01**		488	<0.01**
	Intercept	0.5	1.99	0.06			
Angular Speed	Slope	0.2	2.44	0.02*		518	<0.01**
	Intercept	0.02	2.25	0.035*			
Time rotating	Slope	0.51	5.58	<0.01**		396	<0.01**
	Intercept	5.79	2.04	0.06			

behaviour and interact with a virtual world in closed-loop. Using this paradigm, we show that smaller and lighter ants tend to walk faster and longer distances than their bigger and larger counterparts and that the distance ants walk correlates with both median velocity and time spent walking. We have also found that wood ants display individual consistent behaviour when placed twice in the virtual environment, walking similar distances, at equivalent speeds and repeatable amounts of time. We used this repeatability of behaviour to investigate in which cues wood ants relied on for integrating distance, speed and amount of time walking. This consistency in behaviour is present in the absence of visual cues. However, distance and time spent walking can be modified depending on the velocity of self-induced (closed-loop) optic flow when present, which is not true for median velocity. Conversely, median velocity and distance walked are increased to a much greater extent in the presence of external (open-loop) optic flow, while time walking remains constant. Furthermore, turning behaviour did not show the same consistency between trials and tended to elevated on the first trial compared to the second.

Validation of methodologies in the context of insect VR

Demonstrating that an ant is behaving naturally when restrained has only been achieved, to our knowledge, by Dahmen and colleagues (2017), who designed the trackball system for desert ants, *Cataglyphis fortis*, upon which ours is inspired. They showed that ants placed on a trackball at the position of a feeder reproduced a similar path on their homeward bound journey to the one they would produce if freely walking. We followed several of the considerations suggested by these authors, including the use of a light polystyrene ball, although not hollowed as they suggested, a metallic ball support that reduces friction, and a tether and holder combination that allows ants to self-adjust their height from the ground.

While we have used virtual reality, the behaviour investigated in Dahmen and collaborators' (2017) study was dependent upon the path integration vector ants stored on their outward journey and was not tested on a virtual world. For assessing an animals' interaction with a virtual world, one requires a clear behaviour that can be evaluated both in real and virtual arenas. For instance, cockroaches (*Periplaneta americana*) walking on top of an air supported trackball navigate around virtual trees, using visual information to avoid collisions, even though they do not receive mechanosensory information from the virtual objects they avoid (Takalo *et al.*, 2012). Another example is the case of honey bees, which can transfer behaviours they have learned in a real Y maze into a virtual reality system with visual cues displayed in the same manner (Buatois *et al.*, 2018). However, in this virtual reality set-up the honey bees rotational movements were in closed-loop with the visual display, whereas translational walking movements operated in open-loop and, therefore, bees were not able to truly navigate in an immersive virtual environment. Other attempts to compare behaviour between a virtual and a real arena have been made using fruit flies, which demonstrated that walking on top of a trackball produces some changes in walking behaviour (da Cruz, 2013). However, some of flies' walking behaviours do remain similar between real and virtual reality contexts, such as object fixating and approach, as well as walking speed (Haberkern *et al.*, 2019). In our system, ants were allowed to navigate around the virtual world both in translation and rotation. We did not

compare the ants walking behaviour in a real arena with equivalent visual cues to the ones we displayed in our virtual world directly. However, we used object approach and fixation, behaviours that wood ants perform while freely walking (Graham *et al.*, 2003) and are comparable between the virtual and real worlds in flies (Haberkern *et al.*, 2019), to demonstrate that they do, indeed, interact in a similar manner with our virtual object.

Multiple considerations are needed when developing virtual reality set-ups, namely how fast the animal's visual system integrates information, its visual field, how a designed 3D virtual world is represented in 2D and how changes in the virtual environment are changed by the animals' movement. Wood ants extract information of their visual surroundings using compound eyes with hundreds of facets (Perl and Niven, 2016). Although the properties of the wood ant visual system have not been described yet, inferences based on other ant species can be made. We took into consideration the characteristics of ant vision by displaying the visual world at around 120 Hz, above the frequency at which we assume they integrate visual information based on one study with other insect species (Miall, 1978). This avoided any side effects that may be caused by flickering of the visual world.

To our knowledge, the visual fields of wood ants have also not been described to date. However, Australian desert ants (*Melophorus bagoti*) are known to possess a horizontal field of view of around 300 degrees (Schwarz *et al.*, 2011), which is larger for species of the genus *Cataglyphis* (Zollikofer *et al.*, 1995). Wood ants, being slightly smaller and with smaller eyes but similar facial display (for comparisons see Perl and Niven, 2016; Schwarz *et al.*, 2011; Zollikofer *et al.*, 1995), may have similar (potentially smaller) horizontal field of view. We displayed our virtual world onto a cylindrical surface surrounding the ant horizontally almost 360 degrees, but the projection covered only 120 degrees. Although this does not fully cover the ants' horizontal visual field, the lateral posterior, posterior and dorsal sides of the eye not covered by the projection were covered by static cues (tracing paper, ant holder and camera, respectively), thus cancelling any external motion of the real world that could be perceived. Furthermore, we reduced visual motion cues from the ball by covering a portion of the ball with a custom made floor. However, taking

into account the blind region of *Cataglyphis* ants' eyes (Zollikofer *et al.*, 1995), wood ants were still able to perceive the motion of the ball below them with the ventral portion of their eye. Difficulties in creating a more immersive virtual world arose from using the software VIRMEn. Although different transformations of a 3D into a 2D virtual world were possible to achieve, they all produced some distortions such as slight looming on the extremities of the projection, which were worsened by increasing the field of view using two projectors or a projector placed above covering most of the cylinder wall, floor and ball. Thus, we reduced the ants' field of view to minimize the distortions caused by projecting onto a curved surface and the software itself. Confounding effects could arise from the mismatch between the motion of the virtual world and the motion of the ball, particularly when investigating the use of proprioceptive and optic flow cues to integrate distance, speed and time spent walking (discussed below), and from slight looming effects still present. Nevertheless, we did observe interactions with a virtual object and changes in walking behaviour due to changes in the virtual optic flow despite the faults in our set-up.

We also calibrated the ants' the visual feedback derived from their rotatory movements on the ball so that an ant rotating 360 degrees would observe a 360 degrees rotation of the world. Although the correspondence between virtual and real distance was not calibrated, it was calculated posteriorly and analysis was performed using the real distances ants walked. Effects of this on the perception of optic flow are discussed below. Furthermore, we recorded the trackball's movement using a single optical mouse, placed behind the ant aligned with the trackball's meridian and equatorial lines and with the ant's holder, as opposed to two orthogonal optical mice as used by Dahmen and colleagues (2017). Thus, ants translational movements forwards and backwards and rotational behaviour could be captured by our recording device, but translational movements sideways could not be recorded. A movement sideways is typically performed by some insect species to infer distance from an object based on the visual motion it produces, termed motion parallax (Collett, 1978; Zeil, 1993; Zeil *et al.*, 1996). This behaviour is not commonly seen in wood ants (Nicholson *et al.*, 1999) but they do similar saccade-like body turns when

approaching the edge of an object (Lent *et al.*, 2010), which could be misrepresented here. Thus, the use of two optical mice or the camera positioned above for closing the loop with the visual display, increasing precision of the movement readings, could be beneficial. Indeed, honey bees change their behaviour depending on which sensor is used for recording their movement on a trackball (Taylor *et al.*, 2015). Ants also had to be able to initiate turning, which led to slight differences in facing direction on top of the ball and, therefore, of the ball's movement when the ant intended to walk forward. Although this could generate distortions when the ant intended to approach an object or extract information about its self-generated optic flow, we still observed significant approach behaviour and perception of different velocities of self-induced optic flow.

Validation of methodologies in the context of distance, speed and time integration

Our virtual reality system yields multiple advantages for the study of optic flow in comparison with previous systems. Previous research using walking ants relied mainly on tunnels with patterned walls and floor and manipulated self-generated optic flow by changing the width of the channels (Ronacher *et al.*, 2000) or moving those patterns (Ronacher and Wehner, 1995). Although moving patterns do modify the self-induced optic flow perceived by the animal, they also impose an external optic flow, which could influence behaviour. Indeed, several species of flying and walking insects respond to external motion of the world by moving in the opposite direction. This phenomenon is termed the opto-motor response and it is thought to be related to compensations for external forces that could divert an animal from its intended route, thus producing motion of the visual environment that was not generated by the animal (reviewed in Srinivasan *et al.*, 1999). In our virtual reality set-up, we can fully manipulate self-generated optic flow, in closed-loop, to produce larger or smaller changes for each step, without imposing additional motion of the virtual world. We can also display external optic flow, in open-loop, to compare the effects of external and self-generated visual motion. Furthermore, this can be achieved with such precision that all ant's visual input is equivalent between an open-loop and a

closed-loop trial, varying only on being externally imposed or self-generated. Indeed, we showed that ants' responses to external or self-induced optic flow are fundamentally different (discussed below). Although we did not separate here rotatory and translational external visual motion of the world, our set-up also allows for such comparison. Lastly, our virtual world could contain a combination of external and self-induced visual motion, by allowing ants to navigate in closed-loop but adding an external constant in our movement function, either in the translational or angular motions, although this was not performed.

Experiments using free moving animals also pose disadvantages when analysing animals behaviour in detail. One could use a tracking device that extracts an animal's position on an arena, as used for many studies in wood ants (Lent *et al.*, 2013; Buehlmann *et al.*, 2016; Woodgate *et al.*, 2016), which allows for a precise representation of the distance animals walked, the speed at which they walked and the time they spent walking. However, to capture the whole animal's path, such recordings are typically too far from the animal to be able to capture behaviours in greater detail. To do this, video recordings need to be performed in highly restricted areas, thus showing the animal in detail but missing the overall path. This approach has been used, for instance, by Wittlinger *et al.* (2007), to further analyse the length of desert ants' strides when leg length was manipulated, showing that stride length varied. In our set-up, detailed recordings are possible using a high-speed video camera connected to a macro lens throughout the whole trial. This would permit detailed analysis of stride length at all time points and relationships between speed and distance with stride length, as well as how it is affected by manipulations of the self-generated or external optic flow.

Previous experiments on the investigation of distance estimation have made use of social insects and their ability to store distance information as they travel from their nest (e.g. Ronacher and Wehner, 1995; Ronacher *et al.*, 2000; Wittlinger *et al.*, 2006; Esch and Burns, 1995; Srinivasan *et al.*, 1997). The study of speed integration, however, makes use of a characteristic feature of several walking and flying insects, the ability to control velocity. These insects show consistent average velocity while travelling, which allows

for changes in animals' behaviour due to manipulations of the environment to be inferred at real time (e.g. Zanker and Collett, 1985; Zollikofer, 1994; Baird *et al.*, 2005; Baird and Dacke, 2012). In our system, we found that naïve wood ants not only show consistent median velocity when placed twice in the same virtual environment but they also walk repeatable distances and for similar amounts of time. Thus, distance, speed and time integration can be studied using wood ants naïve behaviour, without training to a specific location, which drastically reduces time and complexity of experiments and confounding effects generated by the formation of specific memories.

However, because in our virtual reality paradigm ants were likely to perceive ventral optic flow from the ball's movement, we could not establish a scenario where purely pro-prioceptive cues could be tested. To truly cancel ventral optic flow, the ventral portion of the eye could be painted, as performed in *Cataglyphis* desert ants by Wolf *et al.* (2018). Furthermore, using a gray trackball instead of white could reduce contrast created by any undesired cues such as dirt or the light patterns on the polystyrene material. Because we did not yet perform this and, therefore, do not know to which extent wood ants would change their behaviour depending on the amount of ventral OF they perceive, manipulations of the optic flow generated by the virtual floor which are not perceived by the ventral eye could lead to some confounding effects: they create a mismatch not only with proprioceptive cues but also the ventral OF from the ball. Projecting the virtual world not only on the cylinder walls but also on the custom made floor and ball could also override optic flow generated by the ball, allowing us to evaluate more precisely how OF manipulations change ant's walking behaviour. Nevertheless, we did observe differences between a normal and slow virtual optic flow, suggesting that the high contrast of the VR still overrides, at least partially, the ventral OF from the low contrast cues on the ball.

Finally, as mentioned above, ants were allowed to rotate slightly on top of the track-ball, which disrupted the alignment between their facing direction (and forwards motion) with the optical mouse recorder. This could compromise the separation between translational movement forwards and sideways, and generate distortions when investigating ants behaviour due changes in self-generated optic flow. The fact that there were no differ-

ences between walking in normal and fast self-induced optic flow could be related to the miscalibration of the movement function in respect to real distance walked and distance moved in the virtual world. One unit distance on the virtual world corresponded to 0.71 cm of the ball's meridian circumference. To calibrate distance, we could have used the average speed of a freely walking wood ant (Holt, 1955) and how many centimeters of the ball it would take for the ant to move in the corresponding displacement in the virtual world. Nevertheless, we did observe differences between ants that walked with a slow and normal self-induced optic flow, suggesting that they do perceive this changes despite this miscalibration.

