SEX AND EXPERIENCE RELATED NEURAL AND BEHAVIOURAL PLASTICITY IN ANTS

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Declaration page for the Masters of Research thesis

This thesis is written in the form of a journal article from **The Journal of Comparative Physiology A**

Declaration

All research described in this report is my own original work.

This work has not been submitted for a higher degree to any other university or institution.

Duncan Douglas Jaroslow

04/05/2018

Contents

Acknowledgements – 5

Abstract – 6

Introduction – 7

Materials and Methods – 10

Results – 15

Discussion – 28

Conclusion – 31

References – 32

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Abstract

Ants utilise age- and caste-based division of labour, providing an opportunity to investigate behavioural and neural plasticity. Newly eclosed workers perform tasks, such as nursing, and initiate foraging as they mature. Alate males only search for mates and do so exclusively on the wing. In worker ants, age and initial light exposure induce neural changes to the mushroom bodies, higher-order sensory processing regions. However, synaptic neuroplasticity has not been directly linked to the behavioural transition into forager life. In hymenopterans, males demonstrate neuroplasticity associated with initial flights or mating, yet it is unknown how age affects neural circuitry. I documented the navigational behaviour of naïve ants as they exited the nest on consecutive trips. I then compared the structure and organisation of olfactory and visual neuropils within the mushroom bodies with behaviour and foraging experience. Ants leaving the nest for the first time carry out learning walks during which they regularly look back towards the nest. As animals gained experience, their path towards the feeder became increasingly direct. I found no evidence of neuroplasticity among workers with different experience levels. Comparing males that were 2 and 11 days old, I found that the volume and number of synaptic profiles increased in the visual neuropils of mushroom bodies. However, a similar comparison in workers revealed no such differences. I discuss how these differences in neuroplasticity in each caste have evolved to suit their respective lifestyles and ecologies.

Key words: Learning walks, neuroplasticity, sensory experience, behavioural maturation, mushroom bodies

Introduction

A prominent feature of social organisation in eusocial insects is division of labour, which is the task specialization of individuals within a group. Division of labour is largely responsible for the ecological success of ants and honeybees (Hölldobler & Wilson, 1990; Szathmáry & Smith, 1995; Robinson *et al.*, 2009). Ants, in particular, have a well-defined social structure that utilises caste, sex, and age-related division of labour and provides an excellent opportunity to investigate the relationship between behavioural maturation and development (Tripet & Nonacs, 2004; Bernadou, *et al.*, 2015). A colony's reproductive duties are carried out by the male drone and winged female castes (Hölldobler & Wilson, 1990; 2009). Winged, or alate, males do little more than search for mates after leaving their nest of origin and do not forage during their entire lifespan, whereas alate females, after leaving their nest of origin, mate, de-alate and establish a new colony (Hölldobler & Wilson, 1990; Keller, 1998). The non-reproductive caste, termed workers, perform almost all of the colony's labour. When workers are newly eclosed from the pupal stage, they perform tasks inside the nest, such as nursing, but tasks performed outside the nest, such as foraging, are carried out by older individuals who have reached the appropriate age and level of behavioural maturity (Gordon, 1996; Tripet & Nonacs, 2004; Bernadou, *et al.*, 2015).

These variations in societal role can result in different sensory experiences among sexes and age groups (Hölldobler & Wilson, 1990; Ehmer & Gronenberg, 2004). Here we focus on males and female workers, which lead a life exclusively on the wing and pedestrian, respectively. Alate males have the primary goal of leaving their nest and searching for a mate which requires extensive visual sensory information to track females during mating flights (Gronenberg, 2008; Narendra, *et al.*, 2016). In contrast, a worker's role in the colony is determined by an age-based division of labour, making sensory experience more variable compared to males (Robinson, 1992; Theraulaz, *et al.*, 1998). Workers utilise olfactory sensory information to identify and recruit nest mates (van Zweden & d'Ettore, 2010; Sharma, *et al.*, 2015), and utilise visual information to assist in navigation during foraging activity (Wehner, *et al.*, 2004; Narendra & Ramirez-Esquivel, 2017). However, foraging activity only occurs once the appropriate age and level of maturity has been reached (Tripet & Nonacs, 2004; Korczyńscka, *et al.*, 2014). During a worker's first nest emergences and prior to the commencement of foraging, they perform learning walks, a behaviour which may help ants acquire information required during subsequent foraging trips (Wehner, *et al.*, 2004; Fleischmann, *et al.*, 2016). These learning walks involve the ant circumnavigating the nest entrance and repeatedly gazing towards the nest entrance. This is thought to allow ants to memorise nest-specific landmark information that would allow them to pinpoint home from subsequent foraging trips (Müller & Wehner, 2010; Fleischmann, *et al.*, 2016). Although foraging does not occur during this activity,

learning walks or flights (for flying insects) are considered critical to their foraging success (Philippides, *et al.,* 2013; Stürzl, *et al.,* 2016; Fleischmann, *et al.,* 2017).

Due to variations in life style and colony roles, each caste has adapted their sensory processing systems to the sensory requirements of their colony roles. In comparison to workers and reproductive female castes, male ants have much larger eyes relative to their body size and more ommatidia (Baker & Ma, 2006; Narendra, *et al.,* 2010), which are optical units that make up the compound eye. These adaptations help to accommodate the visual requirements of mating flights (Narendra, *et al.,* 2016). On the other hand, workers need to invest in both the olfactory system, for pheromone communication between nest mates (Gronenberg, 2008), and visual system, which allows foragers to navigate effectively (Wehner, 2008; Narendra & Ramirez-Esquivel, 2017). This is evident in *Camponotus floridanus* where workers have relatively large antennal lobes and antennal segments for olfactory sensory processing, and smaller optic lobes for visual processing, in comparison to male conspecifics (Zube & Rössler, 2008; Mysore, *et al.,* 2010). Thus, there are distinct size differences in the visual and olfactory detection systems between the males and workers. Are these differences reflected in the higher order information processing centres?

One such higher order information processing centre in the ant brain is the mushroom body (MB). This region is implicit in memory, learning and processing information from multiple sensory inputs (Heisenberg, 1998; Gronenberg, 2008). Specifically the MB calyx lip region, which receives olfactory input (Müller, *et al.,* 2002), and the calyx collar region, which receives visual input (Fig. 1; Ehmer & Gronenberg, 2002). Both the lip and collar are known to increase in volume with age (Kühn-Bühlmann & Wehner, 2006; Yilmaz, *et al.,* 2016). Furthermore, both age and light have been shown to affect microglomeruli, which are synaptic complexes comprised of sensory projection neuron boutons surrounded by MB Kenyon cell dendrites (Yasuyama, *et al.,* 2002; Frambach, *et al.,* 2004). Age processes can greatly influence the density and number of microglomeruli in both the lip and collar (Seid, *et al.,* 2005; Stieb, *et al.,* 2010). However, the association between light exposure and microglomeruli pruning seems to be restricted to the collar (Stieb, *et al.,* 2010; Scholl, *et al.,* 2014). The impact of age and light on ant neural structure is not consistent (Yilmaz, *et al.,* 2016) and is often dependent on life history (Seid & Wehner, 2009; Stieb, *et al.,* 2012). As such, these types of neuroplasticity would likely arise based on individual life experiences. For example, workers who have substantial foraging experience compared to workers with little to no experience or non-foragers (Gronenberg, *et al.,* 1996; Kühn-Bühlmann & Wehner, 2006). It has been shown recently that when newly eclosed workers emerge from the nest for the first time, it coincides with learning walk activity (Fleischmann, *et al.,* 2016). Light exposure experienced during learning walks has also been linked to organisational changes in neural structure

(Grob, *et al.*, 2017). Whilst the effect of age, light and foraging experience on the MB in worker ants is clear, there have been no studies that have directly compared MB volume and synaptic plasticity in relation to learning walks. Many of these studies have assumed ants to be naïve if animals had not carried out any foraging over a 3 day period (Fleischmann, *et al.*, 2016; Grob, *et al*., 2017; Fleischmann, *et al.*, 2018). Furthermore, male ants have been largely absent from studies addressing neuroplasticity.

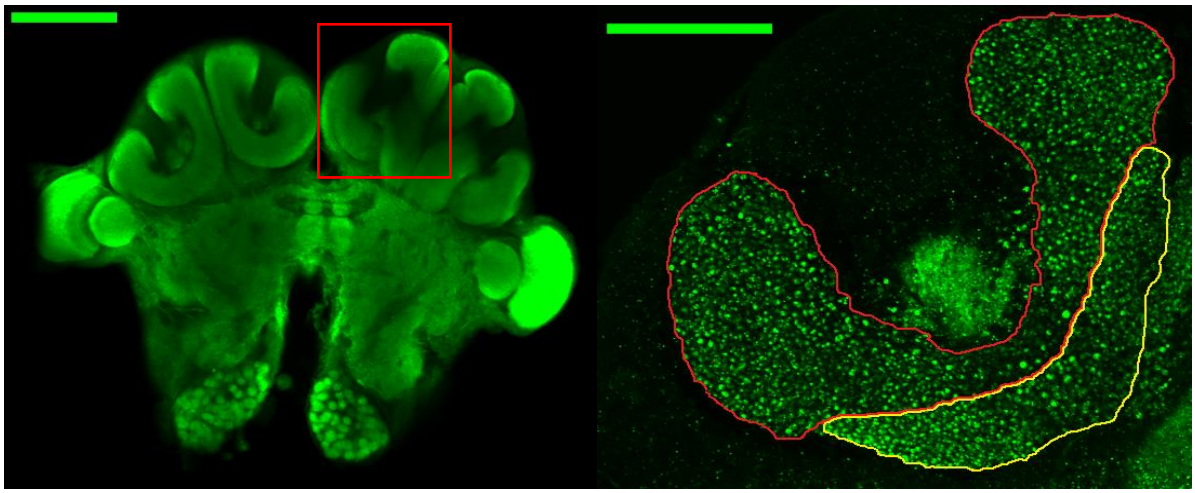

Fig. 1. *Confocal images of C. consobrinus labelled with anti-synapsin; (left) whole brain using a 10x objective, with the MB calyx region highlighted in the red box, scale bar = 200µm; (right) MG in the right medial calyx using a 60x objective, scale bar = 50µm, lip and collar regions have been circled in red and yellow respectively.*

To determine if differences in life histories between ant sexes are reflected in neural development, I first looked in detail at the transition to exterior life in *Camponotus consobrinus* (Erichson, 1842) and related neural changes (in lip and collar regions of the MB) as animals gain experience. Here I observed the transition of newly eclosed interior workers to experienced exterior foragers, and expected navigating towards a foraging goal to become more efficient with external nest experience. Volume increases and synaptic reorganisation in the lip and collar region were predicted to occur by the time of their first nest departure, and this restructuring was expected to continue with additional exterior nest experience. This study is the first in attempting to elicit learning walks in truly naïve ants and in laboratory conditions.

I then compared synaptic and volumetric changes in two age groups in males and workers of *C. consobrinus*. In paper wasps, age-based neuroplasticity in MB calyx lip and collar volume differs between sexes (Molina & O'donnell, 2008). So, here I predicted that MB lip and collar regions in *C. consobrinus* males would undergo significant neural reorganisation in the first 11 days post-eclosion and this neural reorganisation would differ from workers. This study is also the first to investigate synaptic plasticity in an ant reproductive caste.

Materials & Methods

Animals

One colony of *C. consobrinus* workers and brood were collected from Macquarie University's vegetable garden, Sydney, NSW, Australia (33°46'1.5" S, 151°6'46.0" E) and one colony from Mount Majura, Canberra, ACT, Australia (35°14'45.3" S, 149°10'8.5" E). Colonies were housed and all experiments were conducted in a temperature and humidity controlled room (25°C; 63% relative humidity). I used metal halide and halogen lamps, with a combined illuminance (lux) of 600lx, set to an artificial 12-12hr day/night cycle with an evening and morning twilight period. During summer months, *C. consobrinus* typically begins foraging close to sunset time (Schultheiss, *et al.,* 2015). Hence the room's lighting was set to start dimming at 6:30pm to simulate the start of evening twilight, and turned off by 7:30pm, to simulate night. Collected brood was checked once per day in a dark room with red light (>640nm), which ants did not seem disturbed by. Ants generally lack photoreceptors for the red visual spectrum (Ogawa, *et al.,* 2015; Yilmaz, *et al.,* 2017; Aksoy & Camlitepe, 2018) and thus red light should not induce light-related neuroplasticity. No collecting permits were required because all colonies were collected on University property and in local parks. Animal ethics clearance is not required for working with ants under Australian Code for the Care and Use of Animals for Scientific Research.

Experimental design

Comparison of foraging experience in workers

Ants were housed in nest boxes with multiple chambers that were carved into an autoclaved aerated Hebel block (20cm X 10cm X 30cm, purchased from Bunnings), with a foraging arena (32 X 21cm X 12cm) at the top. A small opening (1.5cm diameter) was made in the foraging arena floor to allow access between the nest and foraging arena. Within the foraging arena, a sugar feeder was provided at 20cm away from the nest entrance. I provided distinct visual landmarks on the outside wall of the foraging arena as a series of irregular patterns, to act as permanent and stable landmarks. Some foragers were observed laying down pheromone trails, possibly to help with navigation. So, the foraging arena floor was covered in paper towel, which was removed and replaced each day. This encouraged ants to utilise visual cues and prevented them from solely relying on olfactory pheromone trails. Two sub colonies of *C. consobrinus* were created from each of the source colonies. Each sub colony comprised 50 mature *C. consobrinus* workers, 75 pupae, 30 larvae, and 1 teaspoon of eggs (~80-100). A dot of orange paint was put onto the pronotum of the mature workers, distinguishing them from any ants reared from brood within in the sub colony. Mature

workers with their pronotum painted orange were added to the sub colonies from the same host colony when necessary to ensure the number of mature ants at any time remained at 50. Ants of known age that eclosed within these colonies were given a unique colour code on their pronotum to enable individual identification. Three groups of known-aged workers that eclosed within the sub colony with different foraging experiences were compared: 1) A control group of newly eclosed workers <24hrs old with no nest emergences; 2) ants that had completed 1 emergence from the nest; and 3) ants that had completed 10 emergences. A nest emergence was defined as when ants entered the foraging arena and travelled at least 5cm away from the nest entrance and until they returned to the nest entrance.

The nest entrance was blocked at all times, except between 4:00pm and 7:30pm, during which time any ant in that colony was able to access the foraging arena. This time frame was chosen because of the natural foraging habits of *C. consobrinus*, who leave to forage as the sun begins to set, and few individuals leave the nest after dark (Personal observation, Schultheiss, *et al.,* 2015). When the entrance was open, behaviour of ants in the foraging arena was recorded with a Sony HDR-CX700VE camera at 25 frames per second. Due to the foraging arena's small size and limited foraging options, the colony's foraging requirements could be met by a few individuals, discouraging other ants from entering the arena. Hence, ants of unknown age were removed from the colony if they were consistently the only foragers for more than 4 days. This was done to encourage those that eclosed within the colony to forage.
It took an average of 26 days for each ant to become sufficiently mature to leave the nest, and during this period more than 90% of animals died for unknown reasons before reaching this level of maturity. Moreover, only 1 or 2 animals typically carried out the bulk of foraging. To encourage other animals to forage I removed the regular foragers, which led to only 11 individuals foraging over a 10 week period, 6 of whom survived to complete their respective treatments. Unfortunately, owing to time constraints I could not increase this sample size.

Sex comparison

Pupae of *C. consobrinus* were collected and immediately placed into dark boxes with 10 accompanying mature workers to assist with eclosion. Once workers or alates eclosed, they were separated into another dark box for either 2 or 11 days in total darkness, and collected for immunohistochemistry after the treatments were completed. Each box housed eclosed workers/alates of interest, contained a small sugar station and 10 mature workers from the same source colony, to minimise neural variation arising from social influences (Seid & Junge 2016; Giraldo *et al.,* 2016).

Sample size for the males was relatively low since it was not possible to collect pupae that eclose as males only. Moreover, pupae that become males are present for a short duration between November-December. Among the several nests I harvested, a small proportion of the collected pupae eclosed as males.

Behavioural Analysis

I used QuickTime v7.7.9 to trim the video recordings of foraging activity and prepare them for further analyses. Only footage of the outbound trips (nest to foraging station) was analysed. Footage of one ant from the ten-emergence group and two ants from the one-emergence group were of sufficient quality to be further analysed. Footage quality was compromised due to animal behaviours that were difficult to film, such as ants walking on ceiling of foraging arena. Using custom written software (© Jan Hemmi & Robert Parker) in Matlab vR2015b (MathWorks, Nattick, USA), the x, y coordinates of the head and mesonotum on each frame (40ms inter-frame interval) were digitised. Using custom written Matlab scripts, the data was used to map paths, analyse gaze directions and generate retinotopic views.