Wood ants show repeatable naïve walking behaviour

In our experiments, wood ants showed individual repeatable behaviour. Ants that walked longer distances on the first trial tended to walk longer distances when placed again on the virtual world, while ants that walked shorter distances did so in both trials. This was also observed for median speed and time spent walking, with ants walking at consistent median velocities and for similar amounts of time on both trials.

Repeatability of individual behaviour is commonly found in many vertebrate species and is typically termed 'personality' (or behavioural syndrome) (reviewed in Gosling and John, 1999; Dall *et al.*, 2004). More recent studies have found that this feature is not exclusive to vertebrates; repeatable individual behaviour has been found in molluscs, crustaceans, cnidarians, spiders and insects (reviewed in Mather and Logue, 2013; Kralj-Fišer and Schuett, 2014). In social insects, studies on repeatable behaviour have focused primarily on colony level 'personalities' rather than in individual consistency (not discussed here, but see Wray and Seeley, 2011; Wray *et al.*, 2011; Modlmeier *et al.*, 2012; Pinter-Wollman *et al.*, 2012; Scharf *et al.*, 2012). However, individual consistent behaviour, distinct between individuals, could confer advantages by maintaining variability within colonies without the energetic constrains of every individual being able to switch between behavioural types (Jandt *et al.*, 2014). Indeed, individual repeatable behaviour has been found in some species of social insects. For instance, individual honey bees display a series of characteristic behaviours, including propensity to perform trophallaxis or to

attack another bee, that are repeated over time in similar manner, regardless of the context in which these behaviours are performed (Walton and Toth, 2016). Similarly, bumble bees differ between each other in their foraging strategies and how they respond to the threat of predation, but these behaviours are consistent within individuals over time (Muller *et al.*, 2012). Individual differences in bumble bees were also found in learning tasks, ability that is consistent across time and contexts (Muller, Chittka *et al.*, 2012). Although colony level consistency has been studied in ants (Modlmeier *et al.*, 2012; Pinter-Wollman *et al.*, 2012; Scharf *et al.*, 2012), to our knowledge individual repeatable behaviours have not been reported to this date. In our experiments we only tested walking behaviour twice, separated by 2 to 4 hours, and did not access other behaviours in different contexts. Thus, we cannot claim that wood ants have individual personalities. Nevertheless, we do provide the first observation of repeated behaviour in ants.

Smaller and lighter ants travel further, at higher speeds and for longer time periods

Studies focusing on correlations between body size and foraging distances in social insects have mainly observed animals freely walking in their natural habitat. Typically, smaller species tend to forage shorter distances than bigger species, which has been reported for bees (Gathmann, 2002) and ants (Davidson, 1997). The same trends are visible when investigating individuals within the same species, with larger individuals foraging further from their nests than their smaller counterparts (Rissing, 1984; Shutler, 1991; Wright *et al.*, 2000; Greenleaf *et al.*, 2007).

In the wood ants *Formica rufa*, the correlation between body size and distance has been studied previously by Wright *et al.* (2000). He found that bigger ants forage for aphid honeydew in trees that are further away from their nest and carry heavier loads on their return trips compared to smaller ants of the same colony. In our virtual world, however, we observed smaller and lighter ants walking longer distances. These differences could be due to the small variation of body sizes we've sampled because we selected mainly large ants (for body size comparison see Perl and Niven, 2016). However, we did

observe a strong negative correlation of both hind femur length and body weight with distance walked. Supposing this correlation would hold if selecting a vaster range of body sizes, one could hypothesize that these differences could be accounted for the task not being the same between the two studies; our ants were not necessarily foraging, while in Wright *et al.* (2000) ants were observed in specifically foraging contexts. Furthermore, two of the colonies we selected were kept indoors for several months prior to experiments, where they were fed *ad libitum* without the need to forage. Although they do not lose the ability to do so, as seen in studies with trained wood ants (e.g. Graham and Collett, 2002; Buehlmann *et al.*, 2016; Woodgate *et al.*, 2016), colonies might lose their size-based stratification of foraging behaviour.

Distance estimation strategies of wood ants are similar to the ones of desert ants

We made use of the wood ants' consistent walking behaviour to investigate which cues they relied on to integrate the distance they walked. Ants were able to walk consistent distances in the absence of any virtual visual cues that could present self-induced optic flow. However, ants could perceive the trackball with their ventral eye and it is possible that the low contrast dirt and patterns of the polystyrene material created ventral self-induced optic flow. If ventral optic flow from the ball is not perceived, these findings are similar to previously described in foraging desert ants, who estimated the distance they travelled from their nest correctly when optic flow was not available (Ronacher *et al.*, 2000). If similar mechanisms are used by both ant species, we can infer that wood ants, as desert ants, also rely on a proprioceptive cue (potentially a stride integrator), to estimate the distance they walk (Wittlinger *et al.*, 2006, 2007). Nevertheless, we cannot yet infer the use of proprioceptive cues in our experimental conditions.

To evaluate if optic flow could play a role in ants walking behaviour, we performed three manipulations of self-induced translational optic flow by changing the velocity at which each step forwards, sideways or backwards of the ant changed the visual world. We then allowed ants to navigate without optic flow or with one of the three self-induced

optic flow velocities. Ants that were subjected to a fast or normal optic flow walked similar distances compared to when relying on proprioceptive cues alone (or ventral self-induced OF from the ball). Conversely, ants that were subjected to a slower virtual optic flow walked longer distances compared to when it was not available. We hypothesise that wood ants use proprioceptive cues and possibly ventral optic flow, but that the contrast of the virtual world overrides the low contrast of the ball and perceiving motion of the virtual visual world slower (but not faster) than expected leads to an increase of distance walked. Similar behaviour has been found in desert ants, who do not need to experience optic flow for estimating distance but can use it when available (Ronacher and Wehner, 1995; Ronacher *et al.*, 2000), suggesting two separate integration processes that interact with each other depending on the context (Wolf *et al.*, 2018). Taken together, although previous studies on distance estimation were conducted with trained animals while ours relied on repeatable naïve behaviour, and although we could not test proprioceptive cues alone, our results are in accordance with previous observations in desert ants, with self-induced optic flow being integrated by wood ants to adjust their walking behaviour.

Distance walked correlates with both speed and time

Distance, speed and time are walking parameters intrinsically related to each other. In our experiments, the distance wood ants walked correlated with both distance and time. Furthermore, ants were also consistent on their median velocity and the time they spent walking across all our experiments. Thus, it is difficult to tell apart which components are being inferred by which type of cue. However, we did observe differences between speed and time estimation in the presence of self induced (closed-loop) and external (open-loop) optic flow. We found that slower self-induced optic flow does not disrupt the median speed ants walk at, but external (open-loop) optic flow does. Conversely, time walked is disrupted by slower self-induced optic flow while external flow does not change the time ants spend walking. These results suggest that time and speed integration depend upon fundamentally different mechanisms. Distance integration, on the other hand, was disrupted by both slow closed- and open-loop optic flow. Thus, the mechanisms for distance integration might differ depending on the context. When relying on a stride integrator or

self-induced optic flow, wood ants could integrate distance by estimating time or using similar mechanisms for integrating time and distance separately. However, if an external visual motion is imposed, they could increase the speed they walked at which would consequently change the distance they travelled, or estimate both separately using similar mechanisms.

Our results are fundamentally different from previous findings on foraging insects. All previous studies report that distance estimation was not dependent upon speed nor time spent travelling (Zollikofer, 1994; Srinivasan *et al.*, 1997; Barron *et al.*, 2005; Wittlinger *et al.*, 2006, 2007). This difference in findings could be a result of previous experiments focusing on foragers returning from a feeder to their nest, and thus expecting to walk a certain distance, while we focused on naïve walking with no prior expectation. On the other hand, studies focusing on speed integration also make use of a consistent behaviour shown by both walking (Zollikofer, 1994; Zanker and Collett, 1985) and flying insects (Heran and Lindauer, 1963; Baird *et al.*, 2010; David, 1982; Kern *et al.*, 2012), the ability to control velocity in order to keep it constant. Indeed, flies and bees use optic flow cues to integrate the speed they fly at (Heran and Lindauer, 1963; Baird *et al.*, 2010; David, 1982; Kern *et al.*, 2012). Similar observations have been made for walking honey bees (Schöne, 1996), but walking ladybirds seem to rely on different cues (Zanker and Collett, 1985). We hypothesise here that wood ants, similarly to lady bugs, do not use self-induced optic flow to integrate the velocity they walk at, even when available and reliable. On the contrary, they can use self-generated motion of the visual world to estimate the time they spend walking.

Rotating within a virtual world might be related to 'learning walk' behaviour

In our experiments, walking and rotating behaviour showed fundamental differences. Ants did not rotate consistent angular displacement, at repeatable median angular speed nor for equivalent time periods as observed for translational walking. Instead, they tended to display less rotatory behaviour when placed in the same environment for the second

time, regardless of optic flow being present or not. Wood ants, as other insects, display a characteristic walking pattern, with a heavy rotational component, when experiencing an environment they do not know or a change in a known environment (Nicholson *et al.*, 1999). This is commonly observed in other ant species (Graham *et al.*, 2010) and similar to learning flights in bees and wasps (Zeil *et al.*, 1996), typically in places from which visual characteristics need to be memorized, such as their nest or a feeding place, or when animals are displaced and need to find characteristics of the environment that they are familiar with to locate themselves again. We hypothesise that, when placed in a novel virtual world, wood ants do perform learning walk-type behaviour, rotating their body very often to observe the virtual world in every direction. Thus, when placed a second time in the same environment, this behaviour is less pronounced because the visual world is already familiar.

Interestingly, when ants were placed in two different virtual worlds, their rotatory behaviour was more consistent between the two trials, although not for all parameters in every optic flow speed. Furthermore, ants walking in closed- and open-loop, experiencing the same environment twice but controlling its motion or not, rotate less on the second trial compared to the first (further discussed below). This is in accordance with our hypothesis because ants, placed for a second time in a novel virtual world, would perform learning walks again to acquire information about their surroundings while ants that were again in the same environment would not. Thus, despite the consistency in walking behaviour and its dependence upon possible proptioceptive and optic flow cues, rotatory behaviour seems to be mostly dependent upon how familiar the visual environment is.

Walking wood ants show optomotor-like response

One group of our ants was subjected to a virtual world that moved in closed-loop with the ant's movement on the ball and then to a replay of the visual world they've experienced previously. Ants drastically changed their behaviour, walking faster and longer distances, when they could not control the motion of the visual environment. Similar behaviours have been observed in other species of insects, typically in flying insects (described in Srinivasan *et al.*, 1999), although it is also present in other phyla (e.g. Fite, 1968;

Wiersma, 1974; Maaswinkel and Li, 2003). Although some studies have also found similar responses in walking insects (Kaiser, 1974; Zanker and Collett, 1985; Lönnendonker and Scharstein, 1991), to our knowledge it has not yet been studied in ants.

In species where this behaviour was observed, animals tended to walk in the same direction of the visual motion and adjust the velocity at which they move to match the velocity of the external optic flow. This behaviour has been termed optomotor response, and is thought to be perceived by animals as a deviation from their path due to external forces, such as strong winds or currents (Srinivasan *et al.*, 1999). Although we have not investigated the if direction ants were walking on the ball while perceiving external optic flue matched the direction of the visual motion, we did show that they change their behaviour in response to this external optic flow. Wood ants usually travel on the ground in cluttered environments, but move upwards on trees to feed on aphid honeydew (Skinner, 1948). Here, they are likely to be affected by external wind forces that displace them or impede moving forwards. Therefore, we can hypothesise that wood ants naturally experience similar external forces to the ones of flying insects and that they use external motion of the visual world to adapt their walking behaviour. Comparisons with other walking insects that do not experience such environmental conditions would yield some insight onto the origins of optomotor response.

Nevertheless, we did not observe the same trends for rotating behaviour. Ants rotated at slower angular speeds and spent less time doing so, thus achieving lower angular displacements when they were in open-loop compared with closed-loop. We could have hypothesised that ants would respond to motion of the visual world rotating in one direction by rotating in the same direction to compensate for the observed motion, as observed for other species. In these species, both translational walking and rotating behaviour are used to compensate for external changes of the visual world. However, this is not what we observed, suggesting that optomotor-like response in ants is not necessarily equivalent as found in other species and rotating, as mentioned above, is linked to learning walks alone.

Conclusion

We have observed, for the first time, repeatable walking behaviour in wood ants, consistent with previous studies in insects reporting individual consistency in behaviour, typically termed 'personality'. Walking behaviour is dependent upon size, with smaller and lighter ants travelling further, at higher velocities and for longer amounts of time, opposite to previously observed. This suggests that distance walked is also dependent on the context or that colonies kept indoors loose size-based stratification of foraging. Nevertheless, they use similar mechanisms to integrate distance when compared to studies on desert ants, using mainly proptioceptive cues and, to a minor extent, self-induced optic flow. Contrary to previously observed, distance is correlated both with median speed and time spent walking, which are differently modified by external or self-generated optic flow, respectively. Because wood ants change their behaviour in the presence of external motion of the visual world, as observed in other species of insects, they also perform optomotor-like response. This response differs from the behavioural changes induced by self-induced optic flow. Consequently, the mechanisms for responding to closed- or open-loop motion of the world seem to be fundamentally different.

General Discussion

We intended to investigate the mechanisms of passive and active learning in an insect, the wood ant *Formica rufa*. To this end, we aimed to 1) develop a visual classical conditioning paradigm for wood ants and investigate if this species is able to form visual memories passively; 2) develop a virtual reality paradigm for wood ants to navigate in closed-loop or open-loop with their visual environment; and 3) develop a reward system to incorporate in our virtual reality paradigm to allow ants to associate a visual cue with a sugar reward through classical and operant conditioning, in a comparable form. Here, we discuss the contributions of the Thesis in terms of methodology and findings to answer our main questions and to the various related fields. We also discuss our findings in relation to previous studies addressing the neural pathways for learning and memory formation through classical conditioning and active/passive perception. Furthermore, we propose a model for active and passive learning within the wood ants' brain. Finally, we address open questions left by this Thesis and suggest future experiments that could answer these questions.

Contributions of the Thesis

Methodological contributions

In Chapter 2 we describe a novel protocol for studying visual classical conditioning in wood ants. This new methodology links previous research on visual learning using operant conditioning paradigms with free walking wood ants (e.g. Collett *et al.*, 2003; Harris *et al.*, 2005; Buehlmann *et al.*, 2016; Woodgate *et al.*, 2016) and classical conditioning paradigms designed for investigating olfactory learning in ants (Guerrieri and d'Ettorre,

2010), olfactory learning in bees (based on Bitterman *et al.*, 1983) and the few previously described visual learning classical conditioning studies in bees (Hori *et al.*, 2007; Niggebrügge *et al.*, 2009; Balamurali *et al.*, 2015). Thus, our first methodological aims were met in this chapter.

In Chapter 4 we describe a new virtual reality set-up in which wood ants can navigate in closed-loop within a virtual world. To achieve this goal, we combined methodological aspects of other experimental set-ups designed for other insect species, in particular Dahmen *et al.* (2017), with a virtual reality engine developed for studying navigation in rodents (Aronov, 2014). Using this approach, we show that wood ants respond and interact with a virtual environment while restrained on top of an air supported ball, performing similar approaching and fixating behaviour towards a virtual beacon as they do towards similar real objects (Graham *et al.*, 2003). This experimental set-up allows for a full control over the visual environment the animal is exposed to, as well as a precise and detailed analysis of the animal's behaviour that can be recorded at all times. This represents a significant advance in terms of the development of true virtual reality research in insects in several aspects. Previous research using similar set-ups often implemented experiments in open-loop (Hedwig and Poulet, 2005; Dahmen *et al.*, 2017), or in closed-loop solely for rotational visual motion (Paulk *et al.*, 2014, 2015; Buatois *et al.*, 2018). To our knowledge, only one system has been developed for cockroaches that show natural avoidance collision with virtual objects, in closed-loop with both translational and rotational motion (Takalo *et al.*, 2012). Here we developed a set-up where an insect model for visual learning and navigation, the wood ant, can walk and interact with a virtual world while restrained, in closed-loop in both translation and rotation motion. Thus, this chapter addresses our second methodological aim.

Although our third methodological aim was not met due to difficulties encountered while constructing and testing the virtual reality paradigm and due to time constrains, we made other methodological contributions that had not been envisaged at the outset of the project. In Chapter 3, we adapted the classical conditioning protocol described in Chapter 2 for studying the lateralisation of visual memory formation. We also show that our

methodology allows for evaluating memory formation at different time points, covering short- (10 minutes), medium- (1 hour) and long-term memory (24 hours). Furthermore, in Chapter 4 we show that wood ants display consistent walking behaviour. Because studying insect navigation typically requires a training session that can vary in time length from hours to days (e.g. Collett *et al.*, 2003; Harris *et al.*, 2005; Buehlmann *et al.*, 2016; Woodgate *et al.*, 2016), the use of a consistent naïve behaviour reduces the time necessary for addressing particular questions that do not require a learnt component. Moreover, it allows for behaviours to be analysed free of specific memories, which could introduce confounding effects.

Contributions to the study of passive and active learning

Comparing operant and classical conditioning requires an animal model that can learn in both contexts. Wood ants *Formica rufa* are known for their ability to associate visual landmarks with food sources (Harris *et al.*, 2005) or to directions en route to a feeder or their nest (Collett *et al.*, 2003), when walking freely. These studies show that wood ants can form associative memories in operant conditioning paradigms. However, at the outset of this project it had not been shown that they can form visual associative memories in a classical conditioning paradigm. In Chapter 2, we show for the first time that wood ants can form visual memories through classical conditioning while restrained, which answers our first experimental aim. The differences in methodologies mean that direct comparison of memory formation in these two contexts is not possible. However, we suggest that wood ants can be used as an insect model for the study of passive and active memories. Honey bees (*Apis melifera*) and fruit flies (*Drosophila melanogaster*) are also good models for the study of active and passive learning. Although fruit flies are not known for particularly advanced learning abilities within the insects, they were the insect model used for the only study to date addressing this question (Brembs and Heisenberg, 2000). Experiments with honey bees (Buatois *et al.*, 2017, 2018) have also addressed related questions and they are known for their ability to learn through operant and classical conditioning (Srinivasan, 2010). Furthermore, *Schistocerca gregaria* locusts (Simões *et al.*, 2011) and *Camponotus* spp. ants (Dupuy *et al.*, 2006; Guerrieri and d'Ettorre, 2010) can

also learn in both contexts. Thus, the work presented in this thesis expands the number of insect species in which this comparison can be made, increasing the possibility of understanding the mechanisms of passive and active learning in species that occupy different ecological niches and, potentially, the mechanisms by which different learning mechanisms evolved.

In Chapter 4, we show that wood ants can also be used for experiments within a virtual reality set-up. In this set-up, they can navigate in closed-loop with their environment but the visual display can also be externally manipulated. We validated this set-up by demonstrating that ants do interact with the virtual world by approaching and fixating on a visual landmark, which answers our second experimental aim. Although we were unable to compare wood ants learning abilities through operant (closed-loop) and classical (open-loop) conditioning, we were able to compare walking behaviour in closed- and open-loop. Thus, our findings in regards to visual classical conditioning and active/passive interactions with the visual world while walking allowed us to discuss and hypothesise the possible neural mechanisms by which each form of learning could occur (see below).

Additional contributions to the fields of insect learning, lateralisation and navigation

In Chapter 3, we made use of our classical conditioning paradigm to explore the lateralisation of visual associative learning in wood ants. We showed that visual memories created through classical conditioning in wood ants are lateralised, as has been observed for olfactory memories in eusocial bees (Anfora *et al.*, 2010, 2011; Frasnelli *et al.*, 2011). In our experiments, lateralisation of visual memories was observed despite no manipulations of the ants' visual system. Instead, the ants viewed the conditioned stimulus with both eyes and were rewarded with a sucrose drop that touched one antenna or the other before being presented to the mouth parts. Ants that received a reinforcing touch on the right antenna recalled the visual memory 10 minutes and 1 hour after training but not 24 hours later, whereas ants that were trained with a reinforcing touch on the left antenna only recalled the visual memory after 24 hours. Thus, short- and medium-term memories

are lateralised to the right while long-term memories are lateralised to the left, as is the case with olfactory memories in eusocial bees. This expands the knowledge within the field of insect learning by demonstrating the lateralisation of memory formation in both a novel modality and in a new species of eusocial insects. Therefore, with this piece of research, we also contribute to the hypothesis that lateralisation within neural circuits might arise in parallel with eusociality within the Hymenoptera. These findings also add to our knowledge of lateralised behaviour in wood ants, which also show asymmetries in the context of gap crossing (Bell, 2017) and trophallaxis (Frasnelli *et al.*, 2012), extending the repertoire of lateralised behaviours in a single insect species (reviewed in Frasnelli, 2013; Niven and Bell, 2018).

In Chapter 4, we make use of our virtual reality paradigm and the consistency in wood ant walking behaviour to explore distance, speed and time integration in insects. We have shown that wood ants, like desert ants (Ronacher and Wehner, 1995; Ronacher *et al.*, 2000; Wittlinger *et al.*, 2007), can use self-generated visual motion to estimate the distance they walk, when available and reliable. Furthermore, this provides a specific expectation for walking distance without prior training. This extends the knowledge on the field of insect navigation and path integration by confirming that previous findings obtained from work on desert ants are also applicable to another ant species, and that this ability is not restricted to trained homing ants.

In this chapter, we also investigated the ants' ability to estimate median velocity and time spent walking. Contrary to previous findings (Zollikofer, 1994; Wittlinger *et al.*, 2006, 2007; Srinivasan *et al.*, 1997), we show that the distance naïve wood ants walk in the virtual world correlates both with speed and time. Although both these walking parameters can be estimated without the presence of optic flow, when optic flow is present and reliable it can also be used for integrating the time spent walking. Thus, this chapter also contributes to the field of insect navigation by showing that: 1) walking wood ants can estimate walking time, which has not been shown before in any other foraging insect; 2) distance estimation is dependent upon time integration or these two parameters rely on similar underlying mechanisms; and 3) wood ants do not rely on self-induced optic flow

to estimate the velocity at which they walk like ladybirds (Zanker and Collett, 1985) but not bees (Baird *et al.*, 2005, 2010; Kern *et al.*, 2012) or flies (David, 1982).

Finally, Chapter 4 also addressed changes in wood ant walking behaviour due to external motion of the visual world that is not generated by the ant's movement. We showed that wood ants walk longer distances and at faster speeds when experiencing external optic flow, which could be interpreted as an optomotor-like response. However, we did not observe differences in the time ants spent walking. These findings make additional contributions to the field of insect navigation by: 1) showing that optomotor-like responses, previously observed in other species of walking (Kaiser, 1974; Lönnendonker and Scharstein, 1991) and flying insects (Srinivasan *et al.*, 1999), are also present in a species of ant; and 2) revealing that the mechanisms for speed and time integration can be fundamentally different.

A model for the neural mechanisms underlying passive and active learning in wood ants

The insect nervous system is decentralized consisting of the brain, located within the head, and a series of nerve cord ganglia, located in the thorax and abdomen, connected to each other via connectives (figure 1). The brain is divided in two ganglia, the supra- and the subeaophageal ganglion, which is situated between the supraesophageal ganglion and the nerve cord. The supraesophageal ganglion is subdivided into the protocerebrum (including the optic lobes, mushroom bodies, central complex, lateral protocerebrum and lateral accessory lobes), deutocerebrum (including the antennal lobes and antennal mechanosensory and motor centers) and tritocerebrum (reviewed in Wessnitzer and Webb, 2006; Ito *et al.*, 2014).