Immunohistochemistry

For ants in both the sex and foraging experience comparisons, the olfactory lip and visual collar regions of the MB calyx (*Fig. 1*; Ito *et al.*, 2014) were analysed for structural changes in their volume and microglomeruli density and number. Pre- and postsynaptic microglomeruli profiles were labelled with anti-synapsin antibodies following a protocol modified from Groh *et al.* (2014) and Kamhi *et al.* (2017). After their respective treatments were completed, ants were briefly anesthetised by cooling their body temperature, and decapitated. Brains were dissected in cool ant saline solution (129 mM NaCl, 6 mM KCl, 4.3 mM $MgCl_2$ X H_2O, 5 mM $CaCl_2$ X $2H_2O$, 159.8 mM Sucrose, 274 mM D-glucose, 10 mM HEPES buffer, pH 6.7, 4.0°C). The brain was then immediately fixed with 4% formaldehyde in phosphate buffered saline (PBS, pH 7.1) and left overnight at room temperature. After this, brains were washed in PBS (3x10mins) and then washed in 2% Triton-X in PBS (3x10min), to increase the permeability of the brain tissue. The brains were then washed in 2% normal goat serum and 0.5% Triton-X in PBS (PBSTN) for one hour to block non-target specific antibody binding. The brains were incubated in SYNORF1 (1:50; 3C11, SYNORF1; DHSB) in PBSTN at room temperature for 4 days, to allow SYNORF1 anti-synapsin antibodies to bind to their targets. After this the brains were washed in PBS again (5x10min), transferred to a PBSTN solution containing the secondary fluorescent antibody Alexa Fluor 488

(1:250; Invitrogen), and incubated at room temperature for 3 days. Brains were then washed in PBS again (5x10min) and then dehydrated via washes in an increasing ethanol series (30%, 50%, 70%, 90%, 98%, 100%; 10min each). Brains were then stored in 100% ethanol and transferred to a freezer (-20°C) for storage. To prepare for imaging, I added 4 drops of methyl salicylate to each tube that contained the brains, and gently mixed for 5 minutes. Methyl salicylate clears the brain tissue, increasing the visibility of the secondary antibodies at the primary antibody's binding sites. The brains were then gently transferred from the tube to a slide. Liquid was wicked away with a tissue at this point, and a few drops of methyl salicylate was added. A cover slip was placed over the brain, and the slide was left overnight in the dark to allow excess methyl salicylate to evaporate.

Laser scanning confocal microscope image processing

Brains were imaged using an inverted confocal laser scanning microscope (Olympus Fluo View FV1000© IX81). Whole brains were imaged with optical sections of 3.1µm with a 10x objective (*Fig. 1*). Amira v. 6.0.1 software (FEI Visualization Sciences Group, Düsseldorf, Germany) was used to obtain the volume of the lip and collar region of one MB calyx from these images. The calyx chosen was either the left or right medial calyx and was selected randomly. I also imaged either the left or right medial calyx of the MB in 0.25µm thick optical sections with a 60x objective (oil immersion) to obtain a magnification level sufficient to distinguish individual microglomeruli within the lip and collar regions (*Fig. 1*). ImageJ2 v.1.51u software (Rueden, *et al.*, 2017) was used to determine the average microglomeruli densities for the lip and collar regions, using calyx images taken on the confocal laser scanning microscope with the 60x objective. The calyx chosen here was the same as the calyx used to determine the volumes of each sample. On each image, three 10x10x10µm cubes were drawn onto the dense collar region, and two onto the lip region, modified from Cabirol (2017). The collar microglomeruli density was recorded as the average number of microglomeruli across the three collar cubes, and the lip microglomeruli density was recorded as the average number across the two lip regions. Each cube was located at least 10µm away from each other. The cube's location along the Z-axis was chosen at random. Microglomeruli were counted using the ImageJ *cell counter* plug-in, according to an unbiased cell counting stereology protocol (Williams & Rakic, 1988). All Images were analysed blind to sex, age, or foraging experience. Estimated microglomeruli numbers for the MB lip and collar were acquired by multiplying the average number of microglomeruli per mm^3 by the absolute volume of that neuropil.

Statistical Analysis

First the densities of microglomeruli in the lip and collar regions were considered in relation to the absolute volumes of each neuropil (William & Rakic, 1988).

R v3.4.0 was used to perform all statistical tests. Distributions of all data sets were checked for normality (Shapiro-Wilk procedure, $\alpha<0.05$) and variances across treatment groups were tested for equality (Levene's test). One-way ANOVA was used to compare data with a normal distribution; collar volumes, lip microglomeruli densities and lip microglomeruli counts between the different foraging treatment groups (factorial ANOVA). Kruskal-Wallis tests were used to compare data without a normal distribution; collar microglomeruli densities, collar microglomeruli counts and lip volumes between different foraging experience treatment groups.

Correlations were assessed between head width, age and time spent on foraging arena, and each of the forager's lip and collar data sets. Pearson's correlation tests were used for parametric data, and Spearman's correlation tests for non-parametric data.

Welch's *t*-tests were used to compare data with a normal distribution and unequal variances; the volumes, microglomeruli densities of both the lip and collar between age groups in alate males, and the volumes, microglomeruli densities and microglomeruli counts of both the lip and collar between age groups in workers (Welch's *t*-test). Wilcoxon tests were used to compare data without a normal distribution; microglomeruli counts of the lip and collar regions between age groups in alate males (Wilcoxon). Pearson correlation and Spearman correlation tests were conducted between head width of males and age-grouped workers and each of the lip and collar data sets.

Results

Learning walks in newly eclosed ants

In all cases, ants did not move directly towards the foraging station, and generally first performed small loops close to the nest entrance. Although the paths from the nest entrance to the foraging arena varied in terms of how well directed the ant was, generally the ant spent time exploring the arena (*Fig. 2A*). This exploratory behaviour continued for ant 2, from the one trip treatment group, even after coming into close contact with the foraging station (*Fig. 2A: Ant 2*). There were many cases where the retinal position of ants 2 and 3, from one and ten trip treatment groups respectively, was directed ±30° towards the nest during exploratory activities (*Fig. 2B: Ant 2 & Ant 3*). However, there was a large amount of variation in the initial behaviour between individuals. Ant 1, from the one trip treatment group, rarely looked toward the nest entrance and often faced the opposite direction of the nest (*Fig. 2A & B: Ant 1*).

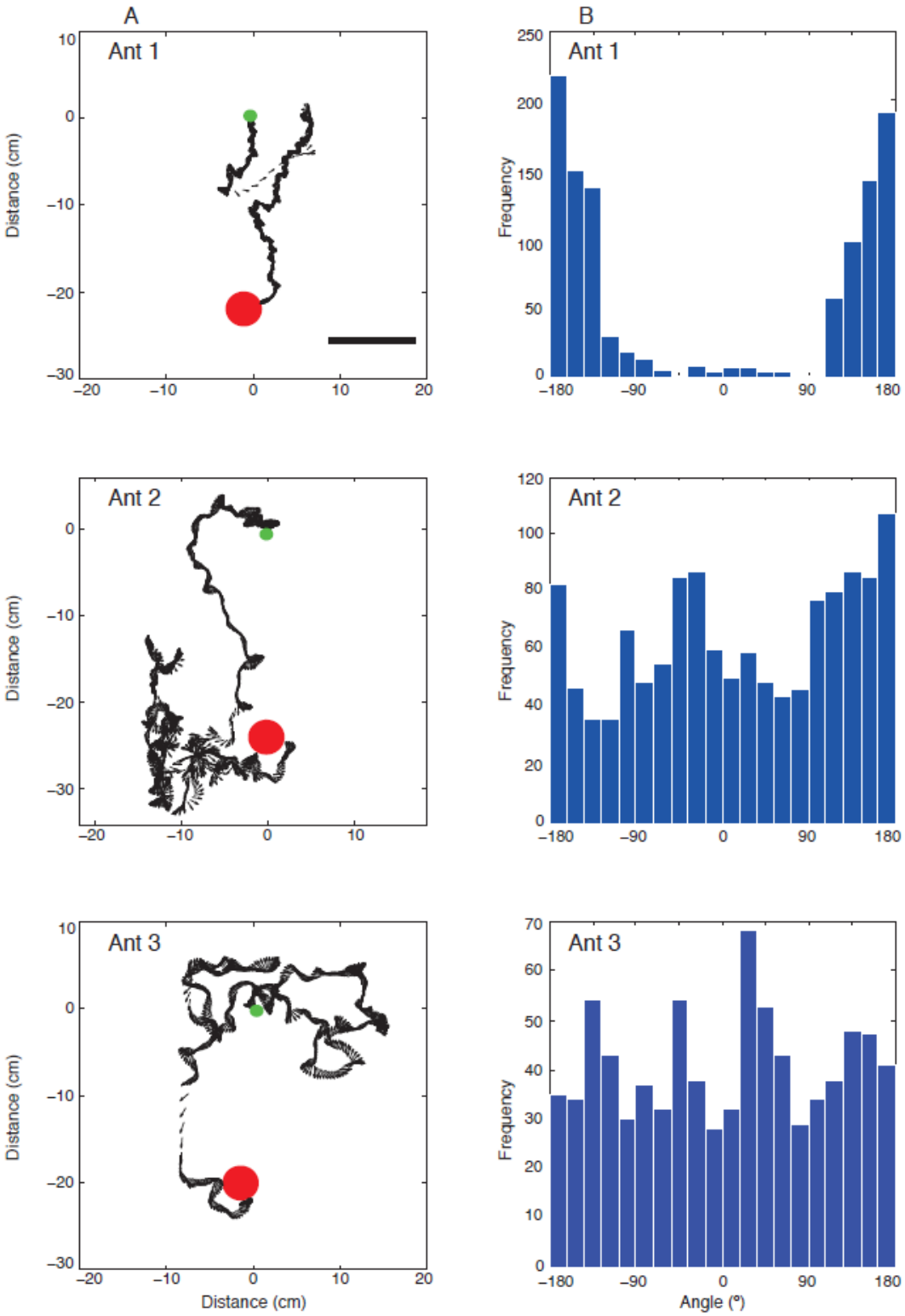

Fig 2. *Gaze direction of three individual ants when first outside the nest. (Column A) Quiver plots show the gaze direction, with each arrow representing the gaze direction of ant on a single frame (40ms inter-frame interval). Gaze direction determined by acquiring x, y coordinates of head and pronotum position for each frame, with the nest entrance set to 0, 0. Scale bar in Column A: Trip 3 is 10cm, and applies to all quiver plots. The nest entrance is at the green circle, and the foraging station is at the red circle. (Column B) Histograms show the frequency of the ant's retinal position, nest position at 0°.*

On the third and fifth excursions of ant 3, the ant was more directed towards the foraging station in comparison to its first trip (*Fig. 3A: Trip 3 & Trip 5; Fig. 2A: Ant 3*). Ant 3 frequently also looked towards the nest entrance during the third and fifth trip (*Fig. 3B: Trip 3 & Trip 5*), but she did so much less frequently than in the first trip (*Fig. 2B: Ant 3*). However, ant 3 was better directed towards the foraging station and looked back at the nest less frequently in her third trip than the fifth trip. In the seventh and tenth excursions, the ant's path was well directed towards the foraging station (*Fig. 3A: Trip 7 & Trip 10*), and very rarely looked towards the nest (*Fig. 3B: Trip 7 & Trip 10*).

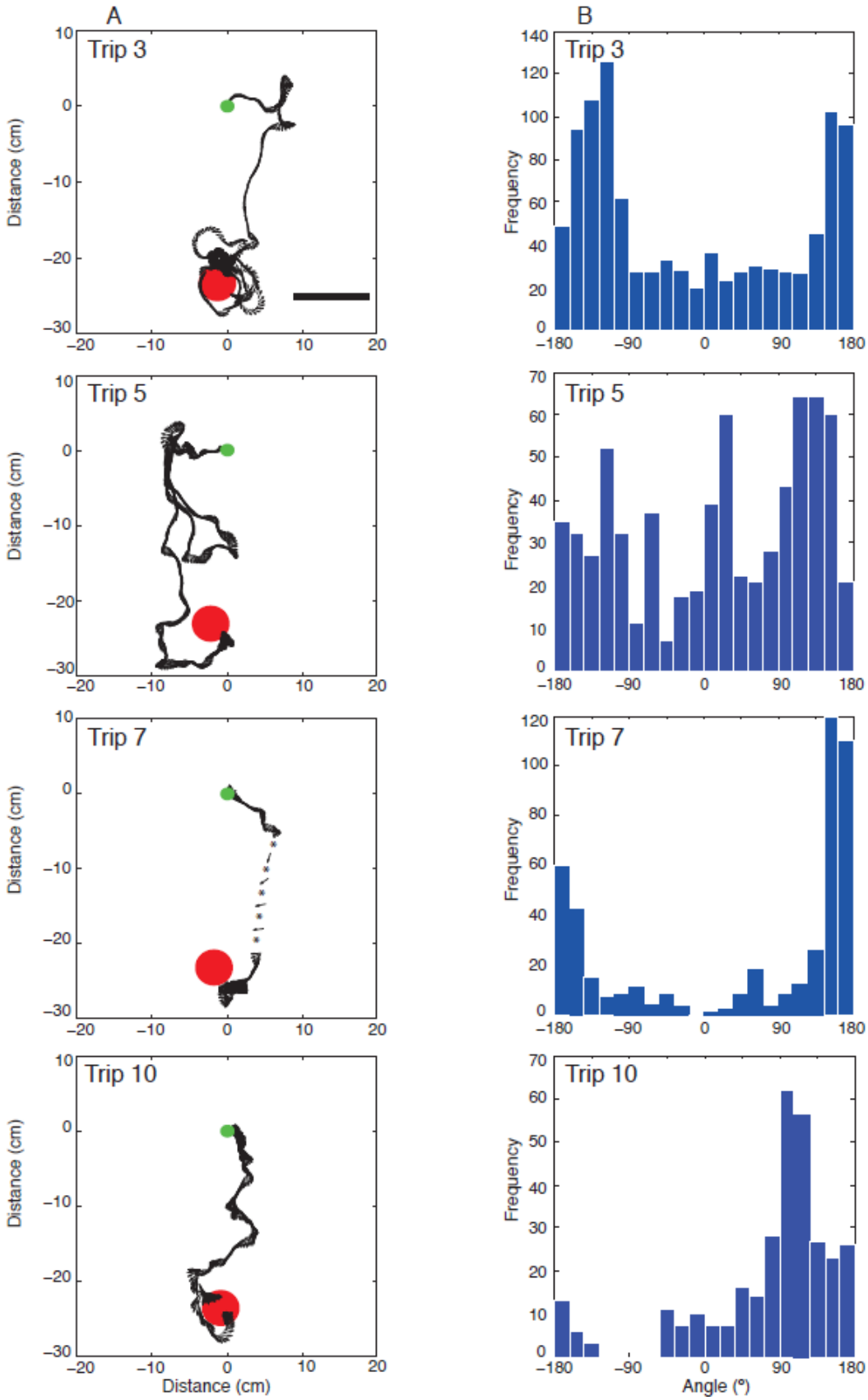

Fig 3. *Gaze direction of ant 3 during her third, fifth, seventh and tenth nest departures. (Column A) Quiver plots show the gaze direction, with each arrow representing the gaze direction of ant on a single frame (40ms inter-frame interval). Gaze direction determined by acquiring x, y coordinates of head and pronotum position for each frame, with the nest entrance set to 0, 0. Scale bar in Column A: Trip 3 is 10cm, and applies to all quiver plots. (*) Indicates frame where ant is*

obstructed. The nest entrance is at the green circle, and the foraging station is at the red circle. (Column B) Histograms show the frequency of the ant's retinal position, nest position at 0°.

Relationship of foraging experience and neural structure

To determine if experience outside the nest had an effect on neural structure in *C. consobrinus* workers, I compared the volume of the MB calyx lip (received olfactory input) and MB calyx collar (received visual input) regions relative to head width between treatment groups. Worker body sizes varied, so I asked if lip and collar volumes changed relative to head width. The relative volume of the collar and lip regions was not significantly different between any of the foraging groups (Collar: ANOVA, $F_{2, 8}$=0.309, p=0.742; Lip: Kruskal-Wallis, H=5.212, df=2, p=0.738; 0 excursions group *n*=5; 1 excursion group *n*=3; 10 excursions group *n*=3; *Fig. 4A*). The volume of the MB collar and lip regions did not correlate with head width (Collar: Pearson correlation, *t*=-1.779, *r*=-0.510, df=9, p=0.109; Lip: Spearman correlation, *rho*=0.036, S=212, p=0.924), age (Collar: Pearson correlation, *t*=-0.420, *r*=-0.139, df=9, p=0.684; Lip: Spearman correlation, *rho*=0.022, S=224.03, p=0.923) or time spent outside the nest (Collar: Pearson correlation, *rho*=-0.191, S=261.95, p=0.574; Lip: Spearman correlation, *rho*=-0.029, S=226.29, p=0.934).