In the subsequent section, we will use some of the above nomenclature in discussing the neural basis of visual learning and antennal projections to central areas of the brain based on previous studies in other insect species. We will relate these to our findings from Chapters 2 and 3, where we investigated visual associative learning through classical
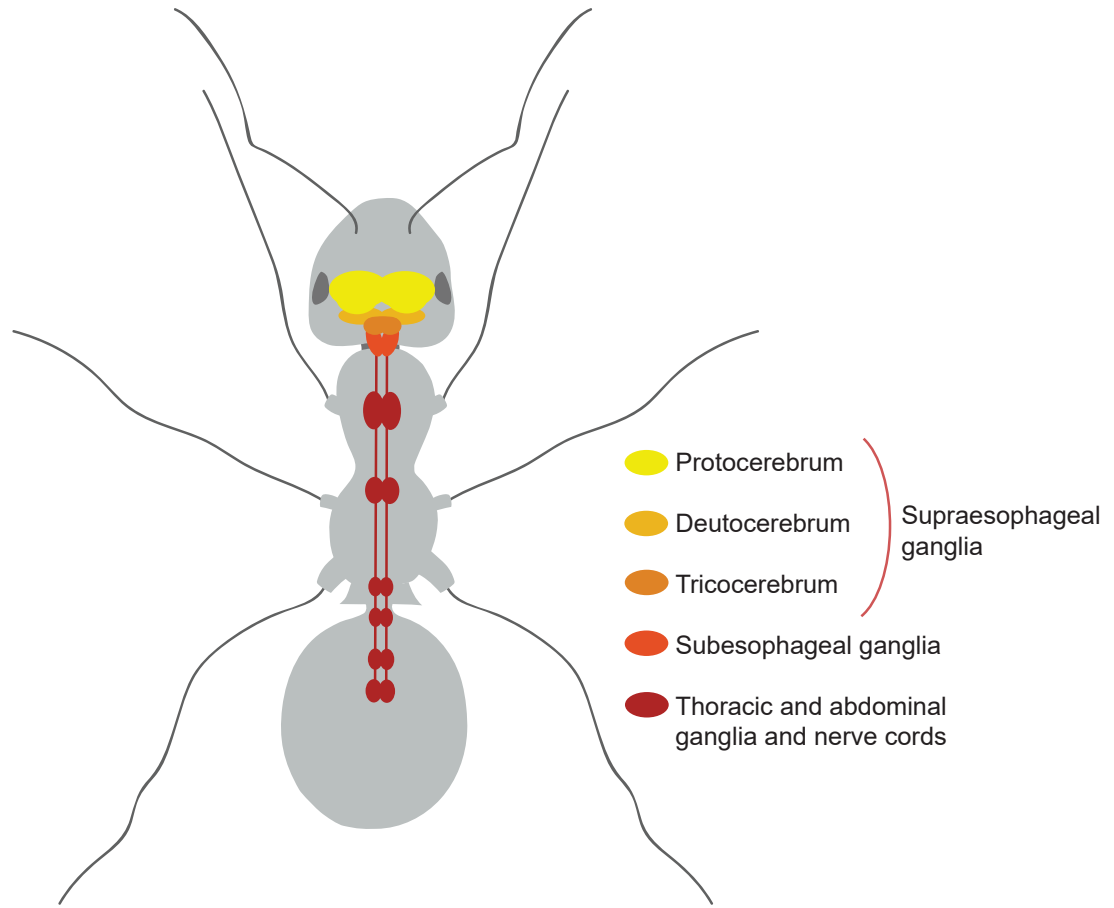
Figure 1: Schematic diagram of a wood ant nervous system. The supraesophageal ganglia is composed by the protocerebrum (yellow), deutocerebrum (light orange) and tritocerebrum (dark orange) and is connected to the subesophageal ganglia (light red). Nerve cords connect the brain to the thoracic and abdominal ganglia (dark red). Figure adapted from Wessnitzer and Webb (2006) and Choi and Vander Meer (2012).

conditioning and lateralisation of short-, medium- and long-term visual memories due to asymmetrical antennal input. We also discuss the candidate regions for integrating internal and external signals and to drive the motor behaviours we have investigated in ants walking in our virtual world. Finally, we propose a model for the neural mechanisms underpinning active and passive learning.

Candidate neural circuits underlying passive visual learning in wood ants

Visual information is sensed primarily by insects' compound eyes through the photoreceptors and transmitted to the optic lobes (OL). In the Hymenoptera, the optic lobe is formed of three primary layers, the lamina, proximate to the photoreceptors and receiving input directly from them, the medulla that receives the majority of its inputs from the lam-

ina, and the lobula (reviewed in Ito *et al.*, 2014). In flying insects such as bees and flies, the optic lobes occupy a large proportion of the insect brain. In flightless species, such as ants, the optic lobes are relatively much smaller (Gronenberg and López-Riquelme, 2004).

Both the medulla and lobula send projections to more central regions of the brain, including the mushroom bodies (MBs). The MBs are formed by Kenyon cells that receive inputs at the level of the MB calyces, subdivided into the lip, collar and basal ring, and project to the output regions of the MB, the pedunculus and the lobes (reviewed in Ito *et al.*, 2014). In bees, axons from the medulla and lobula project specifically to the outer ring track around the pedunculus-calyx transition of the ipsilateral MB (Mobbs, 1982; Gronenberg, 1986). Additional projections have been found in bees, connecting neurons from the medulla mainly to the collar region of the ipsilateral MB, with some restricted projections to the ipsilateral basal ring, and from the lobula neurons more predominantly to the basal ring, with some restricted connections with the collar region. These projections also show some overlap with each other (Ehmer and Gronenberg, 2002). In ants, the collar region of the calyces also receives input from both medulla and lobula, and connections between the optic lobes and the basal ring have also been discovered (Gronenberg and López-Riquelme, 2004). Inputs from the OLs to the MBs were subsequently found in other insects, including bumble bees (Ehmer and Gronenberg, 2002), beetles (Lin and Strausfeld, 2012) and cockroaches (Nishino *et al.*, 2012). In fruit flies, Vogt and collaborators (2016) observed two types of visual projection neurons (vPNs) directly connecting the OL and MB. These two tracts were differently required to form visual memories of colour or brightness, which showed independent processing of distinct visual memories (Vogt *et al.*, 2016). Furthermore, the MBs' role in the formation of olfactory memories has been well described in several insect species, especially honey bees (Menzel, 2001) and fruit flies (Davis, 2005), supporting the idea these neuropils could be involved in multimodal learning and memory formation.

The central complex (CX) is another brain region that receives input from the optic lobes. It is divided in four neuropils, the protocerebral bridge (the main input region),

the fan-shape body, the elipsoid body (the output region) and the noduli, connected to each other (figure 2; Loesel *et al.*, 2002; Ito *et al.*, 2014). Neurons of the central complex are known for being sensitive to light polarization, which has been observed in several species including locusts (Vitzthum *et al.*, 2002) and dung beetles (Dacke and el Jundi, 2018). In honey bees, neurons form the medulla involved in coding colour or movement project to the CX (Hertel and Maronde, 1987). In fruit flies, visual short-term memory traces of elevation of the panorama and contour orientation were found in the fan-shape body following aversive classical and operant conditioning in a virtual reality paradigm in open- or closed-loop, respectively (Liu *et al.*, 2006). Furthermore, asymmetries within the flies' fan-shape body, namely at the level of the asymmetrical body neuropil (typically found on the right hemisphere; Wolff and Rubin, 2018), are thought to be necessary for the formation of long- but not short-term olfactory memories through aversive classical conditioning (Pascual *et al.*, 2004).

Inputs from the OLs are also received by the lateral protocerebrum of honey bees (Maronde, 1991) and bumble bees (Paulk *et al.*, 2009). Furthermore, the lateral protocerebrum also shares connections with the MBs and is part of the olfactory pathway, particularly at the level of the lateral horn, although it does not seem to be necessary for olfactory memory formation (Hammer and Menzel, 1998).

Because of the role of the antenna in our visual classical conditioning experiments, it is important to consider where mechanosensory and gustatory inputs from the antenna project within the insect brain. Mechanosensory antennal inputs are not well known but have been described in some insect species (reviewed in Homberg *et al.*, 1989), particularly in the cockroach, where they were found in the antennal lobes (ALs) and antennal mechanosensory and motor centers (AMMC; Zeiner and Tichy, 1998; Burdohan and Comer, 1996; Farris, 2008). Projections from the AMMC to the lateral protocerebrum have also been found (Farris, 2008). Likewise, antennal gustatory pathways in insects are not fully described yet, although some projections to the subesophageal ganglion and tricocerebrum have been identified (Ignell and Hansson, 2005; Jørgensen *et al.*, 2006), as well as indirectly to the ALs (Ignell *et al.*, 2005). Antennal projections involved in

the processing of odours and olfactory memory formation have, however, been well de-
scribed. Olfactory neurons from the antenna project to the ipsilateral ALs. The ALs
are composed of numerous glomeruli that send projections to the ipsilateral MBs, at the
level of the calyxes. Olfactory projections target the lip region of the calyxes rather than
the collar where the OLs project to, but the basal ring neuropil of honey bees and ants
receives inputs from both sensory modalities (Gronenberg and López-Riquelme, 2004).
Convergence between the olfactory and visual pathways has also been described in other
regions of the honey bee brain, including the lateral protocerebrum (Maronde, 1991).
Thus, although this concerns neurons that are responsible for processing olfactory rather
than touch and taste information, there may be similar regions where gustatory and/or
mechanosensory antennal inputs converge with visual inputs within central areas of the
insects' brain.

When considering learning through appetitive classical conditioning, as performed in
our experiments, it is also necessary to understand the reward neural pathway. In olfac-
tory learning, the processing of appetitive US information is thought to be mediated by
a group of neurons located in the subesophageal ganglion (SOG). Indeed, excitation of a
single honey bee neuron located at the SOG, the ventral unpaired median x1 (VUMx1),
is sufficient to substitute the presentation of the US inducing memory formation when
paired with the presentation of the olfactory CS (Hammer, 1993). Similar VUM neurons
and their involvement in the reward pathway have been described in other species, par-
ticularly in fruit flies, and are known to innervate the ALs, MBs and lateral horn (of the
lateral protocerebrum) (Hammer, 1993) but not the central complex (reviewed in Perry
and Barron, 2013). Although experiments addressing the reward pathway have typically
focused on olfactory rather than visual classical conditioning and have not been investi-
gated in ants, it is reasonable to assume that food reward would be similarly integrated
in the ant brain through VUM-like SOG neurons. Thus, although the central complex is
involved in the processing of visual information and formation of aversive olfactory mem-
ories, it may not be directly involved in the formation of an associative memory between
a visual stimulus and a food reward through classical conditioning.
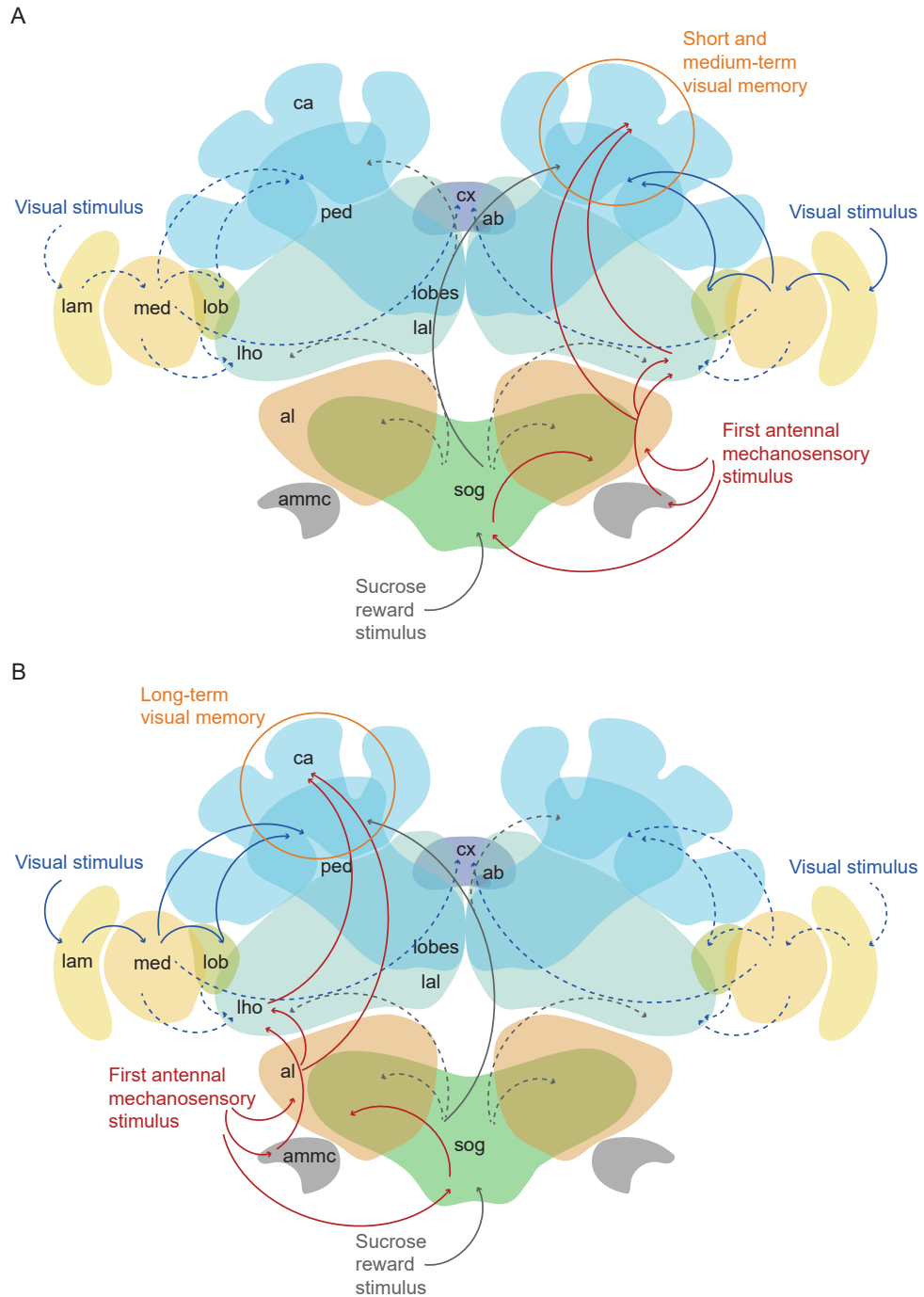
Figure 2: Schematic diagram of the neural pathways underlying the formation of an associative memory between a visual cue and a sugar reward through classical conditioning with lateralised antennal reinforcement. The visual pathway (blue lines) includes the optic lobes, constituted of lamina (lam), medula (med) and lobula (lob), the calyces (ca) of the mushroom body, the lateral horn (lho) of the lateral protocerebrum, and the central complex (cx). The antennal mechanosensory and gustatory pathways (red lines) include the antennal mechanocensory and motor centre (ammc), the antennal lobe (al), lateral protocerebrum, and calyces. The reward pathway (gray lines) includes the subeasophageal ganglia (sog), the antennal lobes, lateral protocerebrum and mushroom bodies (adapted from Yamagata *et al.*, 2007; Ito *et al.*, 2014). The circuits most likely to be included in the formation of A) short- and medium-term and B) long-term memories are represented in full lines. Dashed lines represent circuits that could be also present but not directly involved in this memory formation. Further crosstalk between neuropils is also likely to exist (not represented). Other neuropils are also visible, such as the asymmetrical body of the central complex (ab), the pedunculus output region of the mushroom bodies (ped) and the lateral accessory lobes (lal).