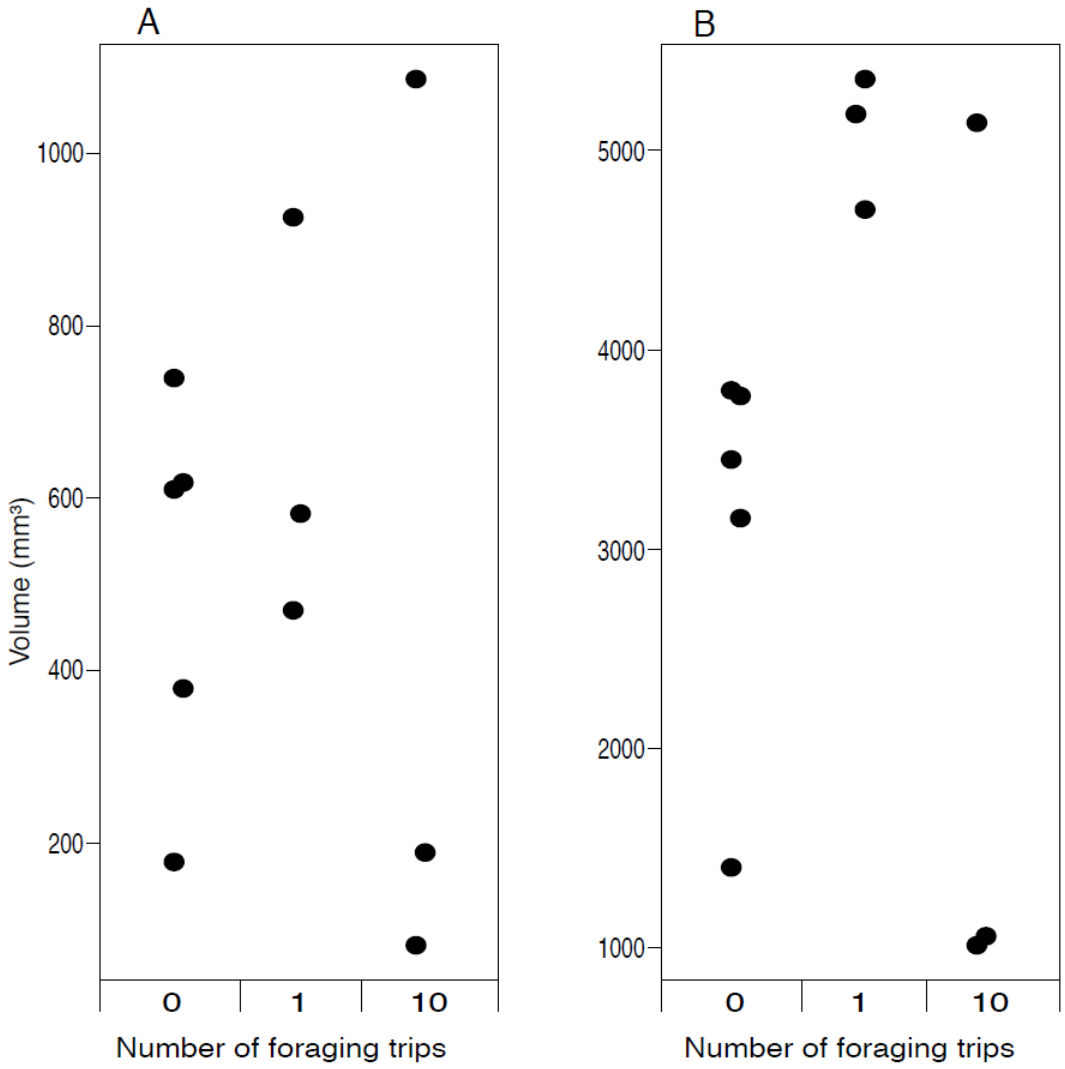

Fig 4. *Relationship of the relative volumes of the MB calyx collar (A) and MB calyx lip (B) with number of nest departures in workers of C. consobrinus.*

Next, I compared the microglomeruli densities of the MB calyx collar and MB calyx lip regions between treatment groups to determine if there was a change in neural organisation at the synaptic scale (*Fig. 5*). As with the relative collar and lip volumes, there was no significant difference between treatment groups in the density of microglomeruli for either the collar or the lip (Collar: Kruskal-Wallis, H=0.170, df=2, p=0.918; Lip: ANOVA, $F_{2,8}$=3.406, p=0.085). Here, microglomeruli densities in the collar and lip regions did not correlate with head width (Collar: Spearman correlation, *rho*=0.159, S=184.92, p=0.640; Lip: Pearson correlation, *t*=-0.353, *r*=-0.117, df=9, p=0.733), age (Collar: Spearman correlation, *rho*=-0.062, S=233.67, p=0.856; Lip: Pearson correlation, *t*=-2.042, *r*=-0.563, df=9, p=0.072) or time spent outside the nest (Collar: Spearman correlation, *rho*=-0.062, S=233.67, p=0.856; Lip: Spearman correlation, *rho*=-0.496, S=329.08, p=0.121).

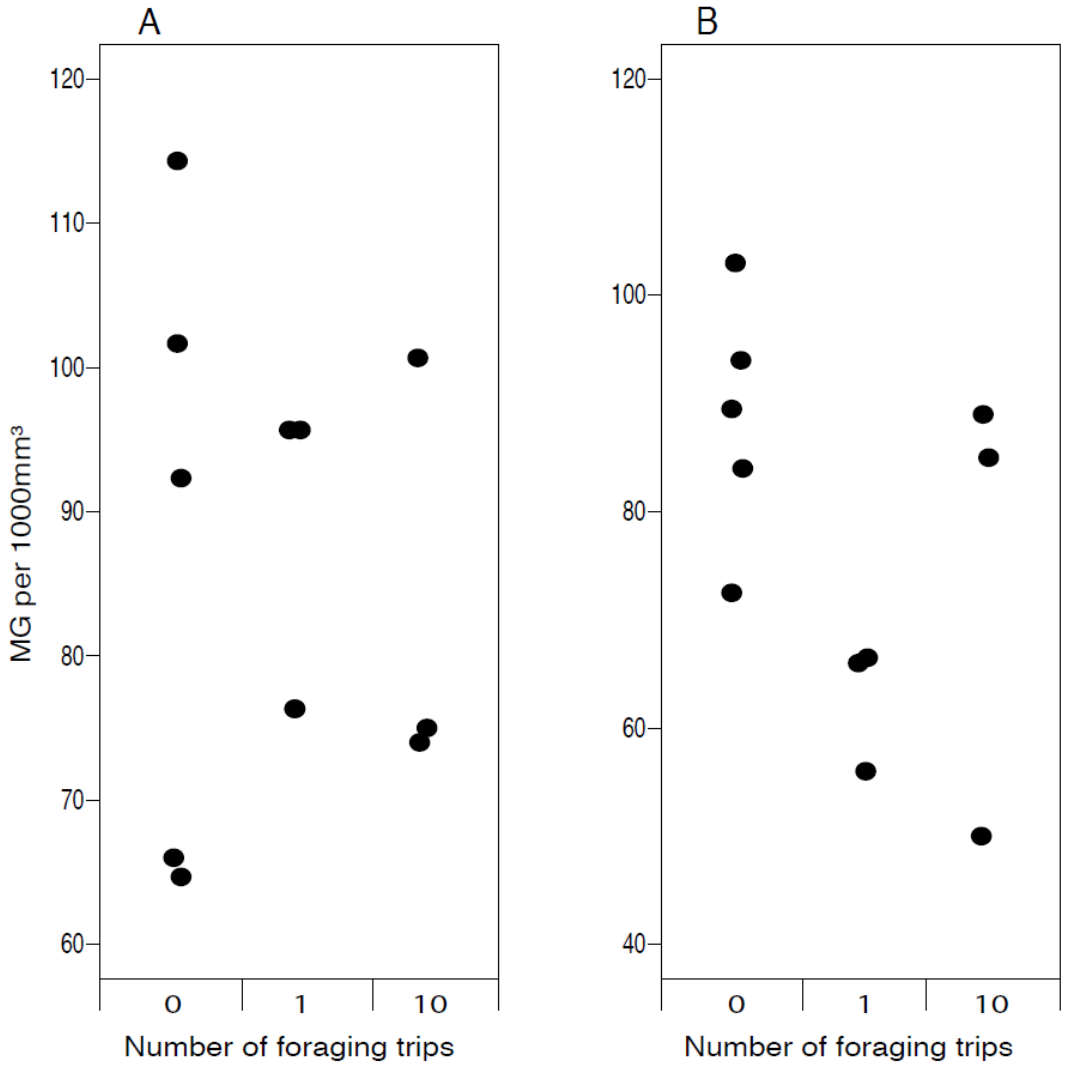

Fig 5. *Relationship of the average microglomeruli (MG) densities of the MB calyx collar (A) and MB calyx lip (B) with number of nest departures in workers of C. consobrinus.*

The microglomeruli counts for the MB calyx lip and MB calyx collar regions relative to their absolute volumes were compared (*Fig. 6*). The estimated microglomeruli counts did not differ between foraging experience treatment groups in the lip (ANOVA, $F_{2, 8}$=1.26, p=0.334) or in the collar (Kruskal-Wallis, H=1.527, df=2, p=0.466). Also, estimated microglomeruli counts for the lip and collar did not correlate with head width (Collar: Spearman correlation, *rho*=-0.191, S=262, p=0.576; Lip: Pearson correlation, *t*=-0.869, *r*=-0.278, df=9, p=0.407), age (Collar: Spearman correlation, *rho*=-0.029, S=226.29, p=0.933; Lip: Pearson correlation, *t*=-0.842, *r*=-0.270, df=9, p=0.421) or time spent outside the nest (Collar: Spearman correlation, *rho*=-0.057, S=232.59, p=0.867; Lip: Spearman correlation, *rho*=-0.038, S=228.39, p=0.911).

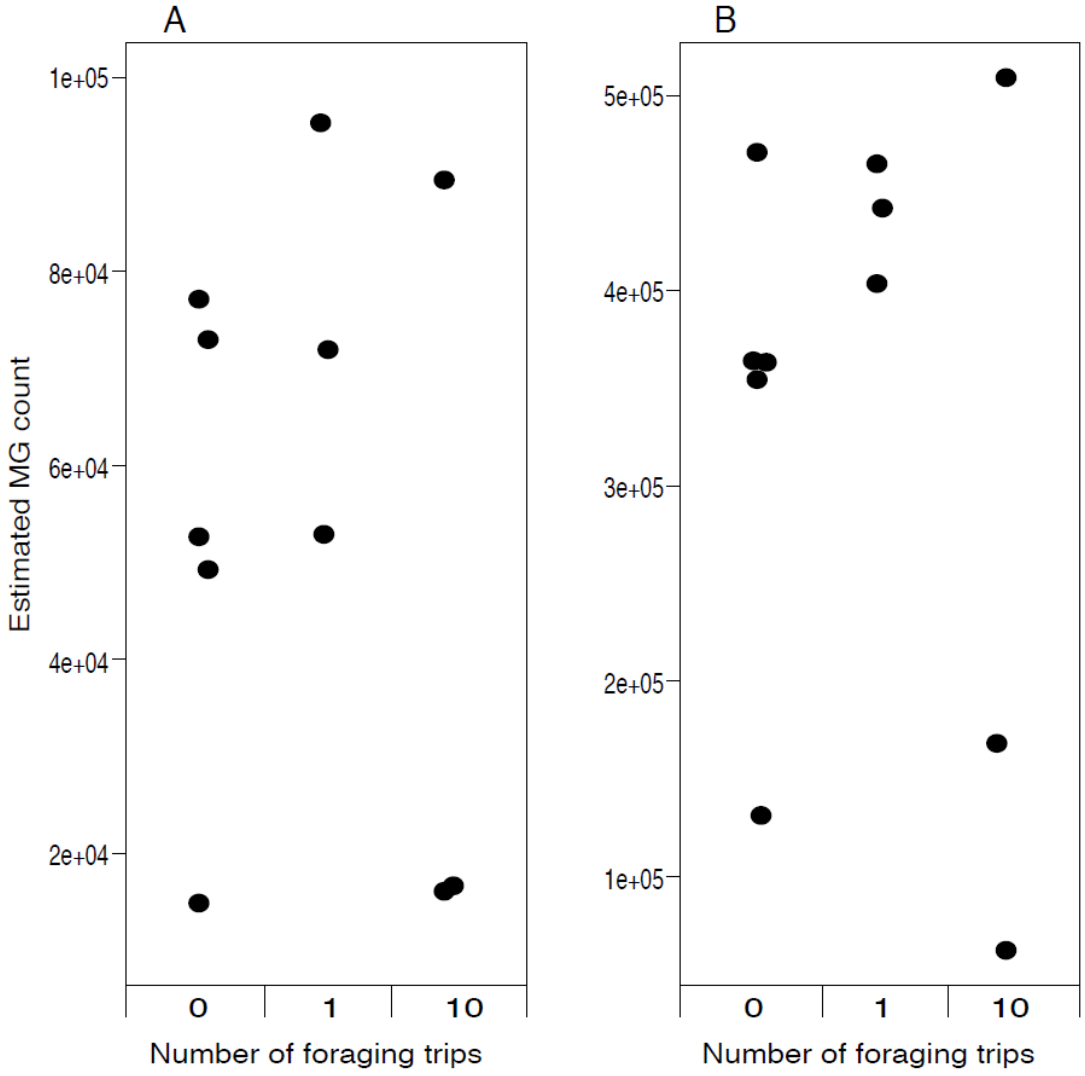

Fig 6. *Relationship of the estimated number of microglomeruli (MG) in the MB calyx collar (A) and MB calyx lip (B) with number of nest departures in workers of C. consobrinus.*

Sex-based neuroplasticity

Males that were 11-days old and reared in total darkness had significantly larger MB calyx collars, compared to males that were 2 days old and reared in total darkness (Welch's *t*-test, t=3.151, df=4.082, p=0.033; 2 day group *n*=4; 11 day group *n*=5; *Fig. 7A*). In the MB calyx lip region there was no significant difference in volume between age-groups (Welch's *t*-test, t=1.369, df=6.812, p=0.213; *Fig. 7B*). Head widths of male ants did not differ between age groups (Welch's *t*-test, t=-0.183, df=6.368, p=0.861), and was not correlated with the volumes of either the collar or lip regions (Collar: Pearson correlation, *r*=-0.003, *t*=0.007, df=7, p=0.994; Lip: Pearson correlation, *t*=-0.537, *r*=-0.199, df=7, p=0.608). In contrast to the males, the workers did not demonstrate a significant difference in the volume between age groups for either the collar or lip regions (Collar: Welch's *t*-test, t=-0.359, df=5.739, p=0.733; Lip: Welch's *t*-test, t=1.051, df=7.229, p=0.327; 2 day group *n*=5; 11 day group *n*=5; *Fig. 7*). Neither the collar nor lip region's volume for workers had a correlation with head width (Collar: Pearson correlation, *t*=-0.040, *r*=-0.014, df=8, p=0.969; Lip: Pearson correlation, *t*=1.816, *r*=0.540, df=8, p=0.107), but head width did differ between worker age groups and was significantly larger in the 11 day old group (Welch's *t*-test, t=3.790, df=5.131, p=0.012).

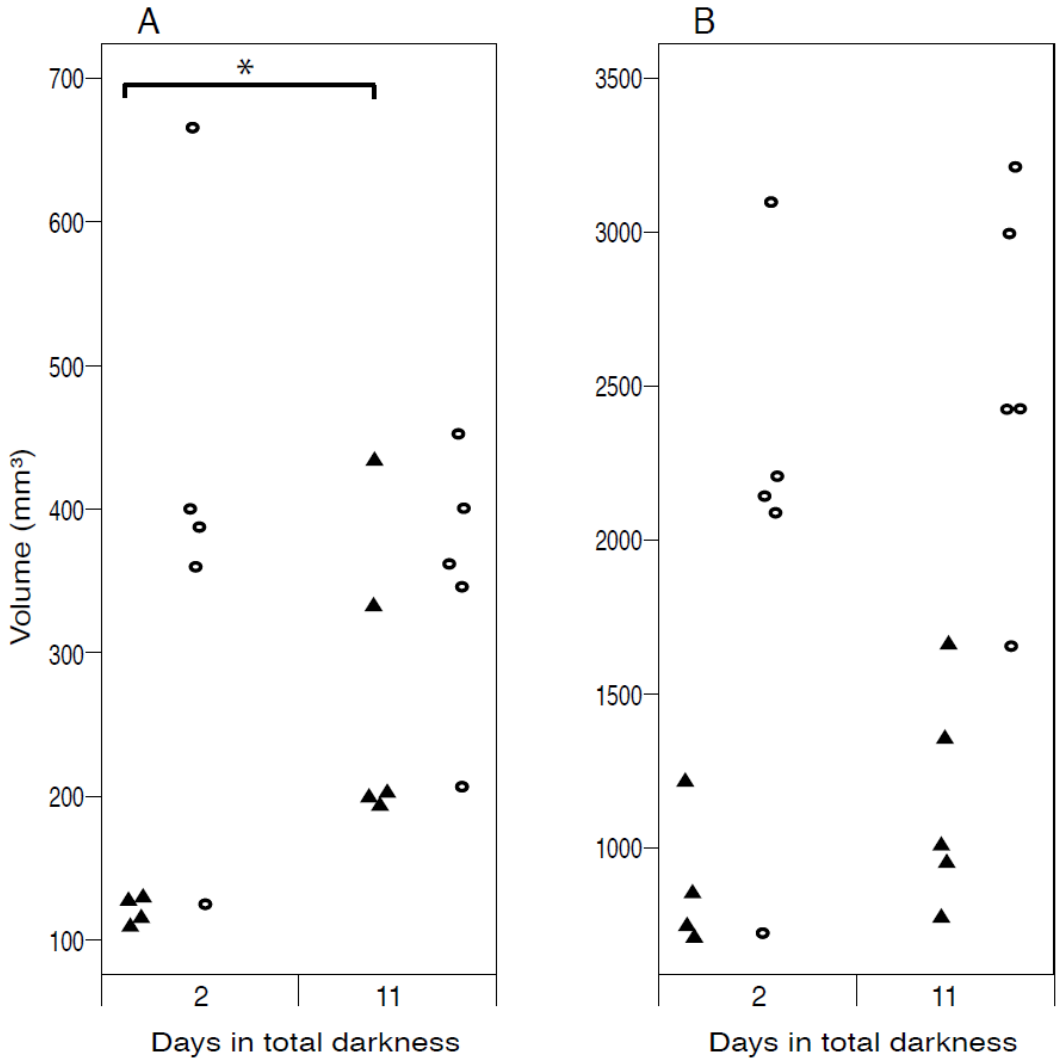

Fig 7. *Relative volumes of the MB calyx collar (A) and the MB calyx lip (B) regions in males (triangles) and workers (circles). Asterisk indicates significant difference between male age groups.*

After determining the difference in MB calyx collar and MB calyx lip volume between treatment groups, the microglomeruli densities were compared to provide insight into any changes occurring at the synaptic scale (*Fig. 8*). The microglomeruli densities of the collar and lip regions did not differ between the treatment groups in either the males (Collar: Welch's *t*-test, t=-0.871, df=6.564, p=0.415; Lip: Welch's *t*-test, t=-0.389, df=6.679, p=0.709) or workers (Collar: Welch's *t*-test, t=-1.539, df=7.779, p=0.164; Lip: Welch's *t*-test, t=-0.457, df=6.791, p=0.662). Additionally, collar and lip microglomeruli densities were not correlated with head width in males (Collar: Pearson correlation, *t*=1.132, *r*=0.393, df=7, p=0.295; Lip: Pearson correlation, *t*=-1.127, *r*=-0.392, df=7, p=0.297) or workers (Collar: Pearson correlation, *t*=-1.710, *r*=-0.517, df=8, p=0.126; Lip: Pearson correlation, *t*=0.143, *r*=0.051, df=8, p=0.890).