Based on our results and previously published studies, we propose a model of visual memory formation in the wood ant brain, reinforced by lateralised antennal contact with the sugar reward. Ants receive visual information about the CS through both compound eyes. This information is transmitted via the small OLs to central brain regions, including the MBs, central complex and lateral protocerebrum. The reward value of the food is acquired during feeding and processed by VUM-like neurons of the SOG, and transmitted to ALs, MBs and lateral protocerebrum neuropils. In our model, both visual and food reward information are paired in the MBs. However, short- and medium-memories are formed in the right hemisphere, while long-term memory is formed on the left hemisphere, which could be advantageous, particularly in relatively small brains, for reducing redundancy within the two brain hemispheres (Niven and Laughlin, 2008; Burns *et al.*, 2010; Niven and Farris, 2012). At the same time, a lateralised reinforcement is applied: the first contact with sugar while visualizing the CS is made either with one or the other antenna. The gustatory inputs from the antennae are conveyed in the tricocerebrum and SOG and, posteriorly, in the ALs. The mechanosensory antennal pathway also involves the ALs and AMMC and is paired with the visual pathway at the level of the MBs and the lateral protocerebrum. As described above, the lateral protocerebrum is also involved in the olfactory pathway but not involved in memory formation. Furthermore, the CX also receives input from the visual pathway and it seems to be involved in aversive olfactory learning, but the SOG reward neurons do not enervate it. Thus, we will focus specifically on the MBs hereafter. The gustatory and mechanosensory antennal signals on the MBs arrive first in one hemisphere then the other depending on which antenna is stimulated first. The timing of this association is, therefore, critical to the pairing of the reward with the visual pathways in the MBs: the first MB that receives antennal input becomes sensitized to the inputs from the SOG while inhibiting SOG projections on the other MB. Consequently, memory is formed within the ipsilateral brain hemisphere to the touched antenna and inhibited in the contralateral, each responsible for memory formation at different time points (figure 2).

Candidate sensorimotor integration centers during walking in wood ants

How animals perceive their surroundings and how that drives behaviour as been subject of debate for several decades. Experimental studies, such as our classical conditioning experiments discussed above, typically address perception as an open-loop system, in which information from the environment is captured by sensory organs and transmitted to the brain (exafference) to produce a behavioural response (efference). However, such approach neglects the changes produced in the environment by the animal's own behaviour, which are also perceived (reafference) and need to be distinguished (efference copy) from external events (von Holst and Mittelstaedt, 1971). Thus, to truly understand perception and animal behaviour, they need to be viewed as a closed-loop process (Powers, 1973; Beer, 2000; Ahissar and Assa, 2016).

For animals to behave, their nervous system depends upon sensorimotor integration centers that can integrate the exafference, reafference and efference copy signals and drive (efference) motor commands. Exafference and reafference signals can be visual, acoustic, olfactory, amongst other exteroceptive stimuli, or proprioceptive (the perception of self movement and position). In turn, the efference copy is idealised as an exact copy, sent from the central movement command generators, of what reafference stimuli the motor output should produce (the expected outcome), which inhibits the sensory pathway from responding to them. Hereafter, the efference copy is described in similar manner, although how it is combined with the reafference signals has been subject of debate (Feldman, 2016; Buckley and Toyoizumi, 2018). A classical example of sensorimotor integration in insects comes from the cricket auditory system. The cricket's thoracic neurons involved in song production (efference) send a copy of the motor commands to other regions of the nervous system that suppress auditory recognition (efference copy), allowing the cricket to respond to external acoustic signals (exafference), such as other individuals' songs, but not their own (reafference) (Poulet and Hedwig, 2003). Sensorimotor integration is also necessary for numerous insect behaviours described in Chapter 4, including locomotion-based behaviours (reviewed in Webb, 2004; Wessnitzer and Webb, 2006; Huston and

Jayaraman, 2011).

In Chapter 4, we studied the wood ants' locomotion behaviour in a virtual world. Although ants were likely to perceive ventral optic flow from the trackball below them, for the purpose of discussing their walking behaviour we will consider hereafter that this was not available and, when virtual OF was not present, they were relying on proprioceptive cues alone. Wood ants showed consistent naïve walking behaviour when placed twice in the same environment, even in the absence of visual cues from the virtual world. They walked similar distances, at consistent speeds and for equivalent amounts of time in both trials, which suggests they have an intrinsic way of integrating these walking parameters, an expectation for them and the ways to control the motor commands necessary to achieve it. Thus, pathways necessary for perception/estimation, expectation and motor control might not always be clearly distinguishable here. Supposing wood ants possess a stride integrator, similar to that described for desert ants (Wittlinger *et al.*, 2006, 2007), estimating distance, speed and time spent walking would consist of a simple closed-loop system between their brain, the motor output (efference), the proprioceptive (reafference) signals and the expectation of what they would be (efference copy). In this simple scenario, ants do not need feedback from their external surrounding to perform this computation (figure 3A). For ants that do have visual information to account for, this system still operates in closed-loop: the brain produces motor commands (efference) that change the visual world (exteroceptive signals) and the proprioceptive signals, which are in turn perceived by the brain (reafference). At the same time, both exteroceptive and proprioceptive sensory pathways are combined with the efference copies of the expected visual and proprioceptive signals. Thus, although the visual information is unnecessary for estimating these walking parameters, it still matches the efference copy information and ants perceive the visual motion (optic flow) of the world as self-induced (figure 3B).

To further investigate how these two interconnected loops coexist, we placed ants in two different environments, with and without optic flow. Furthermore, we divided them into three groups, one in which the perceived self-induced optic flow moved at a normal speed, a second in which it moved 3 times slower, and a third in which it moved 3 times
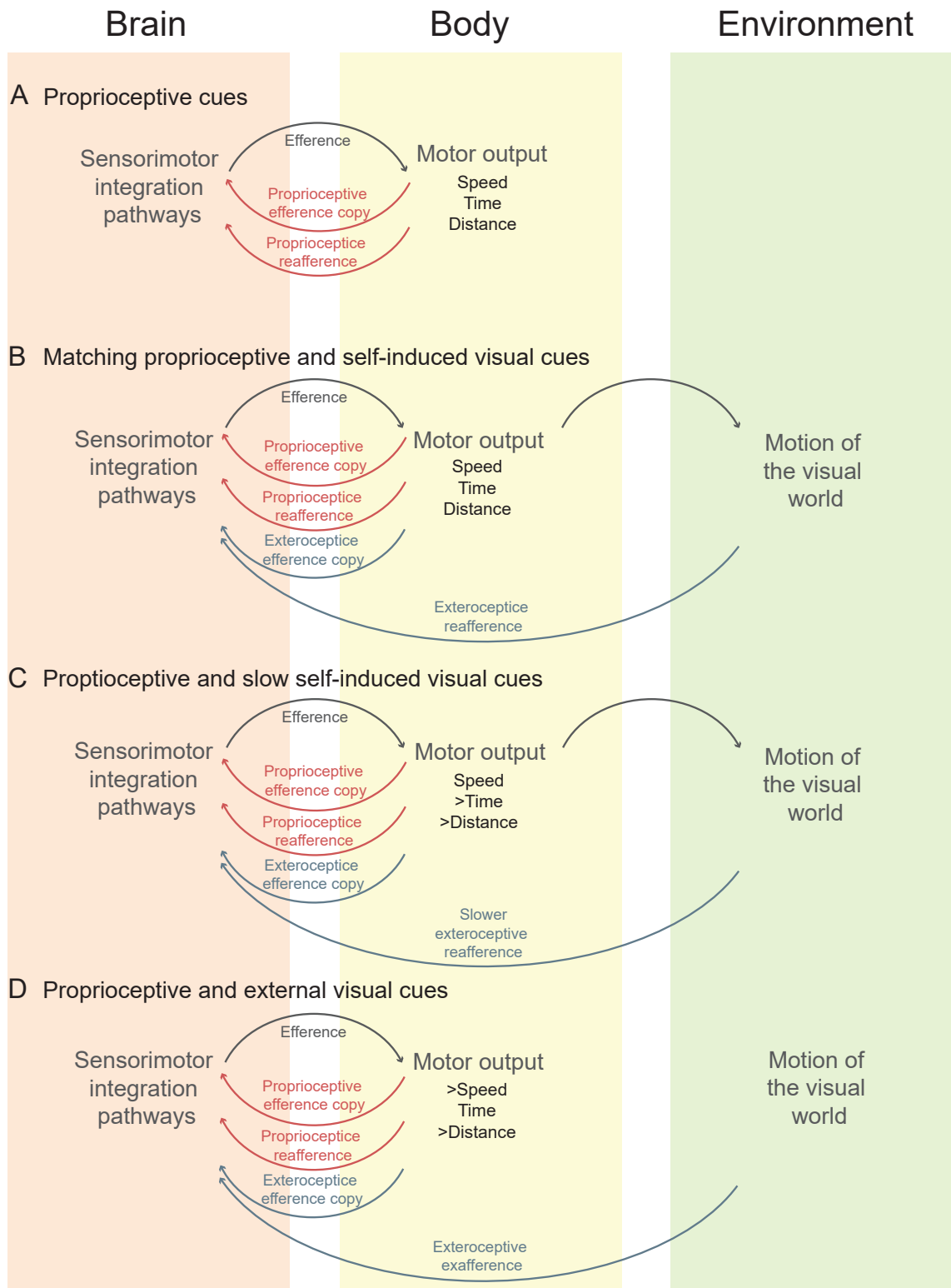
Figure 3: Schematic diagram of closed- and open-loop interactions between brain, body and environment of walking wood ants. Distance, speed and time integration and motor control A) in the absence of visual cues, B) in the presence of self-induced visual motion matching or C) slower than the proprioceptive cues and D) in the presence of external visual cues, in open-loop with self-motion.

faster. At normal optic flow speeds, ants behaved similarly between the two trials, suggesting that were still relying on the proprioceptive reafference signals and the respective the efference copy, although they could integrate the self-induced optic flow cues in one of the trials (figure 3B). However, in the two other groups, although the reafference proprioceptice signals match the expected (efference copy compared to proprioceptive sensory pathways), there was a mismatch between the perceived motion (reafference exteroceptive signals) of the world and its expected motion (efference copy compared to exteroceptive sensory pathways). Note that, although there was a mismatch in the expected and perceived velocity of motion of the world, the direction of the intended movement, walking and resting phases, as well as acceleration and deceleration phases were still the same between the two signals and, therefore, the motion of the world was still perceived as self-induced. Ants that observed a slower optic flow than expected walked longer distances and for larger amounts of time, suggesting they rely on the combination of the efference copy and reafference exteroceptive visual signals to detect deviations from their intended distance and time spent walking. Velocity, however, was still estimated based on the efference copy and reafference of proprioceptive signals (figure 3C). On the other hand, ants that experienced a fast optic flow received a faster reafference exteroceptive input than expected by the efference copy. However, that did not change their walking behaviour, suggesting that ants rely on the proprioceptive loop as long as the exteroceptive loop does not indicate that they might be walking slower than expected.