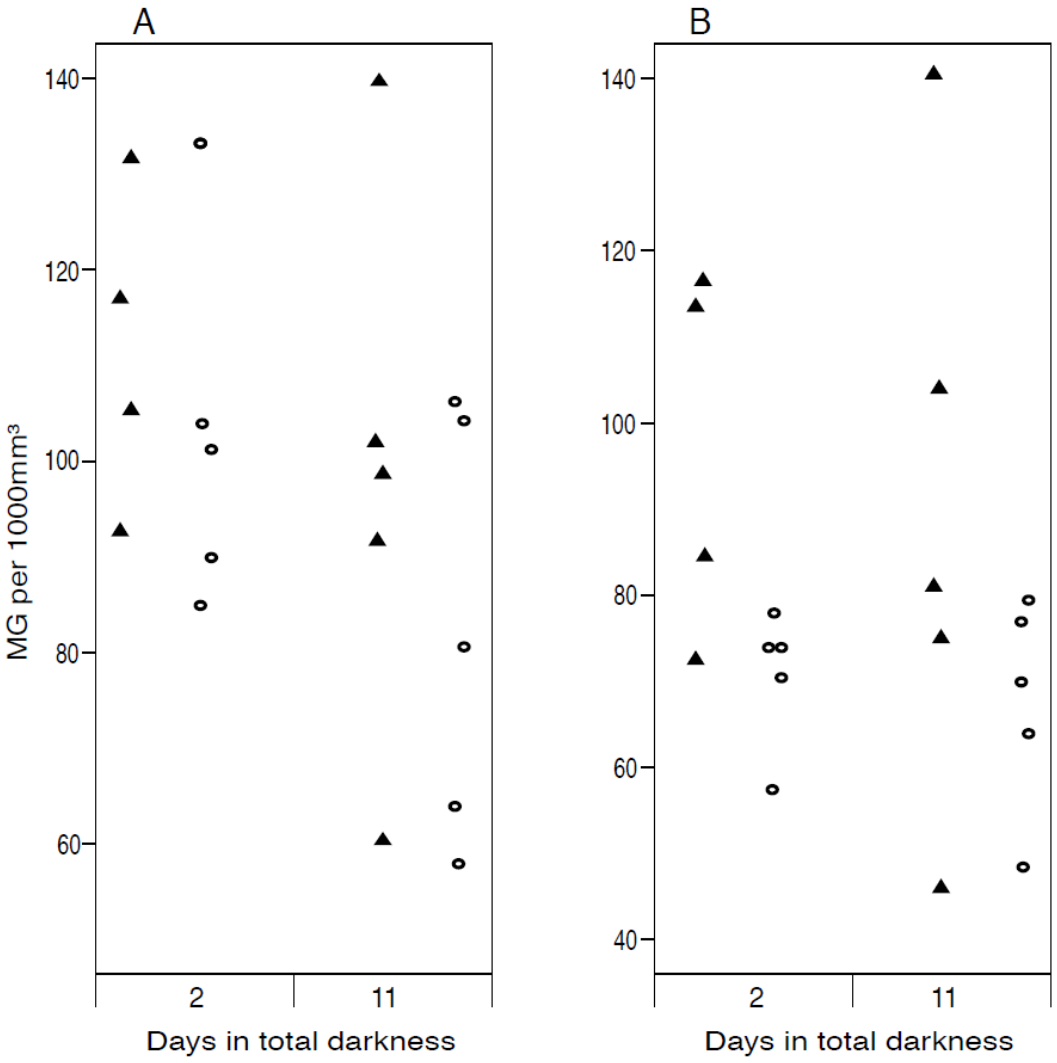

Fig 8. *Average microglomeruli (MG) densities in the MB calyx collar (A) and the MB calyx lip (B) regions in males (triangles) and workers (circles).*

Finally, I examined how the microglomeruli counts in the MB calyx collar and MB calyx lip regions changed relative to their absolute volumes. Estimated microglomeruli counts in the collar region were significantly higher in the 11 day old male *C. consobrinus* group compared with the 2 day old male group (Wilcoxon test, w=19, p=0.032; *Fig. 9A*). In the lip region, there was no significant difference in microglomeruli counts between age groups (Wilcoxon test, w=14, p=0.413; *Fig. 9B*). The microglomeruli counts for the workers did not differ between age groups in either the collar or lip regions (Col: Welch's *t*-test, t=-0.152, df=7.939, p=0.883; Lip: Welch's *t*-test, t=1.839, df=6.455, p=0.112; *Fig. 9A*). Also, the microglomeruli counts in the lip region were positively correlated with head width in workers (Pearson correlation, t=3.694, r=0.794, df=8, p=0.006), but not for the collar and lip regions in males (Collar: Spearman correlation, *rho*=0.383, S=74, p=0.313; Lip: Spearman correlation, *rho*=-0.07, S=204, p=0.433) or collar region in workers (Pearson correlation, t=0.107, r=0.038, df=8, p=0.918).

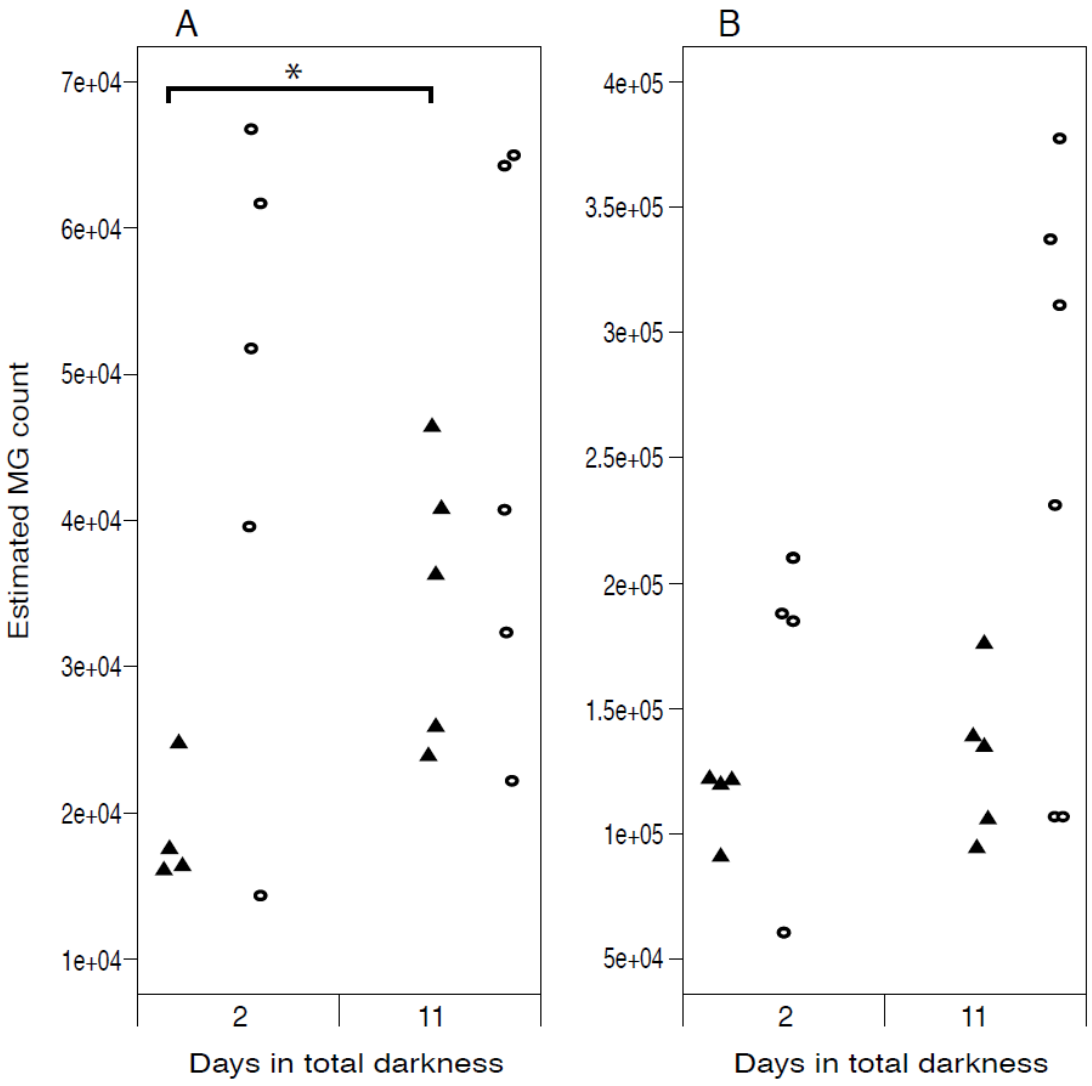

Fig 9. *Estimated number of microglomeruli (MG) in the MB calyx collar (A) and the MB calyx lip (B) regions in males (triangles) and workers (circles). Asterisk indicates significant difference between male age groups.*