In another set of experiments, ants walked in a virtual world with self-induced optic flow but, on the second trial, we replayed the visual information they received on the previous one. In this trial, the ants own movement did not affect the visual motion of the world. Therefore, although the proprioceptice reafference and efference copy could still be combined, the visual input received by their brain was not in closed-loop with the motor outputs it produced (exteroceptive exafference rather than reafference). In this scenario, the efference copy does not match any of the characteristics of the visual input and, therefore, it is perceived as external rather than self-induced. Ants were still able to integrate the time they spent walking, which suggests they still rely on the proprioceptive

loop for time estimation and exafference signals do not disrupt it. However, they walk at increased speeds and for longer distances. Thus, exteroceptive exafference input induces changes in speed estimation or the motor commands necessary to keep a constant velocity (figure 3D).

Our findings suggest that neural activity in closed-loop with the environment differs from that of perceiving changes in the environment occurring in open-loop. Thus, wood ants must be able to integrate exteroceptive and proprioceptive pathways, and the efference copy pathways. They must then be able to use this to adjust the motor efference. The integration of exteroceptive cues has been well described in insects. As mentioned above, visual input travels from the optic lobes to several central neuropils, including the CX, MBs and lateral protocerebrum. Neurons within the CX sensitive to polarized light, necessary for body orientation, have been recorded in locusts (Vitzthum *et al.*, 2002), butterflies (Heinze and Reppert, 2011), bees (Stone *et al.*, 2017) and beetles (el Jundi *et al.*, 2015). Response of CX neurons to external optic flow, generated by moving gratings or looming stimuli, has been observed in locusts (Rosner and Homberg, 2013) and in bees (Stone *et al.*, 2017). Moreover, the response of CX neurons to exteroceptive cues is not limited to a single modality, having been shown for mechanical stimuli to the antennae in cockroaches (Ritzmann *et al.*, 2008) and locusts (Bräunig, 1985). Similar multimodal exteroceptive integration has been observed in the MBs and lateral protocerebrum, which receive input from at least the visual and antennal pathways (described above). Furthermore, neurons of the lateral accessory lobes (LAL) also respond to stationary visual stimuli (Homberg, 1994).

Motor control and descending neurons (efference) from central neuropils to thoracic ganglia have also been extensively described in insects. Several connections between the central complex and thoracic ganglia involved in motor control have been reported (reviewed in Strausfeld, 1999). For instance, neurons sensitive to polarized light connect the central complex to thoracic ganglia in locusts (Träger and Homberg, 2011). In flies, movement initiation (Martin *et al.*, 1999), controlled leg movement to produce appropriate step lengths (Strauss, 2002) and to walk straight towards a target (Strauss and Pichler,

1998) seem to be dependent upon the central complex. In cockroaches, neural activity in a set of CX neurons seems to predict and to drive the motor commands for the animals' walking behaviour (Martin *et al.*, 2015; Bender *et al.*, 2010). The MBs of cockroaches (Okada *et al.*, 1999; Mizunami *et al.*, 1998) and fruit flies (Martin *et al.*, 1998) were also shown to play a role in active walking behaviour and are thought to integrate the movement signals produced by their motor system and the changes caused by the movement in the visual world (discussed in Wessnitzer and Webb, 2006). The LALs are also involved in motor control (Homberg, 1994) and receive input from the central complex (Namiki and Kanzaki, 2016) and the mushroom bodies (Ito *et al.*, 1998). Indeed, the CX's major output neurons enervate the ipsilateral LAL, with some connections targeting the contralateral LAL (Homberg, 1994).

Although the role of central neuropils in the integration of exteroceptive cues of multiple modalities and the role in initiating and controlling movement as been well described, how insects integrate proprioceptive cues remains largely unknown. Proprioceptive signals during locomotion are thought to be sensed by the femoral chordotonal organ (Hofmann *et al.*, 1985; Bässler, 1977). In stick insects, recordings of neurons from these proprioceptive sensory organs show that they respond to both position and velocity of the tibia relative to the femur (Hofmann *et al.*, 1985; Stein and Schmitz, 1999). Ascending neurons from these organs to central brain regions have not yet been described, but Homberg (1994) did show that ascending neurons from thoracic ganglia targeting the LAL respond to proprioceptive signals, as does one recorded neuron from the CX. Based on these findings, Homberg (1994) suggests that the LAL links the ascending (afference/reafference) and descending (efference) pathways of the CX. Furthermore, Seelig and Jayaraman (2015) also observed CX neurons responding to self-motion cues in the absence of visual input suggesting this is accomplished by the integration of proprioceptive cues.

Contrary to afference inputs and motor outputs, to our knowledge there is no direct evidence of the expected sensory inputs (efference copy) in any central brain neuropil. While walking, the expected motion of the visual field and changes in proprioceptive

sensing organs need to be combined with the efference copy in order to be distinguished from external changes. This could occur at the level of central neuropils or directly to the sensory afferents. Studies with cockroaches revealed thoracic ganglia neurons that respond to self extension but not imposed extension of the leg (Delcomyn, 1983). Furthermore, studies on locusts saccadic behaviour have shown that neurons form the optic lobes (and thoracic ganglia) respond to external visual motion but not to motion caused by voluntary movements of the eye (Palka, 1969). This could be accomplished by direct motor inputs to the optic lobe, which have been observed in fruit flies (Kim *et al.*, 2015). Furthermore, different proprioceptive sense organs were also shown to inhibit each other (Stein and Schmitz, 1999). The distinction between exafferent and reafferent signals has also been observed in CX neurons of fruit flies, which are sensitive to translational optic flow during active flight but not during resting phases (Weir *et al.*, 2013). Thus, although at this stage it is not possible to claim the efference copy pathway is integrated directly by the sensory organs or if it's first integrated by central neuropils, such as the CX, the little evidence available supports the former hypothesis.

In our experiments, we did not record neural activity while ants were interacting with the virtual world or perceiving visual motion passively. However, based on previous studies, we can infer which sensory organs and brain neuropils would be involved in each of our experimental conditions described above. Ants walking in the absence of visual cues integrate solely proprioceptive cues at the level of femoral chordotonal-like organs, which could be transmitted to the CX and LAL. Both these neuropils are likely to be involved in driving the motor commands for walking, which descend to thoracic ganglia and appropriate muscles. Proprioceptive signals could be sensed as self-induced due to efference copy integrated by proprioceptive organs. Thus, the reafference proprioceptive input would arrive to the CX and LAL after being modulated by the efference copy (figure 4, red and gray arrows).

This would also occur in the presence of visual cues, but the motor system could also send an efference copy to the visual pathway, possibly at the level of the optic lobes. The motion of the visual world would then be integrated in the MBs, CX and lateral proto-
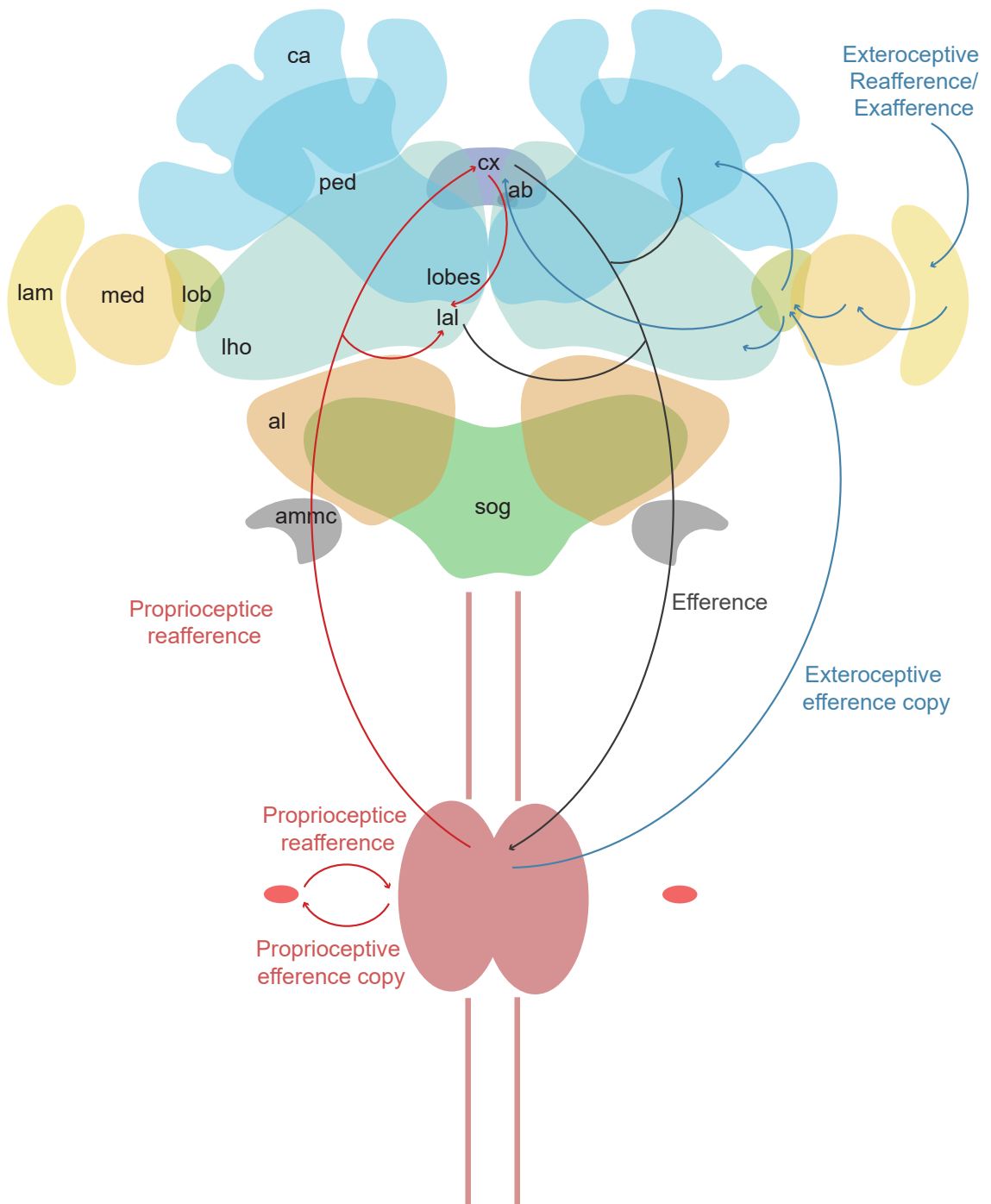
Figure 4: Schematic diagram of the neural pathways involved in walking behaviour of wood ants in closed-loop with their visual surroundings. Neural pathways involved in perceiving the proprioceptive cues (red arrows) and include reafference from proprioceptive sensory organs into the thoracic ganglia and efference copy being integrated at the level of the sensory organs. The reafference is conveyed into the lateral accessory lobes (lal) and central complex (cx). The pathway involved in perceiving the self-induced visual motion of the world (blue arrows) include the optic lobes' lamina (lam), medulla (med) and lobula (lob), the lateral horn (lho) of the lateral protocerebrum, the calyxes of the mushroom bodies (ca) and the cx. The cx, mushroom bodies and lal are involved in initiating and controlling the motor commands (gray arrows). The efference copy is also integrated at the level of the visual sensory organs, in the lobula. Note that this process occurs in both brain hemispheres (represented asymmetrically for better clarity) and in all proprioceptive sensory organs in the legs.

cerebrum after being modulated at the level of the optic lobes. The MBs and CX could, therefore, adapt the motor output during walking based on this feedback loop with the visual system. This would not be necessary in a scenario in which the reafference and efference copy of both proprioceptive and visual systems would produce similar motor output (figure 4, red, blue and gray arrows). However, if the exteroceptive visual reafference was slower than the expectation based on the efference copy, the CX would integrate walking parameters differently based on the visual or proprioceptive signals, and would adapt motor outputs (possibly in conjunction with the MBs and LAL) based on information from the visual system. Conversely, if the reafferent visual stimuli perceived was faster than the expectation, the CX would maintain the adjustment of the motor output based on the proprioceptive signal. Furthermore, the full mismatch between the visual input and the efference copy in the open-loop situation could be integrated by the same neuropils as exafference rather than reafferent visual input, which would drive different motor commands.

Possible mechanisms of passive and active learning

In previous sections we proposed models for visual memory formation though classical conditioning, in open-loop, and for walking behaviour in wood ants in closed- and open-loop with the visual surroundings. In this section, we will address possible neural mechanisms for learning the association between a visual cue and a sugar reward in closed-loop, though operant conditioning, and how it differs from passive classical conditioning. For appetitive learning to occur, the animals state also plays a role and needs to be integrated in the brain (Huston and Jayaraman, 2011). Here, we consider that the hunger state would be similar between the two forms of learning and do not address state integration.

In classical conditioning, both visual cue and food reward are perceived passively, with an interval of only a few seconds, with no influence of the animal's own behaviour. Thus, the visual cue constitutes an exafferent signal and, because animals are typically tethered and unable to move and actively interact with their surroundings, there are no exteroceptive reafferent signals, no motor commands to approach the visual cue, no changes in the proprioceptive input nor efference copy from the motor commands to the proprio-

ceptive and exteroceptive pathways. Conversely, during operant conditioning, animals are freely walking and need to approach the visual landmark to receive the food reward. The visual cue is not perceived solely seconds before the food reward but potentially a long time in advance of ingestion. Thus, the nervous system needs to integrate not only the association between the visual cue and the food reward, but the motor commands necessary for approaching them. While traveling towards the visual cue, they perceive the changes in the visual surroundings, including how distant they are from the visual landmark, as reafferent signals rather than exafferent. Moreover, proprioceptive reafference signals are also integrated, in parallel with the efference copy being combined with both visual and proprioceptive pathways.

In an operant conditioning scenario, another neural representation needs to be formed in the brain: animals approach the visual cue because they have a specific *goal*, to feed. Thus, the memory formed is not only of the association between the food reward and the visual cue, but also of the location at which the animal needs to be in regards to the visual cue to be rewarded. In other words, the memory formed could be as simple as through classical conditioning, of an association between the characteristics of the visual cue and the presence of food, but these characteristics only match the learned association when the animal is located near the cue. Consequently, the efference copy from their motor actions can be compared to the goal location, in addition to the reafferent proprioceptive and visual signals received from visualizing the cue from different locations while approaching. Similar mechanisms have been hypothesised for path integration and the comparison between efference copy signals to the memorized goal of direction and distance vectors (discussed in Webb, 2004; Haferlach *et al.*, 2007; Webb, 2012; Huston and Jayaraman, 2011).

As described previously, visual information is conveyed into multiple central brain regions, including the mushroom bodies, central complex and other neuropils, but the MBs seem to be the primary region for short-, medium- and long-term memory formation during passive learning. We hypothesise that the MBs are still the region in which the associative memory is formed between the characteristics of the visual cue, at a specific

location, and the food reward. The goal location to travel to, in the form of memorized distance and direction vectors, is most likely integrated by the CX, which receives reafference input from both proprioceptive and visual pathways (both involved in estimation of distance, velocity and time in wood ants and appropriate motor control). Walking parameters integrated through visual motion of the world are also memorized by the MBs. Both MBs and CX (via LAL) are then involved in the precise control of motor commands necessary to reduce the difference between the visual and proprioceptive reafferent signals and the memorized location (distance and direction) goal (figure 4).

While actively interacting with their environment, wood ants do not use solely their visual system. Instead, they integrate a multitude of cues, including olfactory, for instance, while following pheromonal trails (Rosengren and Fortelius, 1987), gustatory, while touching food, and mechanosensory cues, while touching food, touching other ants during trophallaxis (Frasnelli *et al.*, 2012) and walking (Bell, 2017). Indeed, many insects use their antennae for actively sampling their environment (Staudacher *et al.*, 2005). Thus, the role of active antennation and the contact of the antenna to the food reward possibly prior to sensing it with the mouth-parts also needs to be considered in our model.

In our classical conditioning experiments (Chapters 2 and 3), antennae were free to move and able, to some extent, to sample their surroundings. However, the timing and duration of the contact between the antenna and the sucrose reward was controlled by the experimenter. Consequently, as described for the visual cue, antennating and receiving an input through the antennae did not match one another. The contact with sucrose was still perceived as an exafferent signal and conveyed to the brain as previously hypothesised (figure 2). However, in operant conditioning ants have the freedom to actively sample the food reward with their antenna. They sense the reafferent gustatory, mechanosensory and proprioceptive inputs, at the level of the antennae, and can combine this with the efference copy sent by the motor neurons controlling antennal movements. This probably occurs also at the level of the antennal sensory organs. The gustatory, mechanosensory and proprioceptice reafference signals are conveyed possibly to MBs and the CX (through ALs, AMMC and lateral protocerebrum). In turn, the same neuropils would be involved

in controlling the motor commands for antennating, with the addition of transmission to the AMMC prior to the antennal muscles.

It is unknown if ants typically touch a sugar reward with one antenna prior to the other while freely moving. If they do so, then they may prioritize the formation of short- and medium- or long-term memories depending on which antenna they use, as observed in our classical conditioning experiments. During trophallaxis, the ant receiving food tends to touch their counterpart more often with the right antenna than the left (Frasnelli *et al.*, 2012). Although this context has a communication component between two individuals, it gives rise to the possibility that ants could also show asymmetries in antennal contacts made to food. One possibility would be ants shifting from using the right antenna first to using the left, if the associative memory between visual cue, location and a food reward would be necessary in the short-term first before committing to form a long-term memory. This could happen, for instance, when visiting a feeder for the first time and requiring a few foraging trips to the same feeder in the same day (dependent upon short- and medium-term memories) before the presence of food at that location and the associated visual cues are deemed sufficiently reliable to be memorized for returning the next day (dependent upon long-term memory).

Although the neural basis proposed for the formation of visual associative memories through classical and operant conditioning in wood ants are still highly speculative, the basic mechanisms of an open-loop and a closed-loop system between brain, body and environment hold true regardless of the neuropils involved. The likelihood of some brain regions having a more important role during operant compared to classical condition-ing does give rise to the possibility that this more complex integration of exteroceptive and proprioceptive cues, as well as the efference copy, facilitates learning, as observed behaviourally by Brembs and Heisenberg (2000).

Open questions and future work

On the comparison of active and passive learning

If wood ants learn the association between a visual cue and a food reward differently depending on whether learning occurs passively (though classical conditioning) or actively (through operant conditioning) remains unanswered. To address this, our virtual reality paradigm could be used with the incorporation of an automatic reward system. This system would deliver a drop of sucrose solution to the walking wood ant when it reaches an appropriate location within the virtual world or at a given time. We can achieve this using a syringe filled with sucrose connected to a picopump that delivers controlled units of air puffs. Installing the tip of the syringe directly bellow the ant's mouth parts, at a reachable distance, would allow precise amounts of sucrose to be delivered to the ant while navigating in the virtual world without radically disrupting its perception of the virtual environment. Because when studying associative learning, the connection between the visual cues and the reward depends upon the timing between one event and the other, for learning to occur in the virtual world the time of presentation of the sucrose reward would have to be fully controlled. In closed-loop experiments where ants would form a visual memory through operant conditioning, they would navigate in the virtual world and only receive a reward when reaching the desired virtual object. In open-loop conditions, in which ants would form visual memories passively through classical conditioning, the visual world would change and only when the desired virtual object would appear in front of the ant the reward would be presented, regardless of the ant's own movement. To achieve this, the changes in the virtual world would have to be connected to the picopump and activate it only when the conditions are met, as previously developed for rodents (Aronov, 2014).

To investigate the neural basis of learning through operant and classical conditioning, an electrophisiology system could be incorporated in our paradigm. To our knowledge, electrophysiology techniques have not yet been used with wood ants. Nevertheless, they have been developed for other insect species. For instance, intracellular recordings of central complex neurons have been accomplished in dung beetles (el Jundi *et al.*, 2015) and patch clamp recordings of neurons of the olfactory pathway have been performed in

honey bees (Kropf and Rössler, 2018). Furthermore, extracellular recordings of local field potentials (LFPs) have been performed in both fruit flies (Paulk *et al.*, 2015) and honey bees (Paulk *et al.*, 2014) on trackball paradigms similar to ours. Imaging tecnhiques, such as *in vivo* calcium imaging, have also been developed for insects that do not possess the genetic tools that research using *Drosophila melanogaster* benefits from, including honey bees (Faber *et al.*, 1999) and other species of ants ants (Dupuy *et al.*, 2010). Thus, these methodologies could also be developed for our insect model and used in parallel with our virtual reality paradigm.

To establish this, ants should have their head fixed while walking on top of the track-ball. Thus, behavioural experiments would have to be performed beforehand to ensure that ants with their heads fixed to their thorax or to the set-up itself would still walk normally and interact with the virtual world. Ants also adjust the height of their body and heads, which is necessary for them to walk on top of the trackball. To account for this movement, the recording electrode could be attached to the ant or pin by which it is held and allowed to move up and down as the ant adjusted its height from the ground. Changes in height could also be recorded and used to move the recording or imaging device in the same axis accordingly, in almost real time (developed by Vähäsöyrinki, M., personal communication to Niven, J.E.).

On the lateralisation of memory formation

Our findings on lateralisation of short-, medium- and long-term memories raised several questions that remain unanswered. Within our model insect, the wood ants, little is known about asymmetric function of the sensory organs in the process of memory formation. To our knowledge, it has not been yet investigated if freely walking ants, in previously described learning arenas (Woodgate *et al.*, 2016; Buehlmann *et al.*, 2016), would display similar memory lateralisation if they detected the sugar with one antenna before the other or if the active movements of the ants on approaching and interacting with the sugar drop would mask this phenomenon. Although we would be unable to control which antenna touches the sucrose in the first place or to test ants at precise time points as in our classical conditioning experiments, we could investigate whether they preferentially use one

antenna before the other in this context, or show other asymmetries as seen in trophal-laxis (Frasnelli *et al.*, 2012). Furthermore, experiments with freely walking ants with one antenna covered could be performed to investigate if long-term memory formation was disrupted by the absence of input from one of the antennae.

We also do not know if lateralisation in memory is restricted to asymmetrical input on the antenna or if similar memory recall would be achieved in ants trained to perceive the visual cue with one eye only. So far, learning but not memory recall has been investigated for vision in harnessed honey bees (Letzkus *et al.*, 2006, 2007) and memory formation has only been addressed for olfactory conditioning (Rogers and Vallortigara, 2008; Anfora *et al.*, 2010, 2011; Frasnelli *et al.*, 2011) or, in our study, in asymmetrical input to the antennae with sucrose. Thus, classical conditioning experiments with tests at different time points ought to be repeated with wood ants with one of the eyes covered, imposing asymmetries within the visual system directly.

Furthermore, our paradigm can be used to investigate the neural basis of visual learn-ing in wood ants and the asymmetries in neural activity caused by asymmetrical training. Mechanical or chemical brain lesions, as performed in honey bees (Komischke *et al.*, 2005) and very recently in wood ants (Buehlmann, 2019), could be combined with our experiments to reveal which regions of the brain are necessary for learning and short-, medium- and long- term visual memory formation. Because ants are restrained, the electrophysiological recordings and imaging techniques described above could also be performed in our classical conditioning paradigm, while an ant is learning and recalling a memory at different time points. Furthermore, in combination with brain lesions, record-ing neural activity could allow us to observe changes caused by absence of activity from the lesioned neurons.

Why some insects show lateralisation of memory formation and others don't remains unclear, although evidence suggests that this phenomenon has evolved in parallel with eusociality (Frasnelli, 2013; Niven and Bell, 2018). To address this, similar classical condition protocols could be used to investigate learning and memory formation in other species of solitary and eusocial insects. A comparative study with different species would

provide a clear understanding of when the lateralisation of memory first arose and which groups of insects may have either never possessed or lost it. Thus, one could relate the several characteristics of a species ecology, including its social behaviour, to the presence or absence of memory lateralisation. Furthermore, when complemented by the study of asymmetries on other lateralised behaviours, this could yield the answer to if memory lateralisation arises because of specific selective pressures or as a byproduct of brain asymmetries specialized in other behaviours.

On distance, speed and time integration

To fully understand which cues wood ants rely on to integrate the distance, speed and time they spend walking, additional analysis and experiments could be performed using our virtual reality paradigm. Here, we will divide the experiments and analysis related to each of the three hypothesis previously raised for distance estimation in foraging insects: optic flow, proprioceptive (idiothetic) cues, and energy expenditure.

In this thesis, we have performed a thorough analysis of how changes of self-induced optic flow changes distance, speed and time integration. We have shown that ants walking once in an environment with virtual optic flow and once without behave differently depending on the velocity of the self-induced motion of the world. To investigate this further, we could make the same ants experience two virtual worlds with self-generated optic flow but differing in velocity. This experiment would provide evidence of the same ants changing behaviour because of the velocity of optic flow they experience, rather than comparing groups of ants that experienced different optic flow speeds. We also investigated how external visual motion of the world changes the ants walking and rotating behaviour by replaying, on the second trial, the exact same visual cues they had experienced in closed-loop previously. Because in these experiments the visual world moved both in translation and rotation, it is important to investigate how each component affects the ants behaviour separately. Furthermore, we could allow ants to run in the virtual environment in closed-loop but adding an externally generated optic flow, either forwards, backwards, sideways or rotating. These experiments would not only reveal how external motion in different directions affects distance, speed and time estimation but also how

they modify ants optomotor-like response.