Discussion

This study is the first to elicit learning walks in laboratory conditions and to record learning walk repertoire on the first trip out of the nest for newly eclosed animals. Furthermore, I show that navigational behaviour in a single ant changes and becomes more directed as foraging experience increases. As workers gained experience and carried out multiple outbound trips, the volume, microglomeruli density and microglomeruli count of the MB calyx lip and collar regions did not differ from naïve animals. Male ants, whose brains are understudied, do exhibit some degree of neuroplasticity. In older males, the volume of the visual input region (collar) was larger and the number of microglomeruli in the collar region was also higher. Here I will discuss the implications of these behavioural and neural changes in these ants.

Learning walk behaviour and experience based directional changes

I mapped and analysed the outbound journey of three ants in a foraging arena. I analysed the first outbound journey in three individuals (*Fig. 2*), as well as the third, fifth, seventh and tenth outbound trips of one of these ants (*Fig. 3*). The results described here show that ants with little to no experience outside the nest frequently gaze towards the nest entrance. There is a high number of nest directed gazes during initial excursions, however there is substantial variation between all three ants. In the ant tracked for 10 consecutive outbound trips, her trips generally had fewer nest directed gazes as experience increased (*Fig. 2B: Ant 3; Fig. 3B*). This ant's gaze also became increasingly directed towards the foraging station as she gained experience. The behaviour described here for initial outbound trips conforms to learning walk behaviour observed in other ants (Fleischmann, *et al.,* 2016; 2017). Directed gazes towards the nest entrance are a key aspect of learning walks/flights in many central-place foragers (Collett, 1995; Philippides, *et al.,* 2013; Fleischmann, *et al.,* 2016). Furthermore, learning walks generally occur during an ant's first outbound trip, and become less frequent as ants develop familiarity with their nests surroundings (Wehner, 2004; Müller & Wehner, 2010).

Neural structure consistencies in foraging workers

I then investigated how foraging experience, measured as the number of departures from the nest, affected the neural architecture of the lip and collar regions within the MBs. However, there was no evidence of a significant difference between foraging experience groups in terms of their neuropil volumes, density of microglomeruli and estimated total number of microglomeruli. These factors

did not differ based on the amount of time spent outside the nest. Results were in stark contrast to initial predictions that neuroplasticity would occur by the first nest departure, and would continue to change neural architecture with further experience outside the nest. Although there was a significant age difference between the naïve group (<24 hours old) and both the 1 and 10 excursion groups (average age of 26 ± 11 days), there were no differences in neural structure or organisation. This is important to highlight as previous studies show that both the lip and collar regions exhibit age-related neuroplasticity in other ants, such as *Cataglyphis fortis*, *Cataglyphis bicolor* and *Pheidole dentata* (Seid, *et al.*, 2005; Kühn-Bühlmann & Wehner, 2006; Stieb, *et al.*, 2010). Yilmaz *et al.* (2016) demonstrated that *Camponotus rufipes* exhibited periodic neural reorganisation, over the first 42 days post-eclosion, in the collar region. *C. rufipes* collar volumes were recorded at both 28 and 29 days old, but volumes were only greater than 3 days old conspecifics at 28 days old (Yilmaz, *et al.*, 2016). This demonstrates that neuroplasticity does not necessarily occur consistently, and this may partially explain why no differences were found in the foragers examined in this study. The ages of ants used in this study at the time of collection were close to the age groups used by Yilmaz, *et al.* (2016), although the ages of *C. consobrinus* foragers used here were determined by the specific behaviour of individuals.

It was also surprising to see that no neuroplasticity between treatment groups because the 1 and 10 emergence groups had been provided with visual sensory stimuli during their trips into the foraging arena. Ants and bees have previously exhibited light-based neural restructuring of the collar region (Stieb, *et al.*, 2012; Scholl, *et al.*, 2014), but the influence of age and light on neural structure and organisation appears to be inconsistent (Stieb, *et al.*, 2010; Yilmaz, *et al.*, 2016). This type of relationship was not observed in the data presented, however it may help to explain why neuroplasticity was not seen to be associated with experience outside the nest. Inducing learning walks and foraging activity in ants within a lab colony had never been done before and was challenging. However, establishing that learning walks can be elicited in laboratory conditions is promising for future behavioural and neurobiological investigations.

Age based neural development in alate males

Male ants are typically visually oriented (Baker & Ma, 2006; Narendra, *et al.*, 2016). I found that in the males of *C. consobrinus* the volume of the MB calyx collar region, which receives visual input and its microglomeruli count were both greater in the 11-day old group compared to the 2-day old ants. This supports the initial hypothesis, which stated that there would be a detectable difference in the neural structure and organisation between the two age groups. In the paper wasp, *Mischocyttarus mastigophorus*, males demonstrate age-related neuroplasticity (Molina &

O'Donnell, 2008). Age related volume expansions in the MB calyx collar of *M. mastigophorus* females was present, however their male conspecifics had larger MB calyx collars, and this difference increased with age (Molina & O'Donnell, 2008). However, in honey bees, MB volume expansions were similar in relative size increase and timing between all sexes, although distinctions between the lip and collar regions were not made in these studies (Fahrbach, *et al.*, 1995; 1997). Similar to the paper wasps, and in contrast to honey bees, neural changes in male *C. consobrinus* were different to what was seen in female workers. Moreover, workers did not demonstrate any significant neuroplasticity between treatment groups. The hypothesis that workers would demonstrate different patterns of neuroplasticity to the alate males is supported, although the absence of age related changes in workers was unexpected.

Yilmaz, *et al.* (2016), showed that significant age-related changes occurs in the collar region of *C. rufipes*, but not in a consistent fashion, and may be associated with pre-nest emergence maturation. Alate males are ready to mate upon being ready to fly (Wilson, 2000) and many male *C. consobrinus* are able to fly at 6-7 days of age (Personal observation), so their brains need to develop to maturity in this time frame. This is much quicker than workers that left the nest to start foraging at an average of 26 ± 11 days (mean ± sd). This may explain why neuroplasticity was not detectable between worker age groups, but was detectable between male age groups. Male alates make significantly larger visual system investments relative to their head size than workers (Hölldobler & Wilson, 1990; Narendra, *et al.*, 2016), and similarly invest more into restructuring and reorganisation of the collar than workers.

In summary, these results indicate that male ants do experience neuroplasticity in the first 11 days post-eclosion. This is consistent with findings in other non-ant hymenopteran males, which also demonstrated neural restructuring during their first two weeks of life (Fahrbach, *et al.*, 1997; Molina & O'Donnell, 2008), and differ to what was seen in workers (Molina & O'Donnell, 2008). In my study, workers did not exhibit the same level or type of neuroplasticity as the males, and in fact did not demonstrate any. The head widths of workers were significantly different between age groups, suggesting that age groups were different in an unidentified way that may have affected neural structure, considering head growth between these age groups is unlikely (Hölldobler & Wilson, 1990). Nevertheless, these results support the initial hypothesis that male *C. consobrinus* experience neuroplasticity between 2 and 11 days of age, and that worker's neural development is different to males. Despite the small sample size, I could demonstrate clear age-related differences in the MB calyx collar region of male *C. consobrinus*. The behavioural implication of these differences in male and worker ants could further our understanding of neural and behavioural maturation in eusocial insects, and therefore deserves further investigation.

Conclusion

I was able to induce learning walks under laboratory conditions for the first time and determine how neuroplasticity is related to foraging experience and sex. The ability to induce learning walks under laboratory conditions allows for more controlled and convenient investigations into the relationship between navigational success and learning and memory. However, neither changes in navigational behaviour nor the presence and level of foraging experience were reflected in the neural development of the visual sensory processing collar or olfactory sensory processing lip regions in workers of *C. consobrinus*. Neural restructuring and synaptic reorganisation in the collar region was detected in male alates only. This may be due to the short lifespan of males in comparison to workers, as well as their extensive use of visual sensory information whilst searching for mates. Thus, I have demonstrated there are distinct sex-specific differences in the neural development of ants, which are likely due to the differences in their lifestyle and ecology.

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