Proprioceptive cues for distance, speed and time estimation have been proposed in this thesis as possible cues integrated by wood ants, as is the case of desert ants (Wittlinger *et al.*, 2006). Although our findings support this idea because ants display consistent walking behaviour in the absence of virtual optic flow, the ventral optic flow from the trackball could still play a role here. Thus, to achieve a test with ants using proprioceptive cues alone, we could cover the ventral portion of their eyes as performed before by Wolf *et al.* (2018) with desert ants. To investigate if ants have a mechanism similar to the desert ants' stride integrator, we could also perform a thorough reanalysis of our videos for all experiments already performed. From these videos, we could extract the number and length of steps that ants perform in each condition. Furthermore, we could manipulate the wood ants' leg length as performed by Wittlinger *et al.* (2006) and evaluate how number and length of steps change in correlation with changes in distance, speed and time integration.

Lastly, although energy expenditure is not thought to be integrated in honey bees and desert ants (Esch and Burns, 1996), only one experiment has addressed this directly by measuring the oxygen consumption in walking honey bees returning to their colonies (Esch *et al.*, 1994). However, this experiment was limited by the short space and, therefore, time that bees walked. Energy expenditure could be tested in wood ants for longer time periods using adaptations of our virtual reality paradigm. To achieve this, we could enclose our trackball system and use a similar metabolic rate machine as used in Esch *et al.* (1994) to measure the ants oxygen consumption while walking on the virtual world.

Additionally, wood ants could be integrating distance, speed and time spent walking over long time periods (10 minutes, in the case of our experiments), or over short consecutive walking bouts. To our knowledge, the time scale of distance, speed and time integration have not been investigated in any walking insect. The characteristics of continuous and interrupted locomotion could be analysed in our virtual reality paradigm for vast ranges of time with enough precision to unravel how walking parameters vary for each walking bout.

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Appendix A

Table A1. Influence of training in ants' responses to the CS, for each type of MaLER. The number of ants (N), degrees of freedom (d.f.), Logistic regression (z) and P-value are shown (*P<0.05, **P<0.01).

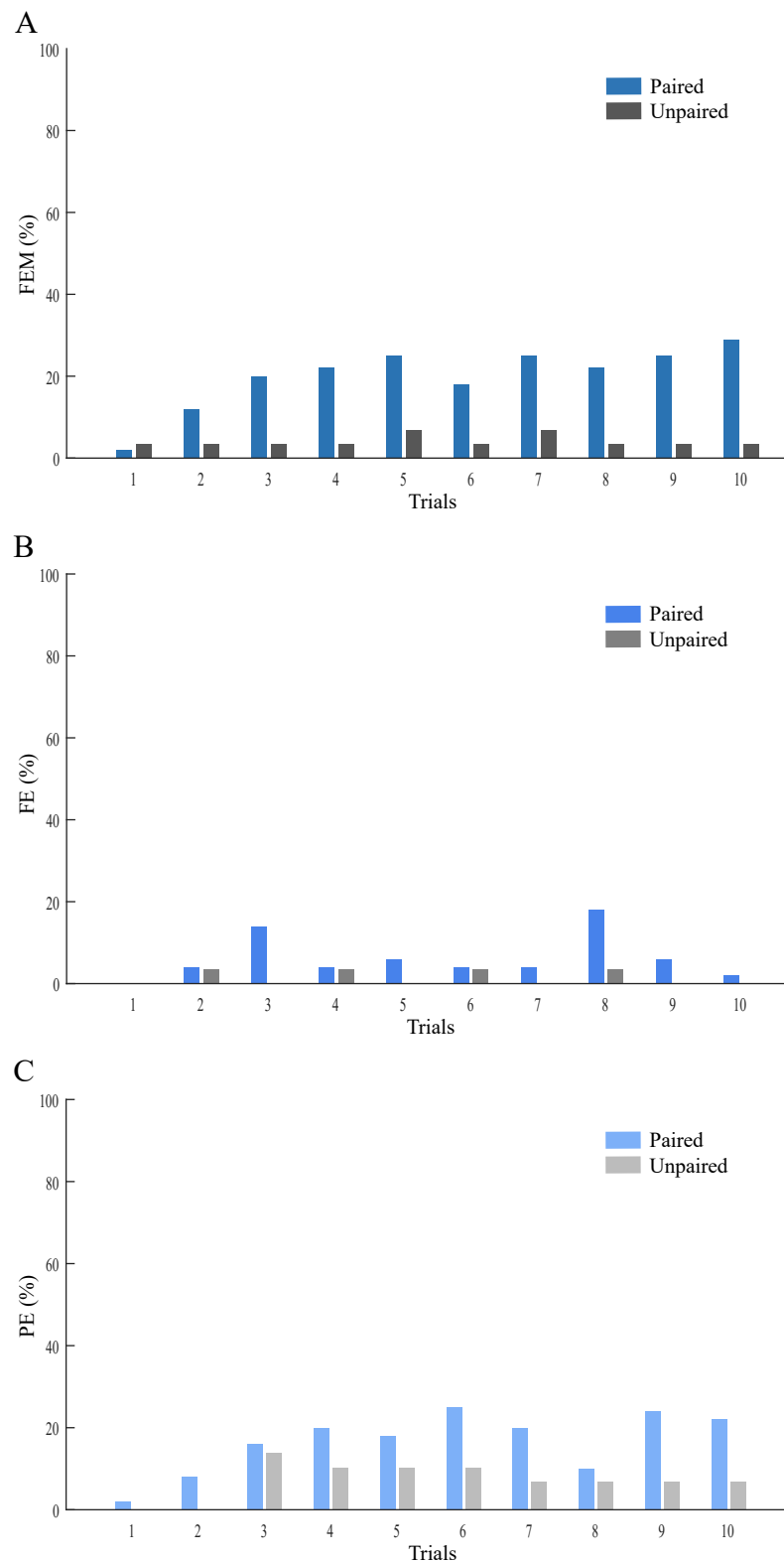| Type of training	Type of MaLER	N	d.f.	z	P
Paired	FEM	51	50	3.87	0.0001**
	FE	51	50	0.84	0.40
	PE	51	50	2.98	0.003**
Unpaired	FEM	29	28	0.13	0.9
	FE	29	28	0.35	0.73
	PE	29	28	0.84	0.4

Figure A1. Learning curves based on each type of MaLER. A) The percentage of paired ants (N=51) performing FEM in response to the CS presentation increased throughout training, but not for unpaired ants (N=29). B) The percentage of ants (paired: N=51; unpaired: N=29) performing FE in response to the CS presentation didn't increase during training for both paired and unpaired ants. C) The percentage of paired ants (N=51) performing PE in response to the CS presentation increased throughout training, but not for unpaired ants (N=29).

Appendix B

(a) Generalised linear mixed models for responses during training. For comparisons between groups, trial number and training type were included as fixed effects, whereas the individual ant was a random factor, using the command `glmer (MaLER ~Trial + Training + (1|Individual))`. For assessing the responses within a training group, we used the command `glmer (MaLER ~Trial + (1|Individual))`.

(b) Barnard tests for comparing MaLER responses during testing. Two-tailed tests were applied for comparisons between two paired groups or between two unpaired or CSO groups. For comparisons between a paired group with an unpaired or with CSO, tests were one-tailed.

(c) Bonferroni corrections were applied for comparisons of the ants' performances during training when appropriate. For the comparison between the PR and PL groups, and between PR, PL and ants that were subjected to a bilateral paired training (PB) the p-value cut-off was 0.025. For the five comparisons between each paired group and its corresponding unpaired group or the baseline CSO, the p-value cut-off was 0.01. UPR and UPL were compared to the CSO with a p-value cut-off of 0.025.

Bonferroni corrections were also applied for comparing the proportions of MaLER performances during testing. The p-value cut-off for the two-tailed test between PR and PL and between PR and PB at 10 minutes was 0.025. For the 1 hour test, two one-tailed and two two-tailed tests were performed, comparing PR and PL with the baseline CSO, and UPR and UPL with CSO, respectively, with an adjusted p-value of 0.025 each pair of comparisons. The same four comparisons were performed at 24 hour test with the same Bonferroni corrections. Two additional tests were performed between RUPL and UPL

and between RUPL and CSO, which were Bonferroni corrected to produce an adjusted a p-value cut-off of 0.025.

(d) To determine whether the direction ants faced (right or left) during training influenced learning, we ran a logistic regression model with the paired groups PR and PL, where trial number, type of training and the direction ants faced were fixed effects and the individual ant was a random effect, using the command `glmer (MaLER ~Trial + Training + Facing + (1|Individual))`.
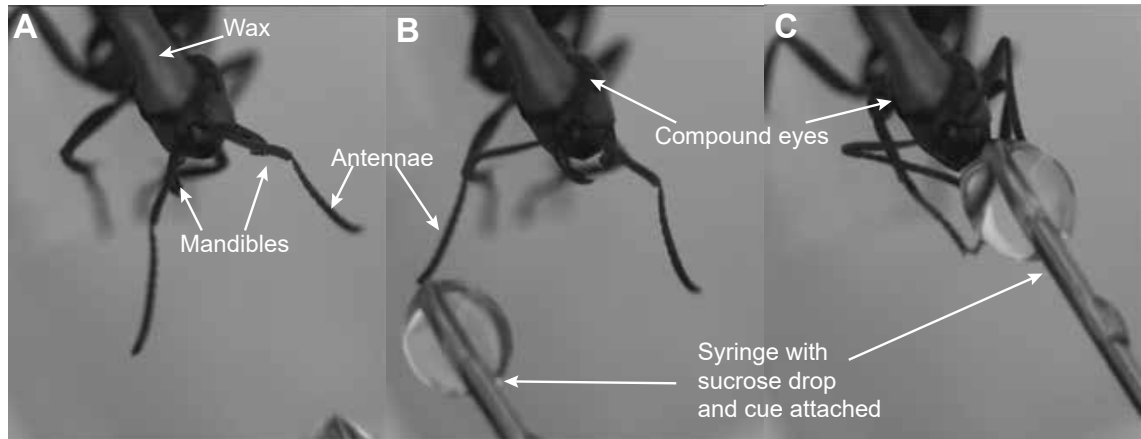
Figure B1: Sequence of events during a paired training trial with a reinforcement on the right antenna. A) 10 seconds of CS visualization, where the ant is shown the visual cue attached to the syringe. B) Drop of sugar touching the right antenna. C) Ant feeding on sugar during approximately 5 seconds. The ants' eyes, antenna and mouthparts, the pin and wax fixing the ant and the needle with sugar droplet are visible in every frame. The CS is attached to the other end of the needle (not visible here).
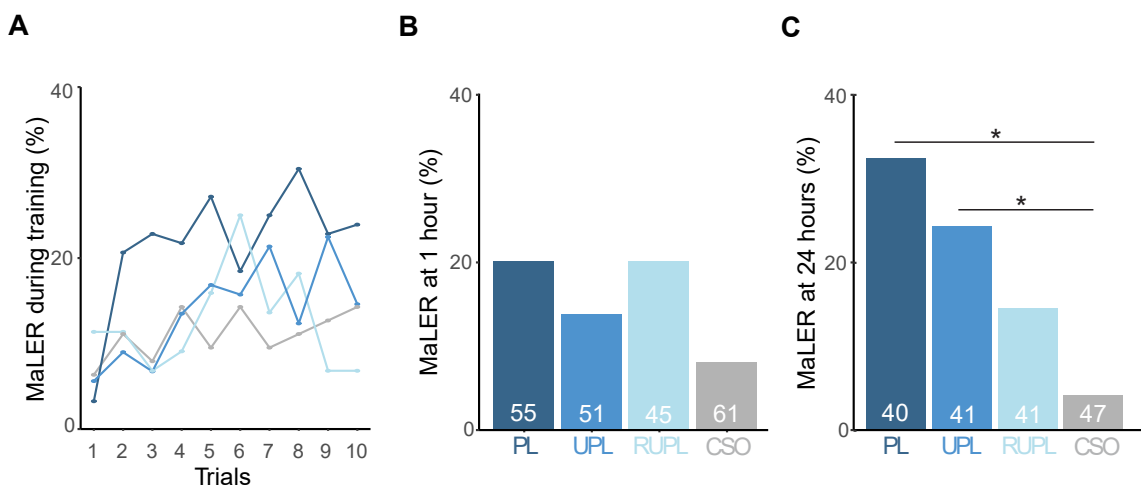

Figure B2: Wood ants recall of a visual memory after 24 hours is less precise than during training. A) Percentage of MaLER responses during training, for all groups reinforced on the left antenna (PL: dark blue; UPL: medium blue; RUPL: light blue) and ants that did not contact with the sugar reward (CSO: grey). Ants subjected to a paired training show a significant increase of responses with training trial, but ants with either type of unpaired training or with a CSO type of training don't. B) MaLER performance of all left reinforcement groups are low after 1 hour with no distinction with the CSO baseline. C) 24 hours after training, MaLER responses are elevated for both PL and UPL groups, which are significantly higher than CSO. RUPL ants did not respond significantly differently than PL, UPL nor CSO. Asterisks indicate significant differences in MaLER percentages during testing, between training types.